© The American Society of Gene & Cell Therapy

Engineering and Visualization of Bacteria for Targeting Infarcted Myocardium

Uyenchi N Le[1], Hyung-Seok Kim[2], Jin-Sook Kwon[3], Mi Yeon Kim[2], Vu H Nguyen[1], Sheng Nan Jiang[1], Byeong-Il Lee[1], Yeongjin Hong[4], Myung Geun Shin[5], Joon Haeng Rhee[4], Hee-Seung Bom[1], Youngkeun Ahn[2], Sanjiv S Gambhir[6], Hyon E Choy[4] and Jung-Joon Min[1,7]

[1]Department of Nuclear Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea; [2]Department of Forensic Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea; [3]Department of Cardiovascular Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea; [4]Department of Microbiology, Chonnam National University Medical School, Gwangju, Republic of Korea; [5]Department of Laboratory Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea; [6]Molecular Imaging Program at Stanford, Department of Radiology & Bioengineering, Bio-X Program, Stanford University, Stanford, California, USA; [7]Bioimaging Research Center, School of Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

Optimization of the specific affinity of cardiac delivery vector could significantly improve the efficiency of gene/protein delivery, yet no cardiac vectors to date have sufficient target specificity for myocardial infarction (MI). In this study, we explored bacterial tropism for infarcted myocardium based on our previous observations that certain bacteria are capable of targeting the hypoxic regions in solid tumors. Out of several *Escherichia coli* or *Salmonella typhimurium* strains, the *S. typhimurium* defective in the synthesis of ppGpp (ΔppGpp *S. typhimurium*) revealed accumulation and selective proliferation in the infarcted myocardium without spillover to noncardiac tissue. The *Salmonellae* that were engineered to express a variant of *Renilla* luciferase gene *(RLuc8)*, under the control of the *E. coli* arabinose operon promoter (P_{BAD}), selectively targeted and delivered RLuc8 in the infarcted myocardium only upon injection of L-arabinose. An examination of the infarct size before and after infection, and estimations of C-reactive protein (CRP) and procalcitonin indicated that intravenous injection of ΔppGpp *S. typhimurium* did not induce serious local or systemic immune reactions. This current proof-of-principle study demonstrates for the first time the capacity of *Salmonellae* to target infarcted myocardium and to serve as a vehicle for the selective delivery of therapeutic agents in MI.

Received 27 July 2010; accepted 26 January 2011; published online 1 March 2011. doi:10.1038/mt.2011.25

INTRODUCTION

Coronary artery disease is a leading cause of morbidity and mortality in the world.[1] The detailed investigation of the molecular pathways involved in the development and progression of the most refractory cardiac disease has led to the identification of numerous causative genes and proteins.[2] Thus, molecular therapy to modify the levels of these proteins through control of their production offers a potential to improve myocardial function in the patients with refractory angina who have failed conventional medical or surgical treatment.[3]

Gene-based treatments involve direct cardiac delivery of potent angiogenic factors using catheter or surgical technique, either in naked form or enclosed in nanoparticles, to stimulate new vessel growth in hypoxic regions.[4] Although the delivery technique *via* direct cardiac delivery may improve cardiac targeting, highly cardiotropic vector would be most desirable. Thus, optimizing the tropism of cardiac delivery vectors would help to improve gene/protein delivery and to reduce unwanted extracardiac transfection. However, no vector to date has sufficient specificity for the infarcted myocardium.[5]

In this study, we examined the feasibility of using avirulent bacteria as drug delivery vehicles with target specificity for the hypoxic/infarcted myocardium based on our previous observations that certain bacteria are capable of targeting the hypoxic regions in solid tumors.[6–9] Most notably, *Salmonella typhimurium* defective in the synthesis of Magic Spot, ppGpp (ΔppGpp *S. typhimurium*)[10] has been shown to exhibit strong tropism for the infarcted myocardium. This feature was exploited for targeted delivery of protein drugs to the infarcted myocardium. Precise triggering of expression could induce greater therapeutic effects of protein drugs while minimizing systemic toxicity.[11] Thus, the *Salmonella* was implemented to equip with an inducible gene expression system that was controlled in remote. Here, we demonstrated a controlled expression of a bioluminescent protein as a model by employing molecular imaging technique.

RESULTS

Selective localization of attenuated *S. typhimurium* in the infarcted myocardium

The Sprague–Dawley (SD) rats with myocardial infarction (MI) were injected with the bacteria listed in **Table 1** through tail-vein and localized in the animal on 1, 3, 5 days post inoculation

Correspondence: *Jung-Joon Min, Department of Nuclear Medicine, Chonnam National University Medical School, 5, Hak 1 dong, Dong-gu, Gwangju 501-746, Republic of Korea. E-mail: jjmin@jnu.ac.kr. Hyon E Choy, Department of Microbiology, Chonnam National University Medical School, 5, Hak 1 dong, Dong-gu, Gwangju 501-746, Republic of Korea. E-mail: hyonchoy@chonnam.ac.kr*

© The American Society of Gene & Cell Therapy

Table 1 Bacterial strains and tropism for myocardial infarction

Strains	Relevant genotype	MI targeting[a]	Injection dose (CFUs)[b]	Reference or source
E. coli, MG1655	Wild type	—	5×10^8	6,31
E. coli, CF1693	Δ*relA*, Δ*spoT* (ΔppGpp)	—	5×10^8	35
S. typhimurium (St) ATCC 14028s	Wild type	—	1×10^7	30
St, A1-R	leu/arg auxotroph	—	2×10^8	36
St, SHJ2165[a]	*aroA*$^-$	—	2×10^8	TT1452 (gifted by J Roth, U.C. Davis)
St, SHJ2176[c,d]	*cya*$^-$	±	1×10^8	This work
St, SHJ2168[c]	Δ*relA*, Δ*spoT* (ΔppGpp)	+++	2×10^8	9,10
St, SHJ2167[c]	*invF*$^-$	±	2×10^8	37
St, SHJ2170[c,d]	*ssrAB*$^-$	—	2×10^8	This work

Abbreviations: CFU, colony forming units; MI, myocardial infarction.
[a]Tropism for MI was evaluated by whole body bioluminescence imaging at 1, 3, and 5 days post inoculation using ***in vivo*** imaging system and enumeration of the bacterial count in the myocardium at 5 days post inoculation. ±, transient (<1 day); +++, sustained (≥9 days).
[b]Injection doses were the tolerable doses estimated based on published papers (sources). However, since no relevant study has been carried out using a rat model, the doses were determined empirically.
[c]The *Salmonellae* strains were derived from 14028s carrying *lux* by P22HT *int* transduction as described in reference.[33] The other strains were constructed by transformation with luminescence-expressing plasmid, pLux, described in reference.[6,7]
[d]These strains were constructed following the method developed by Datsenko and Wanner[38] using lambda Red system. Detail information is provided upon request.

