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Tumor-targeting *Salmonella typhimurium* A1-R Inhibits Osteosarcoma Angiogenesis in the *In Vivo* Gelfoam® Assay Visualized by Color-coded Imaging

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Abstract. *Background: We previously developed a color-coded imaging model that can quantify the length of nascent blood vessels using Gelfoam® implanted in nestin-driven green fluorescent protein (ND-GFP) nude mice. In this model, nascent blood vessels selectively express GFP. We also previously showed that osteosarcoma cells promote angiogenesis in this assay. We have also previously demonstrated the tumor-targeting bacteria Salmonella typhimurium A1-R (S. typhimurium A1-R) can inhibit or regress all tested tumor types in mouse models. The aim of the present study was to determine if S. typhimurium A1-R could inhibit osteosarcoma angiogenesis in the in vivo Gelfoam® color-coded imaging assay. Materials and Methods: Gelfoam® was implanted subcutaneously in ND-GFP nude mice. Skin flaps were made 7 days after implantation and 143B-RFP human osteosarcoma cells expressing red fluorescent protein (RFP) were injected into the implanted Gelfoam. After establishment of tumors in the Gelfoam®, control-group mice were treated with phosphate buffered saline via tail-vein injection (iv) and the experimental group was treated with S. typhimurium A1-R iv Skin flaps were made at day 7, 14, 21, and 28 after implantation of the Gelfoam® to allow imaging of vascularization in the Gelfoam® using a variable-magnification small-animal imaging system and confocal fluorescence microscopy. Results: Nascent blood vessels expressing ND-GFP extended into the Gelfoam® over time in both groups. However, the extent of nascent blood-vessel growth was significantly inhibited by S. typhimurium A1-R treatment by day 28. Conclusion: The present results indicate S. typhimurium A1-R has potential for anti-angiogenic targeted therapy of osteosarcoma.*

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Key Words: Salmonella typhimurium A1-R, tumor-targeting, RFP, osteosarcoma, angiogenesis, Gelfoam®, nestin, GFP, transgenic nude mice.

Osteosarcoma (OS) is the most common primary malignant bone cancer (1, 2) and is most frequent in children and adolescents (3). OS is usually treated with surgery, chemotherapy and radiotherapy. The 5-year survival rate still remains at approximate 60-70% (4). Novel approaches to OS are therefore needed.

The tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) strain was developed in our laboratory (5). *S. typhimurium* A1-R is auxotrophic for Leu–Arg, which prevents it from mounting a continuous infection in normal tissues. *S. typhimurium* A1-R inhibited or eradicated primary and metastatic tumors when used as monotherapy in nude-mouse models of major cancer types (6), including prostate (5, 7), breast (8-10), lung (11, 12), pancreatic (13-17), ovarian (18, 19), stomach (20), cervical cancer (21), glioma (22, 23), as well as sarcoma (24, 25), including osteosarcoma (26-28), all of which are highly aggressive tumor models.

Weiss's group reported that *S. typhimurium* was able to disrupt vascular flow in tumor (29). Liu *et al.* demonstrated that *S. typhimurium* A1-R caused vessel destruction in tumors which depended on the extent of vascularity of the tumor (12).

An *in vivo* angiogenesis assay using implanted Gelfoam® sponges was first described by McCarty *et al.* using agarose and pro-angiogenic factors (30). Our laboratory developed a color-coded *in vivo* Gelfoam® angiogenesis assay in the nestin-driven green fluorescent protein (ND-GFP) transgenic

