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# Salmonella enhance chemosensitivity in tumor through connexin 43 upregulation

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**The use of preferentially replicating bacteria as oncolytic agents is one of the innovative approaches for the treatment of cancer. The capability of *Salmonella* to disperse within tumors and hence to delay tumor growth was augmented when combined with chemotherapy. This work is warranted to elucidate the underlying mechanism of antitumor effects by the combination therapy of *Salmonella* and cisplatin. The presence of functional gap junctions is highly relevant for the success of chemotherapy. Following *Salmonella* treatment, dose- and time-dependent upregulation of connexin 43 (Cx43) expressions were observed. Moreover, *Salmonella* significantly enhanced gap intercellular communication (GJIC), as revealed by the fluorescent dye scrape loading assay. To study the pathway underlying these *Salmonella*-induced effects, we found that *Salmonella* induced a significant increase in mitogen-activated protein kinases (MAPK) signaling pathways. The *Salmonella*-induced upregulation of Cx43 was prevented by treatment of cells with the phosphorylated p38 inhibitor, but not phosphorylated extracellular signal-regulated kinase (pERK) inhibitor or phosphorylated c-jun N terminal kinase (pJNK) inhibitor. Specific knockdown of Cx43 had an inhibitory effect on GJIC and resulted in a reduction of cell death after *Salmonella* and cisplatin treatment. Our results suggest that accumulation of *Salmonella* in tumor sites leads to increase Cx43 gap junction communication and enhances the combination of *Salmonella* and cisplatin therapeutic effects.**

## Introduction

The one limitations of cancer therapy is lack of selectivity of therapeutic agents to tumor cells. Current efforts are focused on discovering and developing anticancer agents that selectively target only tumor cells but spare normal cells to improve the therapeutic index. The use of preferentially replicating bacteria as oncolytic agents is one of the innovative approaches for the treatment of cancer. This is based on the observation that some obligate or facultative anaerobic bacteria are capable of multiplying selectively in tumors and inhibiting their growth. *Salmonella* have been employed as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth.[1–8]

**Key words:** gap junctions, connexin 43, *Salmonella*, cisplatin, tumor
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Gap junctions mediate cell communication by allowing the passage of molecules from one cell to another. The major role of gap junction intercellular communication (GJIC) is considered to be the maintenance of homeostasis in organisms.[9] Gap junctions are formed by two hemichannels, called connexons, each made of six connexin (Cx) proteins. Cx43 is ubiquitous and reduced in a variety of tumor cells.[10] Cx43 may influence the response of tumor cells to treatments by facilitating the passage of antitumor drugs or death signals between neighboring tumor cells.[11] Many tumor cells are characterized by dysfunction of Cx43. *Salmonella* can activate the expression of Cx43 in melanoma cells.[12] However, little is known about the relationship between *Salmonella* and Cx43 expression in molecular mechanisms. Previously, we found that the combination of *Salmonella* and cisplatin exerts additive therapeutic effects on delaying tumor growth and prolonging survival of the tumor-bearing mice.[13] In this study, we want to elucidate the underlying mechanism of antitumor effects by the combination therapy of *Salmonella* and cisplatin.

## Material and Methods

### Bacteria, cell lines, reagents and mice

A vaccine strain of *Salmonella enterica* serovar *choleraesuis* (*S.* Choleraesuis) (ATCC 15480) was obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). This rough variant of *S.* Choleraesuis (S.C.), designated vaccine 51, was obtained by spreading an 18-hr broth culture of

**What's new?**
Bacteria such as *Salmonella* have been studied as antitumor agents, and are known to activate the expression of the gap-junction protein Cx43 in tumor cells. *Salmonella* also enhances the effect of chemotherapy drugs, but the molecular mechanism of this additive effect has not been understood. In this study, the authors found that the bacteria not only cause increased intercellular communication between tumor cells due to Cx43 upregulation, but also increase mitogen-activated protein kinases (MAPK) signaling pathways. *Salmonella* presumably enhances the response to chemotherapy agents by increasing the passage of these drugs between neighboring tumor cells.

the virulent strain 188 of *S.* Choleraesuis strain Dublin over the surface of a dried nutrient agar plate and placing a drop of a suspension of salmonella anti-O phage no. 1, and selecting for a phage-resistant colony after incubation at 37°C for 24 hr.[14] Murine K1735 melanoma, murine melanoma B16F10,[14] murine breast cancer 4T1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/ml gentamicin, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% $CO_2$. Murine k1735 cells were kindly provided by Dr. MC Hung (The University of Texas M. D. Anderson Cancer Center). The working concentrations of the various inhibitors were as follows: 25 μM SB203580 (Sigma-Aldrich, St. Louis, MO), 20 μM PD98059 (Sigma), or 10 μM SP600125 (Sigma). Cells were pretreated with various inhibitors for 1 hr, then *Salmonella* (multiplicity of infection (MOI) = 100) was added to cells for 1 hr. Male C3H/HeN mice at the age of 6–8 weeks were purchased (National Laboratory Animal Center of Taiwan). The animals were maintained in specific pathogen-free animal care facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the China Medical University (the permit number: 99-20-N).