(dpi) by whole body bioluminescent imaging. Wild type strain of *Escherichia coli* and *S. typhimurium* failed to accumulate in the infarcted myocardium (**Supplementary Figure S1**). Subsequently, several mutant *E. coli* and *S. typhimurium* known to be attenuated were constructed and tested (**Table 1**). Surprisingly, the *S. typhimurium* strain defective in ppGpp synthesis (ΔppGpp *S. typhimurium*) showed great accumulation in the infarcted myocardium. The ΔppGpp *S. typhimurium* strain lacks both the *relA* and *spoT* genes that encode ribosome-bound and cytosolic ppGpp synthetase, respectively.[12] The lethal dose, 50% (LD_{50}) of ΔppGpp *S. typhimurium* is 10^6-fold higher than that of the wild type parental strain.[13] For noninvasive visualization, we constructed a variant that expressed bacterial luciferase from the *lux* operon.[6,7,14,15] The *lux* operon encodes all of the proteins (*i.e.*, luciferase, substrate, and substrate regenerating enzymes) necessary to generate bioluminescence.[7] The engineered ΔppGpp *S. typhimurium* was injected intravenously (i.v.) into MI rats ($n = 5$) at a dose of 2×10^8 colony forming units (CFUs). Afterwards whole body bioluminescence imaging was performed at indicated date using *in vivo* imaging system. Bioluminescence was detected in the heart and spleen of MI rats at 1 dpi (**Figure 1a**, top panel). Three days after injection, bioluminescence persisted in the heart but was no longer detected in the spleen and liver. Serial monitoring of the rats revealed an initial increase in cardiac bioluminescence up to 5 dpi, followed by a progressive decline in signal intensity. This decline likely reflected a decrease in the number of bacteria in the heart, presumably due to host immune response. In sham operated control rats, bioluminescence was detected only in the spleen and liver at 1 and 3 dpi (**Figure 1a**, bottom panel), reflecting the reticuloendothelial nature of these organs.

To correlate the imaging data with bacterial load in the heart, spleen, and liver, we counted number of bacteria (CFUs) in the organs of MI rats ($n = 21$) (**Table 2**). Early after injection (12 hours), bacterial load was found primarily in the spleen and liver, most likely captured by phagocytic macrophages,[16] with a relatively small bacterial burden in the heart. After 24 hours, however, the number of CFUs in myocardial tissue increased

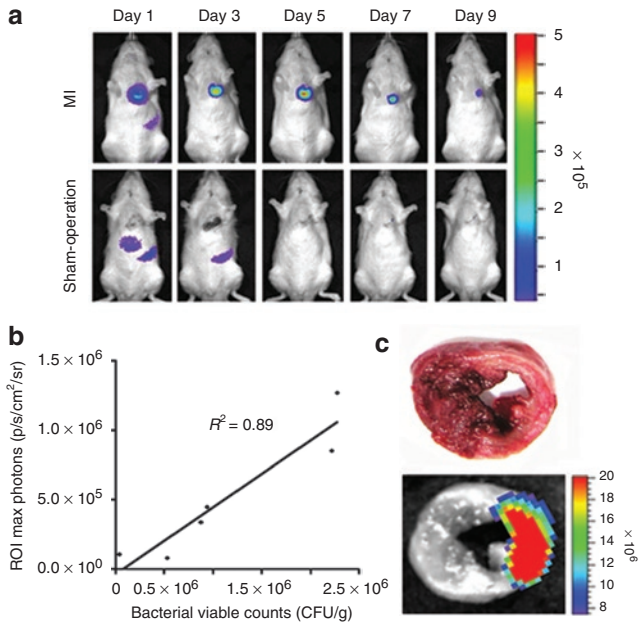

Figure 1 Molecular imaging of bacterial tropism for infarcted myocardium (MI). (**a**) ΔppGpp *S. typhimurium* expressing *lux* (2×10^8 CFUs) was injected through the tail-vein into Sprague–Dawley rats with or without MI ($n = 5$, each group) 6 hours after surgery. (**b**) Direct comparison between photon flux and bacterial colony counts in MI ($n = 21$). A robust correlation exists between bacterial colony counts and bacterial bioluminescence signals ($R^2 = 0.89$). (**c**) Cross-sections of heart from MI rats at 5 days post inoculation of *Salmonellae* injection. Thin slice (1.5 mm) cross-sections were prepared and then subjected to triphenyltetrazolium chloride staining (top). Bioluminescence imaging was performed using a cooled charge-coupled device camera for 30 seconds (bottom). Note the dull white area of reduced dehydrogenase activity. CFU, colony forming units; MI, myocardial infarction; ROI, region of interest.

dramatically, reaching a maximum at 3 and 5 dpi of ~10^6 CFU/g, whereas the bacterial burden in the liver and spleen declined over the same period of time to undetectable levels. No bacteria was detected in the liver and spleen after 5 dpi, confirming the ability of ΔppGpp *S. typhimurium* to specifically target and proliferate in

© The American Society of Gene & Cell Therapy

Table 2 Quantification of ΔppGpp *S. typhimurium* in different organs following intravenous injection in rat myocardial infarction models (n = 21). Numbers represents the number of colony forming units in a gram of tissue (colony forming units/g tissue)

	12 hours	Day 1	Day 2	Day 3	Day 5	Day 7	Day 9
Heart	$4.15 \times 10^4 \pm 1.29$	$7.10 \times 10^5 \pm 1.33$	$1.49 \times 10^6 \pm 0.22$	$2.24 \times 10^6 \pm 0.68$	$2.57 \times 10^6 \pm 0.29$	$8.75 \times 10^5 \pm 1.54$	$5.29 \times 10^5 \pm 0.34$
Spleen	$1.43 \times 10^6 \pm 0.96$	$6.44 \times 10^5 \pm 1.63$	$3.56 \times 10^4 \pm 0.94$	$6.20 \times 10^3 \pm 4.5$	0	0	0
Liver	$2.10 \times 10^5 \pm 1.18$	$4.65 \times 10^5 \pm 0.70$	$2.12 \times 10^3 \pm 0.45$	0	0	0	0

the infarcted myocardium. As shown in **Figure 1b**, the intensity of bacterial bioluminescence from the heart (**Figure 1a**) correlated robustly with the bacterial count (R^2 = 0.89) in the MI region (**Table 2**). Analysis of the myocardial tissue sections from the MI rats revealed that bioluminescence was specifically located in the left ventricular wall (**Figure 1c**, bottom panel), and corresponds to the infarcted region revealed by triphenyltetrazolium chloride (TTC) staining [17] (**Figure 1c**, top panel).

The location of the bacteria within infarcted myocardium was determined by histological analysis (**Figure 2a–c**). The infarcted areas were delineated by desmin immunohistochemical staining[18], which revealed decreased immunoreactivity for desmin in the anterolateral and inferior wall of the left ventricular myocardium (**Figure 2a**). The infarcted area defined by desmin immunohistochemical staining closely correlated with that defined by TTC staining. The location of *Salmonellae* was evaluated at 5 dpi by staining the whole myocardial cross-sections with specific antibody and then analyzing the images (**Figure 2b**). The bacteria were found in the area of infarcted myocardium with decreased desmin immunoreactivity (area 2 and 3), but not in the contralateral normal myocardium (area 1) (**Figure 2c**). Thus, the results from bioluminescence imaging, bacterial enumeration, and histological analyses all strongly suggest that the i.v. administration of ΔppGpp *S. typhimurium* into MI rats leads to selective bacterial colonization at the infarcted myocardium.