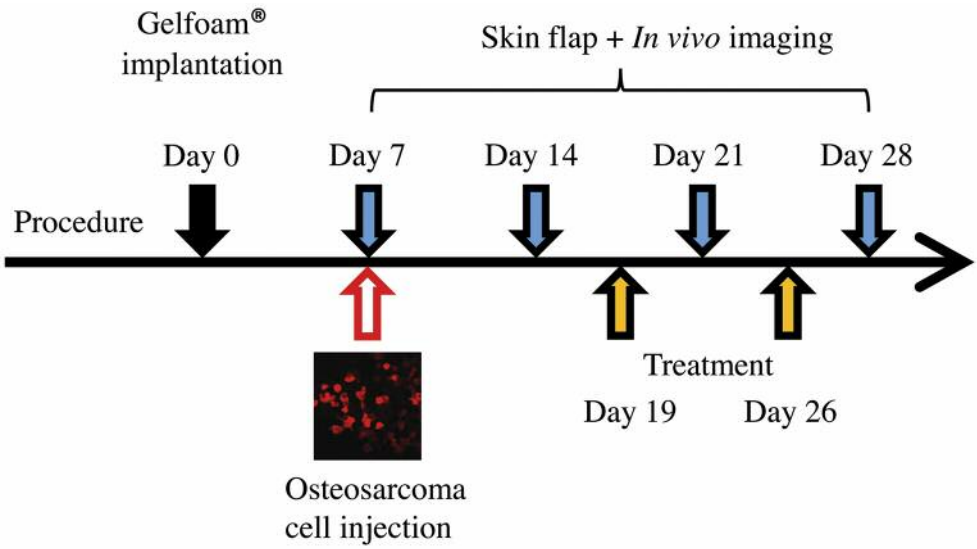

Figure 1. *Experimental schema for Gelfoam® implantation and subsequent cancer-cell injection in nestin-driven green fluorescent protein (ND-GFP) nude mice. Gelfoam® (5×5 mm), hydrated with 300 ng β fibroblast growth factor in 75 ml RPMI-1640 medium, was implanted to the subcutis on the flank of ND-GFP transgenic nude mice. Skin flaps were made at day 7 after implantation under ketamine anesthesia. Angiogenesis was observed by fluorescence in the skin flap using the OV100 Small Animal Imaging System (Olympus Corp.) and confocal fluorescence microscopy with the FV1000 (Olympus Corp.). 143B-red fluorescent protein (RFP)-expressing human osteosarcoma cells (5×10^5 cells/Gelfoam®) were injected in the ND-GFP nude mice (n=6) on day 7 after Gelfoam® implantation. Salmonella typhimurium A1-R (5.0×10^7 colony-forming units) was administered intravenously on days 19 and 26. Control mice were injected intravenously with phosphate-buffered saline. Skin flaps were made day 7, 14, 21, and 28, to observe tumor angiogenesis and tumor size in the implanted Gelfoam®.*

nude mouse in which nascent blood vessels are labeled with GFP. Color-coded imaging enabled determination of the length of nascent blood vessels growing in the Gelfoam® implanted in ND-GFP nude mice (31). Osteosarcoma cells promoted angiogenesis in the Gelfoam® assay in ND-GFP mice (32).

In the present report, we demonstrate that *S. typhimurium* A1-R inhibits nascent vessel growth in osteosarcoma growing in Gelfoam® implanted in ND-GFP nude mice.

Materials and Methods

Cells. Red fluorescent protein (RFP)-expressing 143B osteosarcoma cells were previously established (27). The 143B-RFP cells were maintained in RPMI-1640 medium (Cellgro, Herndon, VA, USA) with 10% fetal bovine serum (Omega Scientific, San Diego, CA, USA) and 1% penicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Mice. Female ND-GFP transgenic nude mice (AntiCancer, Inc., San Diego, CA, USA) were used in this study. Animals were housed in a barrier facility on a high-efficiency particulate arrestance-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO_2 inhalation when they met the following humane endpoint criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop.

Implantation of Gelfoam® (31, 32). ND-GFP transgenic mice, 6–8 weeks old, were anesthetized with the ketamine mixture. Gelfoam (5 × 5 mm) was treated with 300 ng β fibroblast growth factor (βFGF; Chemicon, Temecula, CA, USA) in 75 μl RPMI-1640 medium (Cellgro, Herndon, VA, USA). The treated Gelfoam was then transplanted into the subcutis on both flanks of the ND-GFP transgenic mice under ketamine anesthesia.