### Immunoblot analysis

The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). Proteins were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK) and probed with antibodies against Cx43 (Sigma-Aldrich), ERK (Abcam, Cambridge, UK), phosphor-ERK(Abcam), p38 (Abcam), phosphor-p38 (Abcam), JNK (Abcam), phosphor-JNK (Abcam) or monoclonal antibodies against β-actin (AC-15, Sigma Aldrich). Horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson, West Grove, PA) was used as the secondary antibody and protein-antibody complexes were visualized by enhanced chemiluminescence system (Amersham). The signals were quantified with ImageJ software (rsbweb.nih.gov/ij).

### Analysis of Cx43 transcriptional activity

Cells grown in 24-well plates were cotransfected with luciferase reporter plasmids driven by Cx43 promoters (0.66 μg) and pTCYLacZ (0.34 μg), a β-galactosidase (β-gal) expression plasmid driven by the β-actin promoter, by lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 6 hr post-transfection, cells were treated with *Salmonella* and cell lysates were harvested at time courses. The cell lysates were assessed for their luciferase activities determined by a dual-light luciferase and β-gal reporter gene assay system (Promega, Madison, WI) using a luminometer (Minilumate LB9506, Bad Wildbad). Relative luciferase activity was measured as luciferase activity divided by β-gal activity to normalize transfection efficiency per microgram protein. The protein content in each sample was determined by the BCA protein assay (Pierce).

### Knockdown of Cx43 and Cx43 overexpression

The specific siRNAi oligos of Cx 43 or negative control siRNA oligos was purchased from Invitrogene. The siRNA oligos of Cx43 designed to knockdown gene expression and the target sequences were listed below: Cx43 siRNA73 CCTGCTGATCCAGTGGTACATCTAT and Cx43 siRNA94 GCGTGAAGGGAAGAAGCGATCCTTA. Lipofectamine RNAiMax reagent (Invitrogene) was used for siRNA transfection following the manufacturer's protocol. To overexpress Cx43, cells were transfected with pcDNA-Cx43 by lipofectamine 2000 (Invitrogene). Clonal derivatives were isolated by G418 (400 μg/ml) selection and expanded to independent clones, creating Cx43#4 and Cx43#7cells. Cx43 plasmid was kindly provided by Dr. Shuan-Yow Li (Chung Shan Medical University, Taichung, Taiwan). The specific shRNA of Cx 43 plasmids were purchased from Santa Cruz Biotechnology, (sc-35091-SH, Santa Cruz Biotechnology, Santa Cruz, CA). The K1735 cells were transfected with Cx43 shRNA by lipofectamine 2000 (Invitrogene). Clonal derivatives were isolated by puromycin (2 μg/ml) selection and expanded to independent clones, creating stably silenced Cx43 cells. To inhibit MAPK signal pathway, The p38 dominant negative plasmids, JNK dominant negative plasmids and the ERK2 dominant negative plasmids were transfected with various plasmids by lipofectamine 2000 (Invitrogene). The p38 dominant negative mutant was provided by Dr. J. Han (University of Texas South-western Medical Center, Dallas, TX).[15] The JNK dominant negative mutant was provided by Dr. M. Karin (University of California, San Diego, CA).[16] The ERK2 dominant negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX).[17]

### Scrape loading and dye transfer analysis

Levels of gap junctional intercellular communication in control and treated cell in culture were determined scrape loading and dye transfer technique using a fluorescent dye, lucifer yellow (Sigma-Aldrich). Scrape loading was performed applying cuts on cell monolayer with a razor blade, and then 0.5% lucifer yellow was added to the cells. The dye was rinsed away after 5 min. Cells were washed with PBS, fixed with 4% paraformaldehyde and cells stained with lucifer yellow were detected by fluorescence microscope at magnification of ×200. The dye-spreading area was quantified by measuring the fluorescent area in three flied at the center of the scrape line using QCapture Pro 6.0 (QImaging, Surrey, BC, Canada).