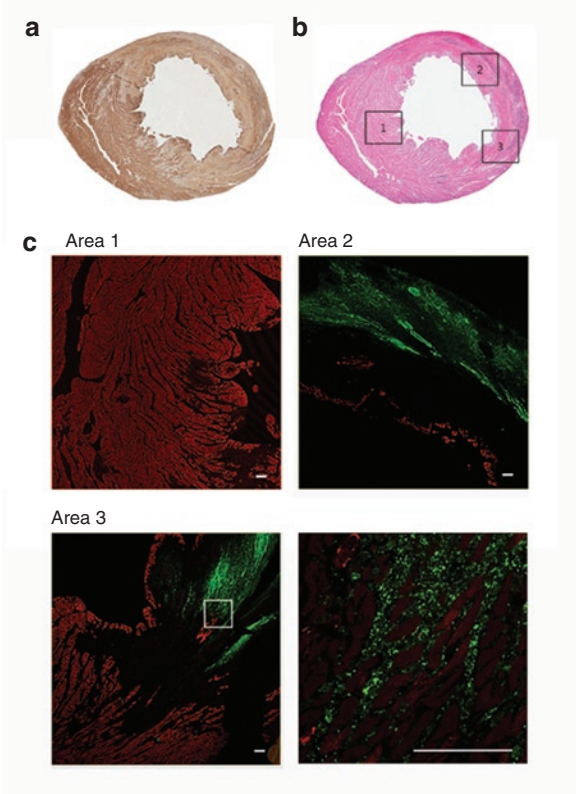

Figure 2 Microscopic features of bacterial tropism for infarcted myocardium. ΔppGpp *S. typhimurium* (2×10^8 CFUs) was injected through the tail-vein into Sprague–Dawley rats with MI (n = 5, each group). (**a**) Distinct delineation between healthy myocardium and infarcted myocardium from heart after immunohistochemical detection of desmin at 5 days post inoculation. Positive detection of desmin corresponds to the healthy region (brown), while negative detection corresponds to an infarcted lesion. (**b**) Hematoxylin-eosin (H&E) staining of a cardiac cross-section that corresponds to desmin immunoreactivity. (**c**) Immunofluorescence staining of indicated H&E stained areas. Sections were stained with antidesmin antibody (red) and anti-*Salmonella* antibody (green). Bottom right panel is a magnification of the boxed area in the bottom left panel (area 3). Bars = 100 µm. CFU, colony forming units; MI, myocardial infarction.

Remote control of protein expression and secretion *in vitro*

We explored the possibility of using ΔppGpp *S. typhimurium* as a vehicle for the targeted delivery of therapeutic proteins to the infarcted myocardium. A bacterial expression plasmid encoding a variant of *Renilla* luciferase (RLuc8)[19,20] was constructed, in which the *pelB* leader sequence[21] and a histidine tag (6× His)[22] were fused to the amino- and carboxy-terminus, respectively, of *RLuc8*. The *pelB* leader sequence consists of the first 22 codons of the gene for pectate lyase B from *Erwinia carotovora* and directs protein secretion into the bacterial periplasm before being cleaved to mature protein. To create an inducible system, *RLuc8* was placed under the control of the P_{BAD} promoter from the *E. coli* arabinose operon (**Figure 3a**), which is inactive except in the presence of the inducer L-arabinose.[9] ΔppGpp *S. typhimurium* was transformed with pBAD-pelB-RLuc8, and the induction of RLuc8 expression in cultured cells in the presence of L-arabinose was examined using a cooled charge-coupled device camera. Light intensity was found to be proportional to the concentration of L-arabinose (R^2 = 0.90), and no light was detected in the absence of inducer (**Figure 3b**). Western blot analysis revealed that a 36.9 kDa protein corresponding to RLuc8[19] was expressed only in the presence of L-arabinose (0.2%). No protein was identified in the absence of L-arabinose, which indicates that the regulation of P_{BAD} is tightly controlled by the inducer. The presence of RLuc8 in both the bacterial pellet and culture medium suggests that RLuc8 was selectively expressed and secreted upon addition of L-arabinose (**Figure 3c**).

Specific delivery of RLuc8 by ΔppGpp *S. typhimurium* in infarcted myocardium

Subsequently, we i.v. administered ΔppGpp *S. typhimurium* carrying pBAD-pelB-RLuc8 (2×10^8 CFUs) to MI rats (n = 6) at 6 hours after MI modeling. L-arabinose was administered on 3 dpi

© The American Society of Gene & Cell Therapy

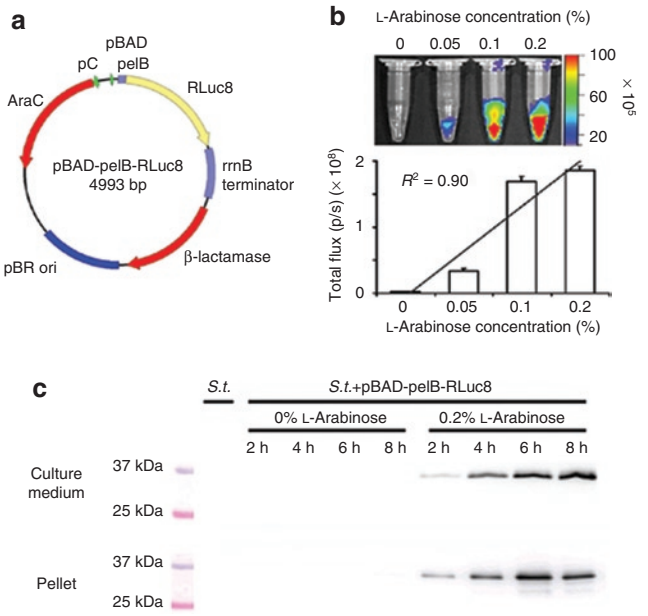

Figure 3 L-arabinose-induced expression and secretion of RLuc8 by attenuated *S. typhimurium*. (**a**) Map of the bacterial expression plasmid containing the *Renilla* luciferase variant (pBAD-pelB-RLuc8). (**b**) ΔppGpp *S. typhimurium* was transformed with pBAD-pelB-RLuc8 and then different concentrations (0–0.2%) of L-arabinose were added when the bacterial culture reached an OD_{600} of 0.5–0.7. After 4 hours incubation, coelenterazine (2 μg) was added to the cultures and bioluminescence was measured immediately using a cooled charge-coupled device camera (top panel). Bottom panel shows the quantification of total photon flux. (**c**) The expression and secretion of RLuc8 (36.9 kDa) was analyzed by western blot using an anti-RLuc antibody. Bacterial pellets (bottom) and culture medium (top) were collected at the indicated times from 2 to 8 hours after treatment with or without 0.2% L-arabinose. *S.t.*+pBAD-pelB-RLuc8 represents *S. typhimurium* (ΔppGpp) transformed with pBAD-pelB-RLuc8. *S.t.* represents ΔppGpp *S. typhimurium* carrying no plasmid (4 hours after fresh culture) as a negative control.