Skin flaps for imaging. ND-GFP transgenic nude mice with Gelfoam implants were anesthetized with the ketamine mixture *via s.c.* injection. An arc-shaped incision was made in the abdominal skin from the axillary to the inguinal region. The subcutaneous connective tissue was separated to free the skin flap without injuring the vessels. Mice were laid flat and the skin flap was spread and fixed on the flat stand for imaging. The skin was closed with a 6-0 nylon suture after observation (33).

Implantation of 143B-RFP cells in the Gelfoam® assay. Seven days after Gelfoam® implantation, RFP-expressing human 143B osteosarcoma cells (5×10^5) were injected with a 0.5 ml 28 G latex-free

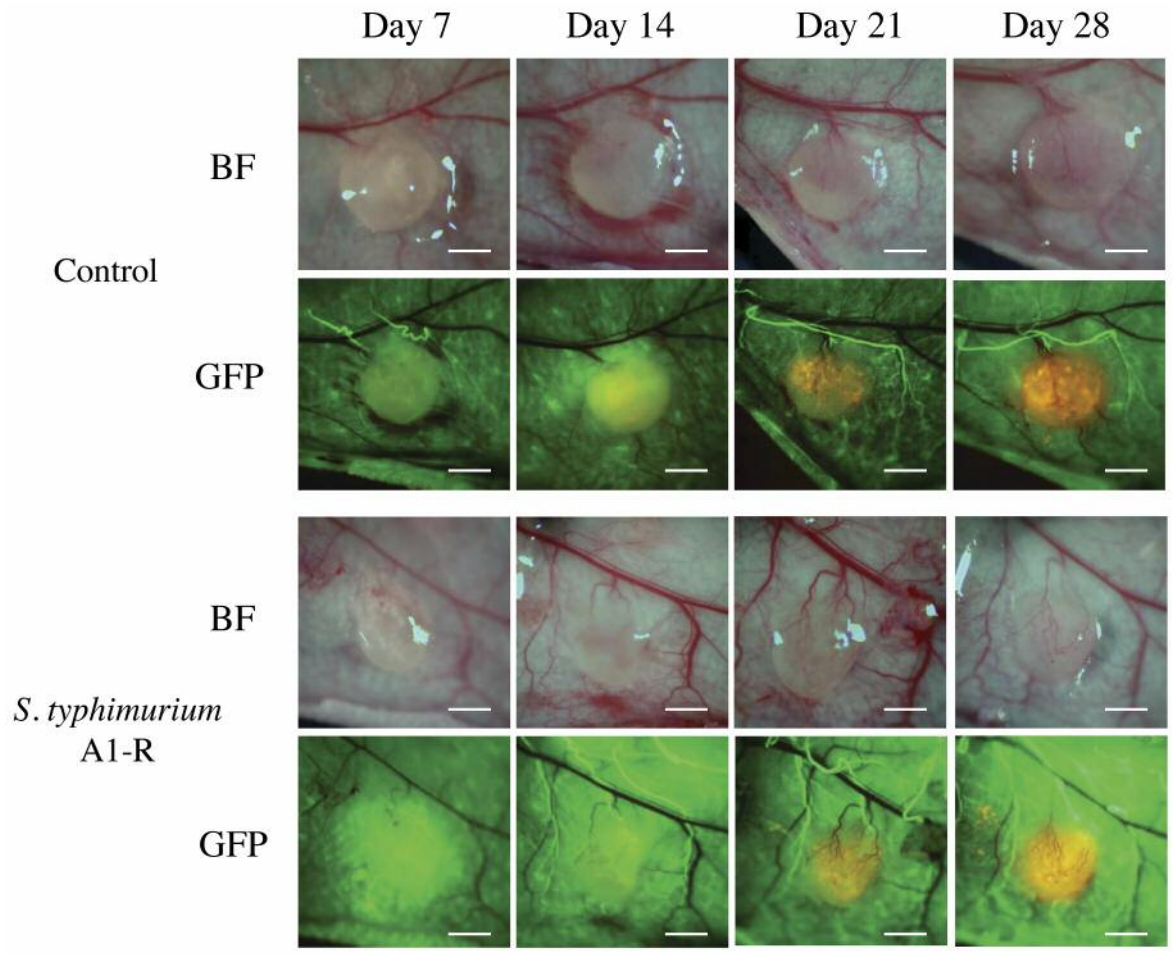

Figure 2. *Time course color-coded imaging of nestin-driven green fluorescent protein (ND-GFP)-expressing nascent blood vessels and 143B-red fluorescent protein (RFP)-expressing human osteosarcoma cells implanted in Gelfoam® in ND-GFP mice. Skin flaps were made and observed with the OV100 in bright field (BF) and with GFP excitation at day 7, 14, 21, and 28. 143B-red fluorescent protein (RFP)-expressing human osteosarcoma cells were implanted on day 7 after Gelfoam® implantation. Salmonella typhimurium A1-R was injected on days 19 and 26 in the experimental group as depicted in Figure 1. Bars=2 mm.*

insulin syringe (TYCO Health Group LP, Mansfield, MA, USA) into the Gelfoam® previously implanted in ND-GFP transgenic nude mice.

Imaging. The Olympus OV100 Small-Animal Imaging System, including an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 charge-coupled device camera (Olympus), was used for imaging in live mice (34). High-resolution images were captured directly on a PC (Fujitsu Siemens, Tokyo, Japan). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell (Olympus Biosystems).

An FV 1000 laser scanning confocal microscope (Olympus) with a XLUMPLFLN 20×W (0.95 numerical aperture [NA]) water immersion objective was used for imaging (35). GFP was excited at 488 nm, and RFP was excited at 559 nm with an Argon laser. Images for vessel length were produced with FV10-ASW Fluoview software (Olympus, Tokyo, Japan) and were not modified beyond the standard adjustment of intensity levels.

Skin flaps were made at 7, 14, 21, and 28 days after Gelfoam® implantation, and the inside surface of the skin flap and Gelfoam® were directly imaged. The skin was closed with a 6-0 suture after each observation (32).

Treatment study design for osteosarcoma in Gelfoam® (Figure 1). All mice were assigned into groups based on GFP-expressing nascent vessel length on day 14 in order to standardize the initial length of each group before treatment. The treatment with *S. typhimurium* A1-R was performed intravenously on day 19, and day 26 for each group. The control mice were injected with phosphate-buffered saline (PBS). The experimental mice were injected with *S. typhimurium* A1-R (5×10^7 colony-forming units).

Measurement of nascent blood vessel length in Gelfoam®. The length of ND-GFP vessels was imaged with the Fluoview FV 1000 laser scanning confocal microscope and measured with FV10-ASW