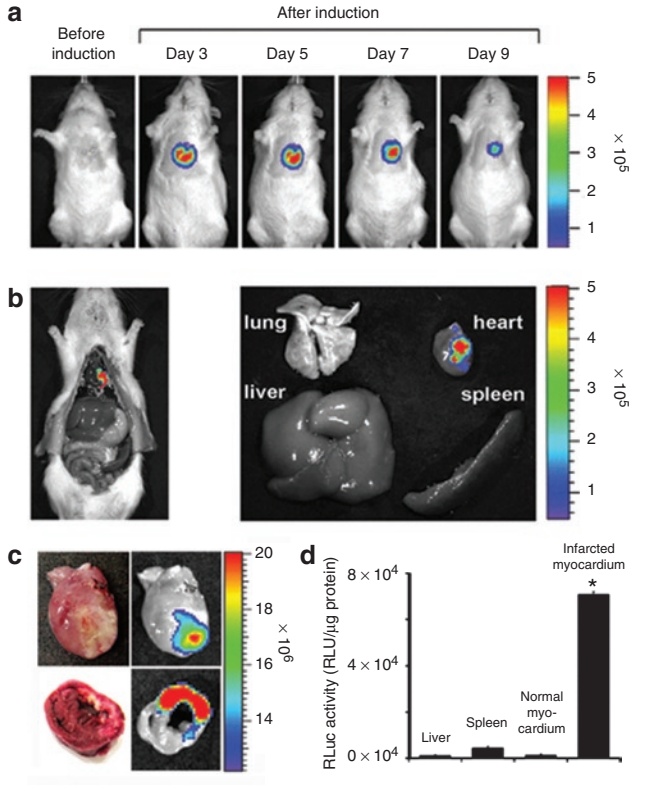

Figure 4 Specific expression of RLuc8 by L-arabinose in myocardial infarction. (**a**) A representative *in vivo* bioluminescence image after tail-vein injection of ΔppGpp *S. typhimurium* carrying pBAD-pelB-RLuc8 (2×10^8 CFUs) into MI rats ($n = 6$). Images were acquired at the indicated day after bacterial injection, before and after (4 hours) intraperitoneal injection of L-arabinose (1.2 g). The image before induction was obtained at 3 days post inoculation (dpi) immediately before administration of L-arabinose. All images were acquired immediately after intravenous injection of coelenterazine (0.7 mg/kg body weight). (**b**) Bioluminescence images of gross necropsy (left) and of the indicated isolated organs (right) from a representative animal (**a**) at 5 dpi. (**c**) Hearts from MI rats ($n = 6$) were excised at 5 dpi and then subjected to triphenyltetrazolium chloride staining (left) and bioluminescence imaging (right). Representative images of the whole extracted heart (top) and cross-sections (bottom) are shown. (**d**) Bioluminescence in infarcted myocardium, noninfarcted myocardium, liver, and spleen was measured using a luminometer at 5 dpi ($n = 6$). The Y-axis indicates relative light units (RLU). Luciferase activity was normalized to total protein concentration in tissue homogenates. *$P < 0.001$. CFU, colony forming units; MI, myocardial infarction.

to induce RLuc8 expression. This induction protocol was based on previous results showing that the number of *Salmonellae* in both liver and spleen declined significantly while reaching a maximum in the infarcted myocardium from 3 dpi (**Figure 1** and **Table 2**). The expression of RLuc8 was monitored using a cooled charge-coupled device camera after daily administration of L-arabinose and the luciferase substrate coelenterazine. Bioluminescence was detected throughout the length of the experimental period (**Figure 4a**) peaking at 5 dpi and declining thereafter, most likely due to the decreased bacterial cell number (**Table 2**). Notably, light signal from the cardiac region was observed only after the administration of L-arabinose, and no bioluminescence was detected in the spleen or any other organ. These results were verified by imaging of gross necropsy and of isolated organs at 5 dpi, confirming that *Salmonellae* had been cleared from organs other than the heart (**Figure 4b**). Examination of the excised hearts at 5 dpi revealed strong bioluminescence in the anterolateral wall of the myocardium, within the infarcted region as delineated by TTC staining (**Figure 4c**). Quantitation of RLuc8 activity in various tissues showed that luciferase activity was significantly higher in the infarcted myocardium than in the contralateral normal myocardium (>50-fold higher, $P < 0.001$) or in any other organ ($P < 0.001$, **Figure 4d**).

We also examined the selective expression of RLuc8 in the infarcted myocardium by immunofluorescence staining. RLuc8 was identified in the infarcted myocardium where bacteria had colonized (**Figure 5a**). Taking these histological findings together with the results presented in **Figure 2**, it was concluded that systemically injected engineered ΔppGpp *S. typhimurium* could specifically target and successfully deliver cargo molecules to the infarcted myocardium. The presence of RLuc8 in the infarcted tissue was further verified by bioluminescence imaging of tissue homogenates, which revealed that light was produced only from infarcted myocardium but not the contralateral normal myocardium (**Figure 5b**). Similarly, western blot analysis revealed the presence of a 36.9 kDa RLuc8 protein specifically in the infarcted myocardium (**Figure 5c**). Taken together, these results suggested

© The American Society of Gene & Cell Therapy

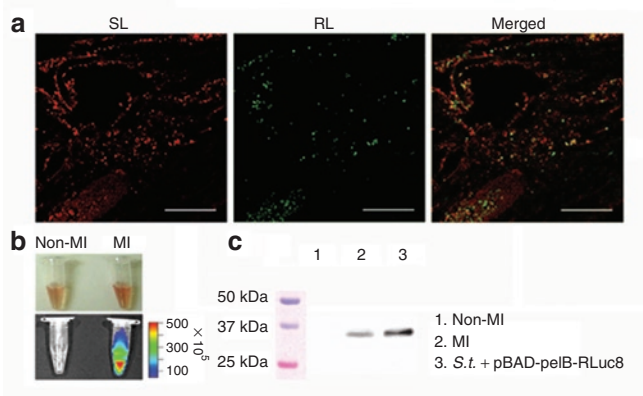

Figure 5 Specific expression and secretion of RLuc8 from attenuated *S. typhimurium* in MI. Infarcted hearts were excised at 5 days post inoculation of ΔppGpp *S. typhimurium* carrying pBAD-pelB-RLuc8 (2×10^8 CFUs) and 4 hours after L-arabinose induction. (**a**) Immunofluorescence staining of *S. typhimurium* (red, SL) and RLuc (green, RL) demonstrated bacterial expression and secretion of RLuc8 respectively. The merged image of SL and RL immunofluorescence is shown (Merged). Bar = 50 µm. (**b**) Bioluminescence in homogenates of infarcted myocardium (MI) and the contralateral normal myocardium (Non-MI) was determined using a cooled charge-coupled device camera after the addition of 2 µg of coelenterazine (top, bright field image; bottom, bioluminescence image). (**c**) The expression of RLuc8 (36.9 kDa) by the *Salmonellae* in the infarcted myocardium (lane 2, MI) was analyzed by western blot using an anti-RLuc antibody. The contralateral normal myocardium (Non-MI) was included as a negative control (lane 1). ΔppGpp *S. typhimurium* expressing RLuc8 (*S.t.*+pBAD-pelB-RLuc8) in the presence of 0.2% L-arabinose in culture medium was included as a positive control (lane 3). CFU, colony forming units.

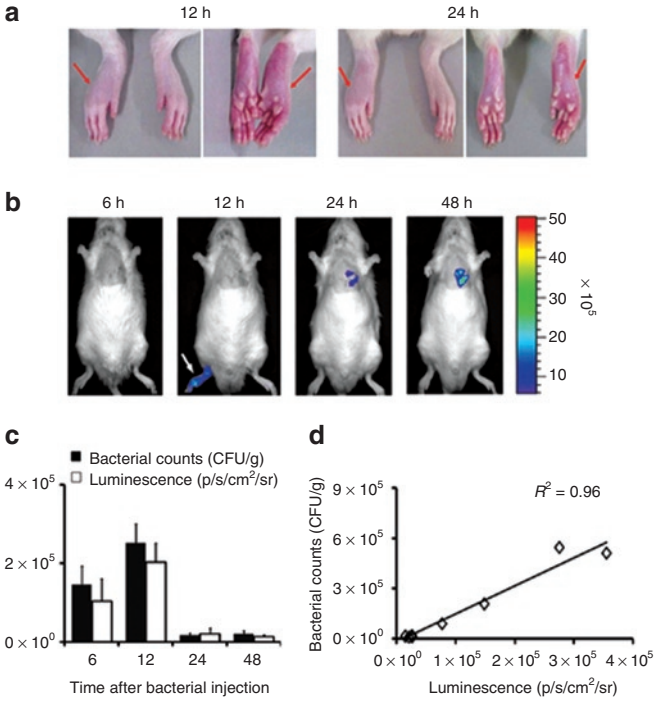

Figure 6 Bacterial tropism for inflammation and infarcted myocardia. An inflammatory reaction was induced by subcutaneous injection of 100 µl of complete Freund's adjuvant (10 mg/ml) in the right hind paw (arrows) of Sprague–Dawley rats ($n = 11$). After 4 days, the animals were subjected to surgical occlusion of the left anterior descending artery. After 6 hours, ΔppGpp *S. typhimurium* expressing *lux* (2×10^8 CFUs) was intravenous injected. Maximum photon intensity was recorded (p/s/cm²/sr). (**a**) Bright field images of inflammation in the right hind paw (arrows). Images correspond to the bioluminescence images of 12 and 24 hours, respectively. (**b**) Bioluminescence images of a representative animal. Images were acquired 6, 12, 24, and 48 hours post inoculation ($n = 3$). (**c**) Direct comparison of the bacterial number and intensity of bioluminescence signal of the inflamed right hind paw ($n = 8$). (**d**) Correlation graph between the bacterial number and intensity of bioluminescence signal of the inflamed right hind paw ($n = 8$). CFU, colony forming units.

a feasibility of engineered bacteria to selectively express a gene of interest in the infarcted tissue.

Bacterial tropism for inflammation and myocardial infarction

To test bacterial tropism toward inflammatory tissue, bioluminescent ΔppGpp *S. typhimurium* (2×10^8 CFUs) was i.v. injected into MI rats in which an inflammatory reaction was induced in the right hind paw by localized injection of complete Freund's adjuvant (CFA) (**Figure 6a**). By using whole body imaging, we observed a similar targeting of the *Salmonellae* to the site of inflammation; however, it was not sustained for >24 hours (**Figure 6b**). The bioluminescent signal from MI was clearly visible at 48 hours. To obtain more detailed information on bacterial accumulation in the inflammatory lesion, we counted the number of bacteria (CFUs) in the right hind paw at different time points and compared it with the intensity of the bioluminescence signal. The two measurements correlated strongly with each other (**Figure 6c,d**). The inflammatory environment may contribute to the targeting by ΔppGpp *S. typhimurium*, although the colonization at the local inflammatory site is only transient.

Toxicity of bacterial infection

The therapeutic administration of live bacteria often raises concerns of potential toxicity. We sought to characterize the acute and short-term toxicity of attenuated ΔppGpp *S. typhimurium* following i.v. injection into rats ($n = 5$ for each group) by monitoring the levels of plasma C-reactive protein (CRP) and procalcitonin (PCT) (**Figure 7a** and **Supplementary Figure S2**). CRP is an acute phase protein that is elevated in plasma and serum as a result of injury, infection, or disease.[23] PCT is a sensitive and specific marker for bacterial infection, particularly sepsis.[24] The plasma levels of CRP in sham operated and MI rats were 95.2 ± 20.7 ng/ml and 115.7 ± 28.5 ng/ml, respectively ($P = 0.035$), which indicated that local inflammatory reactions were induced following surgical induction of MI in rats. However, there were no significant differences in plasma CRP levels after i.v. injection of ΔppGpp *S. typhimurium* (2×10^8 CFUs) into MI or sham rats ($P = 0.358$ for MI rats, $P = 0.12$ for sham rats, **Figure 7a**). Plasma PCT was also undetectable after administration of ΔppGpp *S. typhimurium* (**Supplementary Figure S2**; a positive test band indicates a plasma PCT level above 0.5 ng/ml). In addition, we examined whether or not bacterial localization in myocardium would give rise to further myocardial damage. The size of infarcted area as assessed by TTC staining was compared in MI rats with (ΔppGpp *S. typhimurium*) or without (phosphate-buffered saline (PBS)) bacterial infection (**Figure 7b**). There were no significant differences in the infarct size between two groups as determined at 1 ($P = 0.540$), 3 ($P = 0.189$), and 7 dpi

© The American Society of Gene & Cell Therapy

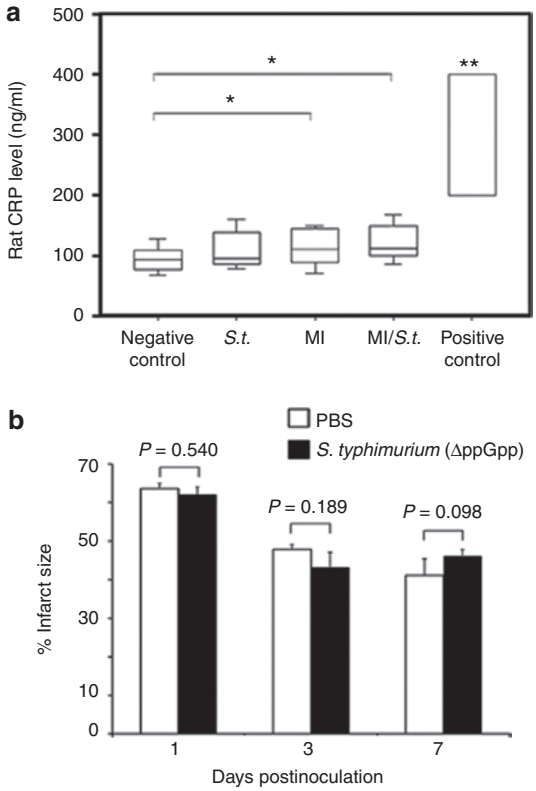

Figure 7 Systemic and local toxicity associated with ΔppGpp *S. typhimurium* injection in rats. (**a**) The levels of C-reactive protein (CRP) after intravenous injection of ΔppGpp *S. typhimurium.* Plasma CRP levels in sham operated and MI rats ($n = 3$ each) before and after the injection of *Salmonellae* (2×10^8 CFUs) were measured at 1 and 5 days post inoculation (dpi). The results are expressed as averages of CRP levels at 1 and 5 dpi. Rats ($n = 3$) were intravenous injected with lipopolysaccharide (LPS, 5 mg/kg for 3 minutes) as a positive control, and blood was drawn 4 hours after injection. Negative control indicates sham operated rats; *S.t.*, sham operated rats with bacterial injection; MI, MI rats without bacterial injection; MI/*S.t.*, MI rats with bacterial injection; positive control, LPS injected rats. Boxes represent the quartiles and whiskers mark the 10th and 90th percentiles. *$P = 0.035$; **$P < 0.01$. (**b**) Change of infarct size associated with ΔppGpp *S. typhimurium* injection in MI rats. Sprague–Dawley rats ($n = 30$) with MI were intravenously injected with PBS or ΔppGpp *S. typhimurium* (2×10^8 CFUs). At 1, 3, or 7 dpi ($n = 5$ for each group), rats were sacrificed and hearts were excised for triphenyltetrazolium chloride staining. The infarct size was measured using image processing software. MI, myocardial infarction; PBS, phosphate-buffered saline.

($P = 0.098$). These results strongly suggest that i.v. administration of attenuated ΔppGpp *S. typhimurium* does not lead to serious local or systemic inflammatory reactions.

DISCUSSION

In this study, we demonstrated for the first time that attenuated ΔppGpp *S. typhimurium* exhibits a specific tropism for infarcted myocardial tissue and can be engineered to secrete a target protein into the infarcted myocardium. This novel finding suggests that the *Salmonellae* can be exploited as a vehicle for the delivery of therapeutic proteins in MI patients. The engineered *S. typhimurium* used in this study exhibited several useful features: (i) an intrinsic tropism for infarcted myocardium, as demonstrated by bioluminescence imaging of reporter gene expression (**Figures 1** and **2**); (ii) inducible gene expression and secretion of proteins into the infarcted myocardium (**Figures 3–5**); (iii) confined gene expression in infarcted myocardium without spillover to noncardiac tissue (**Figures 1a** and **4a** and **Table 2**).