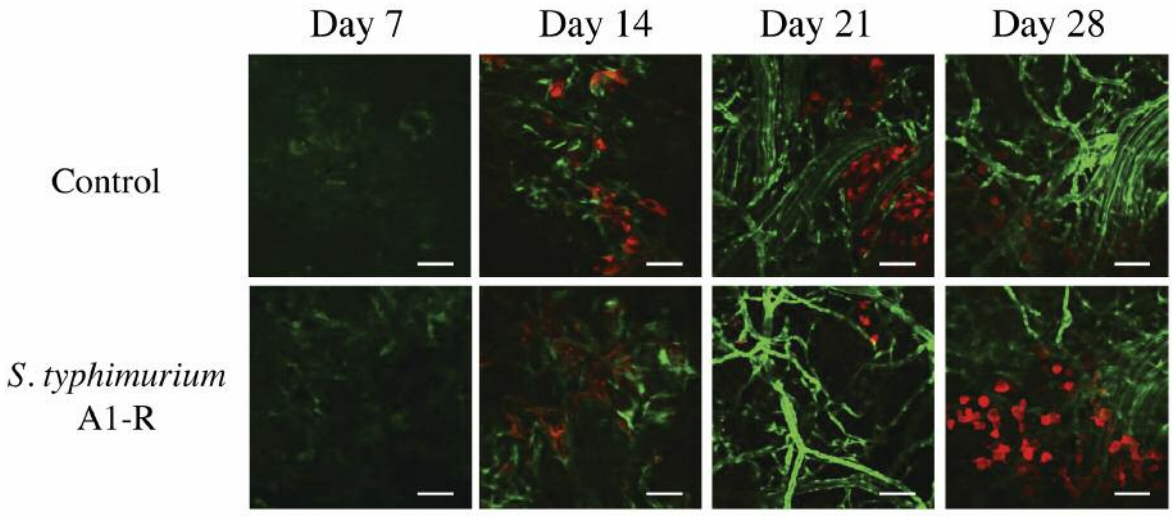

Figure 3. *Real-time confocal imaging of nestin-driven green fluorescent protein (ND-GFP)-expressing blood vessels and 143B-red fluorescent protein (RFP)-expressing osteosarcoma cells in Gelfoam® on mouse skin flap. Skin flaps were made and observed with an FV1000 confocal microscope. 143B-RFP human osteosarcoma cells were implanted at day 7 after Gelfoam® implantation. Salmonella typhimurium A1-R was administered on days 19 and 26 as depicted in Figure 1. Bars=100 μm.*

Fluoview software (Olympus). Three random fields in the tumor area expressing RFP were assessed in each group (32).

Measurement of tumor area in Gelfoam®. The RFP-expressing tumor growing in Gelfoam® of ND-GFP mice was imaged with the Olympus OV100 Small-Animal Imaging System. The fluorescent tumor area was measured with OV100-OV110 software (Olympus).

Statistical analysis. The experimental data are expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test. Values of $p<0.05$ were considered statistically significant.

Results

Real-time in vivo imaging of osteosarcoma formation in Gelfoam® implanted in ND-GFP transgenic nude mice. In order to obtain angiogenesis of Gelfoam® in the ND-GFP nude mouse *in vivo*, Gelfoam® was treated with βFGF, and then implanted into the subcutis on the flanks. Seven days after implantation of Gelfoam®, 143B-RFP human osteosarcoma cells (Figure 1) were injected in the implanted Gelfoam®. Skin flaps were made on day 7, 14, 21, and 28, and tumor angiogenesis was observed with the OV100 Small Animal Imaging System (Figure 2). Nascent blood vessels, expressing ND-GFP vascularized the implanted Gelfoam® in each group. The osteosarcoma cells expressing RFP formed tumors in the implanted Gelfoam® in a time-dependent manner (Figure 2).

S. typhimurium A1-R inhibited angiogenesis in Gelfoam®. Nascent blood vessels expressing ND-GFP vascularized the Gelfoam® in a time-dependent manner in each group (Figures 2, 3). *S. typhimurium* A1-R-treated mice had shorter vessels than those of the control group (Figure 4). The extent of nascent blood vessel growth was significantly inhibited by *S. typhimurium* A1-R treatment (on day 28, $p<0.05$). These results suggest that *S. typhimurium* A1-R was selectively inhibiting tumor blood vessel length in the Gelfoam® model.

The tumor size, which corresponds to RFP fluorescence of the Gelfoam® in both groups, is shown in Figure 5. There was no significant difference in the fluorescent tumor area between two groups.