We showed here that i.v. injected bacteria gained entry and replicated only in the infarcted myocardium, that was followed in real time in live animals through bioluminescence imaging. Due to rapid bacterial replication, light emission originating from the bacteria within infarcted myocardium became easily detectable *in vivo*. No vectors have exhibited specificity for infarcted myocardium through i.v. injection, and therefore inadvertent transfection of nontarget organs is inevitable.[5] Although systemic injection *via* peripheral or central venous system remains, in general, a cornerstone of medical therapy, a trouble remains such as extensive first-pass clearance by the liver, lungs, spleen, and kidney, which would limit systemic injection of drug delivery vector for tissue-specific targeted delivery. Studies have shown poor cardiac uptake of vector i.v. injected, which is not surprisingly, given that only 4–5% of cardiac output passes through the coronary arteries.[25] Nonspecific targeting of drug delivery vector is another potential issue, and therefore, increased exposure to nontargeted organ is problematic. In the context of targeted gene or protein therapy, this can be obviated in some degree with regulating gene expression using cardiac specific promoters[26] and by employing viral vectors that express relative cardiac tropism.[27,28] Nevertheless, most obvious trouble associated with i.v. delivery of such agents is compromised by marked (conspicuous) dilution in the total blood volume.[29] A variety of catheter- or surgical-based techniques also have been developed to directly target the myocardium, but systemic spread of the vector can occur through the bloodstream or the lymphatic system (washout), most often to the liver or spleen.[5] All these problems would be solved by employing ΔppGpp *S. typhimurium,* that specifically localized to regions of MI, and did not leak out from the original site for at least 9 days, and actively proliferated in MI (**Figure 1a** and **Table 2**).

No sign of serious local or systemic inflammatory reactions was noted following i.v. administration of ΔppGpp *S. typhimurium*: the levels of CRP and PCT were not significantly changed (**Figure 7a** and **Supplementary Figure S2**). In most, though not all, diseases, the value of circulating CRP reflects ongoing inflammation and/or tissue damage much more accurately than other laboratory parameters of the acute-phase response.[23] We found that the plasma levels of CRP increased after surgical induction of MI, but did not show a further increase after the bacterial injection. It was speculated that bacterial colonization in the infarcted myocardium induced a minute, localized inflammation that led to the reduction of bacterial number in the infarcted myocardium.

Furthermore, antibiotic treatment (ciprofloxacin) resulted in a complete eradication of the *Salmonellae* residing at the MI site within 2 days (**Supplementary Figure S3**), which means that we could easily terminate bacterial colonization in the infarcted tissue deliberately. These characteristics represent significant advantages of using ΔppGpp *S. typhimurium* as a vector for gene therapy, in that gene expression is confined to the targeted infarct tissue without significant local or systemic inflammatory effects. Furthermore, the ability to manipulate gene expression through

© The American Society of Gene & Cell Therapy

the use of an inducible promoter (P_{BAD}) enables a powerful system for deliberate gene expression *in vivo* (**Figures 3–5**).

The mechanism of bacterial targeting of infarct tissue is an intriguing question. It may be the case that excess infiltration of neutrophils associated with MI might be associated with the tropism of the ΔppGpp *S. typhimurium*. We observed a similar targeting of the *Salmonellae* to sites of inflammation created by localized injection of CFA, although it was not sustained for >24 hours (**Figure 6**). Thus, it could be plausible that inflammatory environment might attract the *Salmonellae*. Another possibility is that the infarct provides a favorable metabolic environment for bacteria to persist and proliferate, although the tropic element(s) has yet to be identified. Interestingly, to date, a strong bacterial targeting to the MI has been observed only with ΔppGpp *S. typhimurium*, and no other *S. typhimurium* strain including A1-R strain that is markedly attenuated in virulence and selected for increased tumor targeting capability[30] or *E. coli* MG1655-strain[31] (**Table 1** and **Supplementary Figure S1**). Thus, mere attenuation of virulence or inflammatory environment of MI would not be responsible for the specific MI targeting by ΔppGpp *S. typhimurium*. The identification of inflammatory cells accumulated in hypoxic sites and also of secreted cytokines in infarcted myocardium may provide clues to the mechanism underlying MI targeting by ΔppGpp *Salmonella*. Whatever the mechanism, the MI targeting is different from the tumor targeting, the latter is due largely to immune privileged microenvironment of tumor, which would provide protection of residing microbes against the host immune system.[14] Thus, at the moment, we can only speculate that altered gene expression in ΔppGpp *S. typhimurium*[32] might be responsible for the MI targeting. Understanding the mechanism by which ΔppGpp *S. typhimurium* targets the infarcted myocardium will require extensive analysis that is beyond the scope of the present work, although nature of which can be exploited to other gene therapy or targeting strategies, if revealed. Overall, the current proof-of-principle study highlights the potential for ΔppGpp *S. typhimurium* to serve as a targeted gene delivery vehicle for the treatment of MI. Further studies are necessary for the translation of this application to other species including humans.

The current study focused on bacterial delivery of a reporter gene *RLuc8*. However, the strategy illustrated here could be applied to any number of therapeutic genes including angiogenic growth factors. Given that the bioluminescence reaction requires oxygen, the detection of RLuc8 activity in MI rats suggests that the engineered bacteria were located in the peri-infarct zone, where oxygen is present. Thus, MI targeting bacteria can potentially deliver therapeutic proteins to salvageable myocardium.

In conclusion, we reported the bacterial strain (ΔppGpp *S. typhimurium*) with specific tropism for MI that may be able to open many new avenues for molecular imaging and therapy, including tissue-specific targeting with signal amplification based on bacterial proliferation, *in vivo* tissue-specific drug delivery, and the design of imageable therapeutic probes. This is a first such demonstration of taking advantage of specific host–microbe interaction for targeted detection and potential treatment of MI.

MATERIALS AND METHODS

Plasmids. The expression plasmid pBAD-pelB-RLuc8 has been previously described.[19]

Bacterial strains. ΔppGpp *S. typhimurium*, SHJ2037 (*relA::cat, spoT::kan*), has been previously described.[10] *Salmonellae* were grown in Luria–Bertani broth medium (Becton Dickinson, Franklin Lakes, NJ) with vigorous aeration at 37 °C. For the imaging of bioluminescence, the bacterial luciferase gene (*lux*) from *S. typhimurium*-Xen26 (Caliper Life Sciences, Hopkinson, MA) was transduced into strain SHJ2037 by P22HT int transduction (SHJ2168).[33] All bacterial strains explored for this study was listed in **Table 1**.

Animal surgery to induce myocardial infarction. Eight-week old, male SD rats (250–260 g; OrientBio, Kyunggi-do, Korea) underwent sham operation (open thoracotomy only) or left anterior descending artery ligation, as previously described.[34] Animal care, all experiments and euthanasia were performed in accordance with protocols approved by the Chonnam National University Animal Research Committee and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85–23, revised 1985). Six hours after surgery, MI rats were injected through the lateral tail-vein with a fresh culture of *Salmonellae* (2×10^8 CFUs) resuspended in 100 µl of 1× PBS, (Gibco/Invitrogen, Carlsbad, CA). Sham-operated rats were injected with bacteria at the same dose.

Optical bioluminescence imaging of RLuc8 expression. Bioluminescence imaging was performed using the *in vivo* imaging system 100 (Caliper Life Sciences). Before imaging, coelenterazine (Biotium, Hayward, CA) dissolved in methanol (stock solution of 2 mg/ml) was injected i.v. at a dose of 0.7 mg/kg body weight in a final volume of 200 µl. Imaging signals were quantified in units of maximum photons per second per centimeter square per steradian (p/s/cm^2/sr) within the region of interest as described.[6,7]

Validation of* in vivo *bioluminescence imaging with* ex vivo *enzyme assays. After whole body imaging, rat organs (spleen, liver, and heart) were extracted and homogenized in lysis buffer (4 ml/g; Promega, Madison, WI), and subjected to five cycles of freezing and thawing. After centrifugation at 18,500*g* for 30 minutes at 4 °C, the supernatants were assayed for RLuc activity. Briefly, 20 µl of sample were mixed with 100 µl of 1× RLuc assay reagent (Promega) in a 96-well plate, and luciferase activity was quantitated immediately using a luminometer (Microlumat Plus LB96V; Berthold Technologies, Badwildbad, Germany). Light intensity was normalized to protein concentration (as measured by the Bradford assay), and expressed as relative light units.

Enumeration of S. typhimurium in different organs. At specific times after bacterial injection, SD rats were euthanized and placed in 70% ethanol for 3 minutes. The heart, liver, and spleen were removed, and placed individually into sterile tubes containing PBS at 4 °C, and weighed. Samples were transferred to sterile homogenization tubes, homogenized, and returned to the original tubes for the preparation of serial dilutions with PBS. Agar plates containing kanamycin (50 µg/ml) were inoculated with the homogenate, and the plates were incubated overnight at 37 °C. Colonies were counted and bacterial load was expressed as CFU/g tissue.

Western blot analysis. Bacterial cell lysate and the culture medium were quantified using the Bradford protein assay. Total protein (40 µg) was analyzed by electrophoresis and blotted, as previously described.[10]

For the analysis of RLuc8 expression in myocardial tissue, homogenized tissue samples were standardized according to protein content, and 200 µg protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% linear gradient gels. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membrane was probed with a mouse anti-RLuc monoclonal antibody (1:5,000 dilution) (Chemicon, Temecula, CA), followed by horseradish peroxidase-conjugated goat anti-mouse IgG (immunoglobulin G) (1:80,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected using luminol reagent (Santa Cruz Biotechnology).

© The American Society of Gene & Cell Therapy

Histological delineation of ischemic lesion. TTC (Sigma, St Louis, MO) staining was performed as previously described.[17] Using image processing software (ImageJ, http://rsb.info.nih.gov/ij/), the size of infarcted area (nonstained, pale white region) was measured on both sides and averaged for each slice, and summed from all slices. The size of the infarct was expressed as the fraction of infarcted region relative to the total size of the myocardium.

Immunostaining for Salmonella and RLuc8 location. The infarcted heart was blocked in cross-section and processed for paraffin embedding. Representative sections were sliced into 4 μm-thickness sections and stained with hematoxylin-eosin. Adjacent sections were processed simultaneously for immunohistochemistry. Pretreatment of the tissues for heat-induced epitope retrieval was performed for 5 minutes in a 125 °C pressure cooker with 10 mmol/l citrate buffer, pH 6.0 for mouse antidesmin (1:100; Dako, Copenhagen, Denmark), rabbit anti-*Salmonella* (1:200; AbD Serotec, Oxford, UK), and mouse anti-RLuc antibodies (1:50; Millipore, Billerica, MA). And then, the slides were incubated with each primary antibody overnight at 4 °C. To detect the damaged myocardium using desmin antibody, streptavidin–horseradish peroxidase detection system was applied and visualized by chromogen reactions of the tissue sections that were initially treated with 0.02% diaminobenzidine.

To detect *S. typhimurium* overlying damaged myocardium, anti-*Salmonella* antibody and antidesmin antibody stained with AlexaFluor 488-conjugated chicken anti-rabbit antibody (Molecular Probes, Eugene, OR) and Cy3-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, Westgrove, PA) to emit green and red fluorescence, respectively. To detect the secretory RLuc protein, anti-*Salmonella* antibody and anti-RLuc antibody stained with AlexaFluor 568-conjugated goat anti-rabbit antibody (Molecular Probes) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) to emit red and green fluorescence, respectively. After a fluorescently labeled secondary antibody, sliced were mounted with 4′,6-diamidino-2-phenylindole/Antifade solution (Millipore). Antibody diluent (Dako) was applied as a negative control stain. Sections were analyzed using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss International, Göttingen, Germany).

Toxicity of attenuated S. typhimurium. Systemic or local inflammation and infection after administration of ΔppGpp *S. typhimurium* were determined by measuring the levels of CRP, using a rat CRP enzyme-linked immunosorbent assay kit (Life Diagnostics, West Chester, PA), and PCT (Brahms PCT-Q; B.R.A.H.M.S Diagnostica, Hennigsdorg, Germany) in rat plasma, according to manufacturers' protocols.

Inflammatory modeling. To characterize the accumulation of bacteria in inflammation, an inflammatory reaction was induced by subcutaneous injection of 100 μl CFA (inactivated *Mycobacterium tuberculosis*, 10 mg/ml, Sigma, St Louis, MO) in the right hind paw of SD rats (n = 6). The left hind paw was injected with physiologic saline as a negative control. After 4 days, the animals were subjected to surgical occlusion of the left anterior descending artery. After 6 hours, ΔppGpp *S. typhimurium* carrying pBAD-pelB-RLuc8 (2×10^8 CFUs) was i.v. injected into inflammatory animals. Images were acquired 4 hours after intraperitoneal injection of L-arabinose (1.2 g).

To quantify viable bacteria which maybe located at inflammatory lesions, other rats (n = 8) with inflamed paws but not infarcted hearts received ΔppGpp *S. typhimurium* carrying pBAD-pelB-RLuc8 (2×10^8 CFUs), simultaneously with 1.2 g/rat of L-arabinose. Bioluminescence imaging was acquired at 6, 12, 24, and 48 hours post bacterial administration, then at each time point, paw muscles (n = 2) were isolated, homogenized, and diluted with 1× PBS. Homogenates were spread onto Luria–Bertani agar plates supplemented with ampicillin (50 μg/ml). Overnight grown colonies were determined and normalized to gram of tissue.

Statistical analyses. Differences in one factor between two groups were determined using the two-sided Student's t-test or by Mann–Whitney U-test (for nonparametric data), and among more than two groups using analysis of variance (ANOVA) with a *post-hoc* test or by Kruskal–Wallis analysis of variance (for nonparametric data). A P value of <0.05 was considered statistically significant for all analyses. Data are expressed as means ± standard deviation (SD).

Additional method. Details of antibiotic treatment is available in the **Supplementary Materials and Methods.**

SUPPLEMENTARY MATERIAL

Figure S1. Tropism of different bacterial strains for infarcted myocardium.
Figure S2. Systemic toxicity associated with ΔppGpp *S. typhimurium* injection in rats.
Figure S3. Clearance of bacterial infection by antibiotics.
Materials and Methods.

ACKNOWLEDGMENTS

This research was supported by the National Research Foundation of Korea (NRF) (No. 2010-0028750) and by Bio R&D program (2010-0020658) funded by the Ministry of Education, Science and Technology, and in part by the Bioimaging Research Center at GIST. Y.H. was supported by the Pioneer Research Center Program "Bacteriobot" through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (No. 2010-0002241). J.H.R. was supported by grant No. RTI05-01-01 from the Regional Technology Innovation Program of the Ministry of Knowledge Economy (MKE). H.E.C. was supported by MOST through KOSEF (No. 2010-0020539). Y.A. was supported by the Stem Cell Research Program of MOST, Republic of Korea (M10641450001-06N4145-0011). H.-S. B. was supported by KOSEF grant funded by MOST, Republic of Korea, through its National Nuclear Technology Program (M20702010003-07N0201-00300).