Discussion

Angiogenesis is required for tumor growth (36) and has been an important therapeutic target for cancer with limited success (37). *S. typhimurium* A1-R previously showed promise to target angiogenesis (29). The present study used a color-coded *in vivo* imaging assay that was minimally invasive (31, 32) to demonstrate the selective anti-angiogenic effect of *S. typhimurium* A1-R. Future studies will combine *S. typhimurium* A1-R with other anti-angiogenesis drugs to obtain synergy. The simple *in vivo* color-coded assay will be useful to screen for such effective agents.

Conclusion

The results presented in this report suggest *S. typhimurium* A1-R has potential for anti-angiogenic targeted therapy for osteosarcoma.

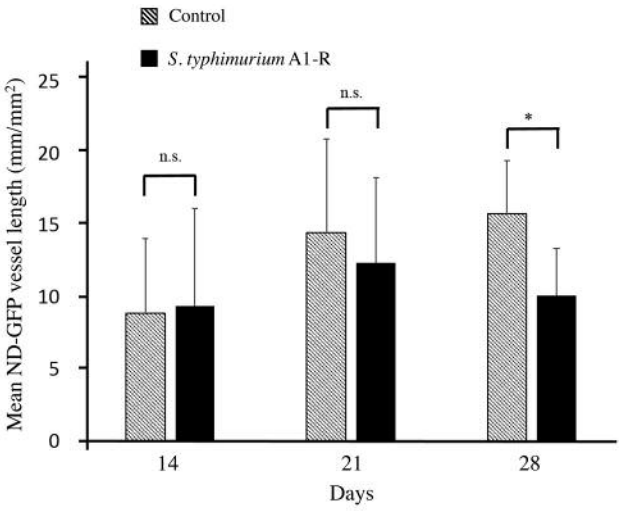

Figure 4. *Quantitative comparison of the mean length of nascent blood vessels expressing nestin-driven green fluorescent protein (ND-GFP) in Gelfoam® implanted with osteosarcoma cells. Vessel length in the Gelfoam® was imaged with the FV1000 confocal microscope and was measured with FV10-ASW Fluoview software (Olympus). Three random fields were measured for fluorescence length of the ND-GFP-expressing blood vessels. The length of nascent blood vessels expressing ND-GFP was suppressed by Salmonella typhimurium at day 28 ($p<0.05$). The experimental data are expressed as the mean ± SD. Statistical analysis was performed using Student's t-test. n.s.: Not significantly different.*

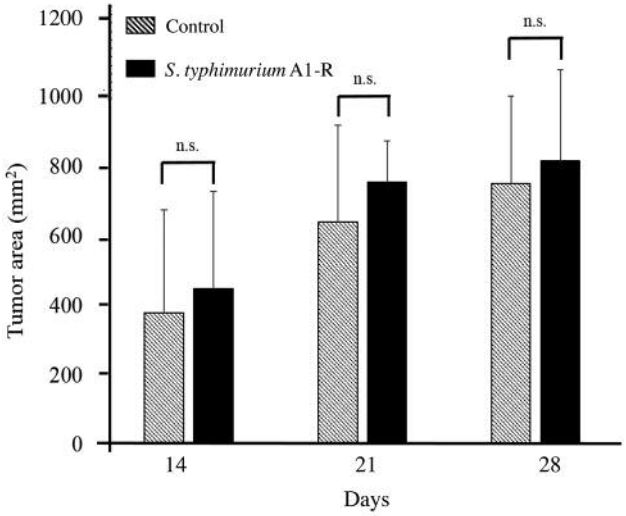

Figure 5. *Comparison of the mean area of the fluorescence area of 143B-red fluorescent protein (RFP)-expressing tumors in Gelfoam®. Tumor area in the Gelfoam® was imaged with the OV100 and was measured with OV100-OV110 software (Olympus). Three random fields expressing RFP were quantified in each group in a time-dependent manner. The experimental data are expressed as the mean ± SD. Statistical analysis was performed using Student's t-test.*

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