REFERENCES

1. Lloyd-Jones, D, Adams, R, Carnethon, M, De Simone, G, Ferguson, TB, Flegal, K *et al.* (2009). Heart disease and stroke statistics–2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **119**: 480–486.
2. Ylä-Herttuala, S and Alitalo, K (2003). Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* **9**: 694–701.
3. Kim, MC, Kini, A and Sharma, SK (2002). Refractory angina pectoris: mechanism and therapeutic options. *J Am Coll Cardiol* **39**: 923–934.
4. Müller, OJ, Katus, HA and Bekeredjian, R (2007). Targeting the heart with gene therapy-optimized gene delivery methods. *Cardiovasc Res* **73**: 453–462.
5. Lyon, AR, Sato, M, Hajjar, RJ, Samulski, RJ and Harding, SE (2008). Gene therapy: targeting the myocardium. *Heart* **94**: 89–99.
6. Min, JJ, Kim, HJ, Park, JH, Moon, S, Jeong, JH, Hong, YJ *et al.* (2008). Noninvasive real-time imaging of tumors and metastases using tumor-targeting light-emitting *Escherichia coli*. *Mol Imaging Biol* **10**: 54–61.
7. Min, JJ, Nguyen, VH, Kim, HJ, Hong, Y and Choy, HE (2008). Quantitative bioluminescence imaging of tumor-targeting bacteria in living animals. *Nat Protoc* **3**: 629–636.
8. Jiang, SN, Phan, TX, Nam, TK, Nguyen, VH, Kim, HS, Bom, HS *et al.* (2010). Inhibition of tumor growth and metastasis by a combination of *Escherichia coli*-mediated cytolytic therapy and radiotherapy. *Mol Ther* **18**: 635–642.
9. Nguyen, VH, Kim, HS, Ha, JM, Hong, Y, Choy, HE and Min, JJ (2010). Genetically engineered *Salmonella typhimurium* as an imageable therapeutic probe for cancer. *Cancer Res* **70**: 18–23.
10. Song, M, Kim, HJ, Kim, EY, Shin, M, Lee, HC, Hong, Y *et al.* (2004). ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J Biol Chem* **279**: 34183–34190.
11. Forbes, NS (2010). Engineering the perfect (bacterial) cancer therapy. *Nat Rev Cancer* **10**: 785–794.
12. Cashel, M, Gentry, DR, Hernandez, VJ and Vinella, D (1996). The stringent response. In: Neidhardt, FC *et al.* (eds) *Escherichia Coli and Salmonella: Cellular and Molecular Biology*, vol. 1. American Society for Microbiology Press: Washington DC, pp 1458–1496.
13. Na, HS, Kim, HJ, Lee, HC, Hong, Y, Rhee, JH and Choy, HE (2006). Immune response induced by *Salmonella typhimurium* defective in ppGpp synthesis. *Vaccine* **24**: 2027–2034.
14. Yu, YA, Shabahang, S, Timiryasova, TM, Zhang, Q, Beltz, R, Gentschev, I *et al.* (2004). Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. *Nat Biotechnol* **22**: 313–320.
15. Yu, YA, Timiryasova, T, Zhang, Q, Beltz, R and Szalay, AA (2003). Optical imaging: bacteria, viruses, and mammalian cells encoding light-emitting proteins reveal the

© The American Society of Gene & Cell Therapy

locations of primary tumors and metastases in animals. *Anal Bioanal Chem* **377**: 964–972.
16. Aderem, A and Underhill, DM (1999). Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**: 593–623.
17. Acton, PD, Thomas, D and Zhou, R (2006). Quantitative imaging of myocardial infarct in rats with high resolution pinhole SPECT. *Int J Cardiovasc Imaging* **22**: 429–434.
18. Dogné, JM, Rolin, S, Pétein, M, Tchana-Sato, V, Ghuysen, A, Lambermont, B *et al.* (2005). Characterization of an original model of myocardial infarction provoked by coronary artery thrombosis induced by ferric chloride in pig. *Thromb Res* **116**: 431–442.
19. Loening, AM, Fenn, TD, Wu, AM and Gambhir, SS (2006). Consensus guided mutagenesis of *Renilla* luciferase yields enhanced stability and light output. *Protein Eng Des Sel* **19**: 391–400.
20. Loening, AM, Wu, AM and Gambhir, SS (2007). Red-shifted *Renilla* reniformis luciferase variants for imaging in living subjects. *Nat Methods* **4**: 641–643.
21. Lei, SP, Lin, HC, Wang, SS, Callaway, J and Wilcox, G (1987). Characterization of the *Erwinia carotovora* pelB gene and its product pectate lyase. *J Bacteriol* **169**: 4379–4383.
22. Arnau, J, Lauritzen, C, Petersen, GE and Pedersen, J (2006). Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expr Purif* **48**: 1–13.
23. Pepys, MB and Hirschfield, GM (2003). C-reactive protein: a critical update. *J Clin Invest* **111**: 1805–1812.
24. Christ-Crain, M, Jaccard-Stolz, D, Bingisser, R, Gencay, MM, Huber, PR, Tamm, M *et al.* (2004). Effect of procalcitonin-guided treatment on antibiotic use and outcome in lower respiratory tract infections: cluster-randomised, single-blinded intervention trial. *Lancet* **363**: 600–607.
25. Lazarous, DF, Shou, M, Stiber, JA, Dadhania, DM, Thirumurti, V, Hodge, E *et al.* (1997). Pharmacodynamics of basic fibroblast growth factor: route of administration determines myocardial and systemic distribution. *Cardiovasc Res* **36**: 78–85.
26. Chen, IY, Gheysens, O, Ray, S, Wang, Q, Padmanabhan, P, Paulmurugan, R *et al.* (2010). Indirect imaging of cardiac-specific transgene expression using a bidirectional two-step transcriptional amplification strategy. *Gene Ther* **17**: 827–838.
27. Pacak, CA, Mah, CS, Thattaliyath, BD, Conlon, TJ, Lewis, MA, Cloutier, DE *et al.* (2006). Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. *Circ Res* **99**: e3–e9.
28. Inagaki, K, Fuess, S, Storm, TA, Gibson, GA, Mctiernan, CF, Kay, MA *et al.* (2006). Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol Ther* **14**: 45–53.
29. Mariani, JA and Kaye, DM (2010). Delivery of gene and cellular therapies for heart disease. *J Cardiovasc Transl Res* **3**: 417–426.
30. Finlay, BB, Fry, J, Rock, EP and Falkow, S (1989). Passage of *Salmonella* through polarized epithelial cells: role of the host and bacterium. *J Cell Sci Suppl* **11**: 99–107.
31. Blattner, FR, Plunkett, G 3rd, Bloch, CA, Perna, NT, Burland, V, Riley, M *et al.* (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1462.
32. Song, M, Kim, HJ, Ryu, S, Yoon, H, Yun, J and Choy, HE (2010). ppGpp-mediated stationary phase induction of the genes encoded by horizontally acquired pathogenicity islands and cob/pdu locus in *Salmonella enterica* serovar *Typhimurium*. *J Microbiol* **48**: 89–95.
33. Davis, RW, Botstein, D and Roth, JR (1980). Advanced Bacterial Genetics: A Manual for Genetic Engineering. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
34. Samsamshariat, SA, Samsamshariat, ZA and Movahed, MR (2005). A novel method for safe and accurate left anterior descending coronary artery ligation for research in rats. *Cardiovasc Revasc Med* **6**: 121–123.
35. Xiao, H, Kalman, M, Ikehara, K, Zemel, S, Glaser, G and Cashel, M (1991). Residual guanosine 3′,5′-bispyrophosphate synthetic activity of relA null mutants can be eliminated by spoT null mutations. *J Biol Chem* **266**: 5980–5990.
36. Zhao, M, Yang, M, Ma, H, Li, X, Tan, X, Li, S *et al.* (2006). Targeted therapy with a *Salmonella typhimurium* leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice. *Cancer Res* **66**: 7647–7652.
37. Jones, BD and Falkow, S (1994). Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. *Infect Immun* **62**: 3745–3752.
38. Datsenko, KA and Wanner, BL (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**: 6640–6645.