### In vitro cell assay

*Salmonella*-treated or nontreated cells were exposed to 0–10 μg/ml of cisplatin under for 48 hr. Cell viability was determined with a colorimetric WST-8 (Dojindo Labs, Tokyo, Japan) assay and expressed as the mean of the percentage of surviving cells relative to that of the cells in the absence of cisplatin. To investigate the sensitivity of cells to cisplatin and their bystander effect, the dead *Salmonella*-treated K1735 cells were cultured with varying proportions of K1735-Luc cells in the presence of cisplatin (5 μg/ml) for 48 hr, and their luciferase activities were assessed as previously described.[18] For the generation of hypoxic condition, cells were cultured in 95% N2 and 5% CO2 (Anaerobic System PROOX model 110; BioSpherix) condition and incubated within the chamber for 24 hr.

### Immunofluorescence staining and Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay

Mice were inoculated with $10^6$ K1735 cells at day 0. Groups of K1735 tumor-bearing mice were injected intravenously (i.v.) with *Salmonella* at day 7 followed by cisplatin (2 mg/kg) treatment at days 15, 17 and 19, or with either treatment alone. Then, tumors were excised and snap frozen at day 20. Cryostat sections were prepared, fixed in 10% formalin, permeabilized with cold acetone, incubated with mouse anti-Cx43 (1:100; Invitrogen), and anti-p-p38 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 40 min and subsequently incubated with Texas Red-conjugated anti-rabbit IgG (1:100; KPL, Guildford, UK, USA) and fluorescein-conjugated anti-mouse IgG (1:100; KPL) and at room temperature for 1 hr. Nuclei were stained with 50 μg/ml of DAPI. The stained tissues were examined under fluorescence microscope at magnification of ×400. TUNEL assay was used to detect cell apoptosis within tumors and was performed according to the manufacturer's instructions (Promega, Madison, WI). Nuclei were stained with 50 μg/ml of DAPI. TUNEL-positive cells were counted under the microscope. We counted three high-power (×200) fields that showed highest density of positive-stained cells per field to determine the average percentage of apoptotic (TUNEL positive) cells in each section.[19]

### Statistical analysis

The unpaired, two-tailed Student's $t$ test was used to determine differences between groups. The survival analysis was performed using the Kaplan–Meier survival curve and log-rank test. Any $p$ value less than 0.05 is regarded statistically significant.

## Results

### Salmonella-induced Cx43 expression in tumor cells

Here, we used *Salmonella enterica* serovar *Choleraesuis* (*S.* Choleraesuis; S.C.) (ATCC 15480) vaccine strain. To examine the effect of *Salmonella* on Cx43 levels in melanoma cells (K1735), K1735 cells were incubated with different MOI of *Salmonella*, and then analyzed by immunoblot analyses. Treatment of K1735 cells with MOI 0.01, 1, or 100 of *Salmonella* caused a dose-dependent increase in Cx43 levels compared with the controls (Fig. 1*a*). We wanted to examine the expression of Cx43 by treatment with *Salmonella*. Treatment of tumor cells with MOI 100 of *Salmonella* significantly induced the expression of Cx43. Previously, we found that MOI 100 of *Salmonella* had the best infective potential.[14] The MOI 100 of *Salmonella* was therefore used in the subsequent analyses. In order to confirm the involvement and activation of transcriptional levels in *Salmonella*-induced Cx43 expression, K1735 cells were transduced plasmid carrying luciferase reporter gene under the control of Cx43 promoter .[20] After treatment with *Salmonella*, the luciferase activity was increased in dose-dependent manner (Fig. 1*b*). In a time-course study, *Salmonella* induced an increase in Cx43 levels at 15 min, which was sustained up to 48 hr (Fig. 1*c* and Supporting Information Fig. S1). Also, *Salmonella* induced a significant response in Cx43 promoter-derived luciferase reporter assay in time-dependent manner. (Fig. 1*d*). We then analyzed whether Cx43 was induced in tumors infected with *Salmonella in vivo*. Mice were inoculated subcutaneously (s.c.) with K1735 cells at day 0 followed by i.v. treatment with *Salmonella* ($2 \times 10^6$ colony formation unit (cfu)/mice) or PBS. Figure 1*e* reveals that *Salmonella* treatment induced the expression of Cx43 in tumor. To evaluate whether this phenomenon was specific of K1735 cells, the two additional murine tumor cells (4T1 and B16F10) showed behavior similar to that of K1735 cells in response to *Salmonella* (Fig. 1*f*). These results suggest that infection of tumor cells with *Salmonella* allowed the expression of Cx43 *in vitro* and *in vivo*.

### Salmonella increased gap junction intercellular communication

To determine the extent to which Cx43 expression was related to GJIC in K1735 cells, we performed scrape lading dye transfer assay using the gap junction permeable fluorescent dye lucifer yellow. The scrape loading/dye transfer assay

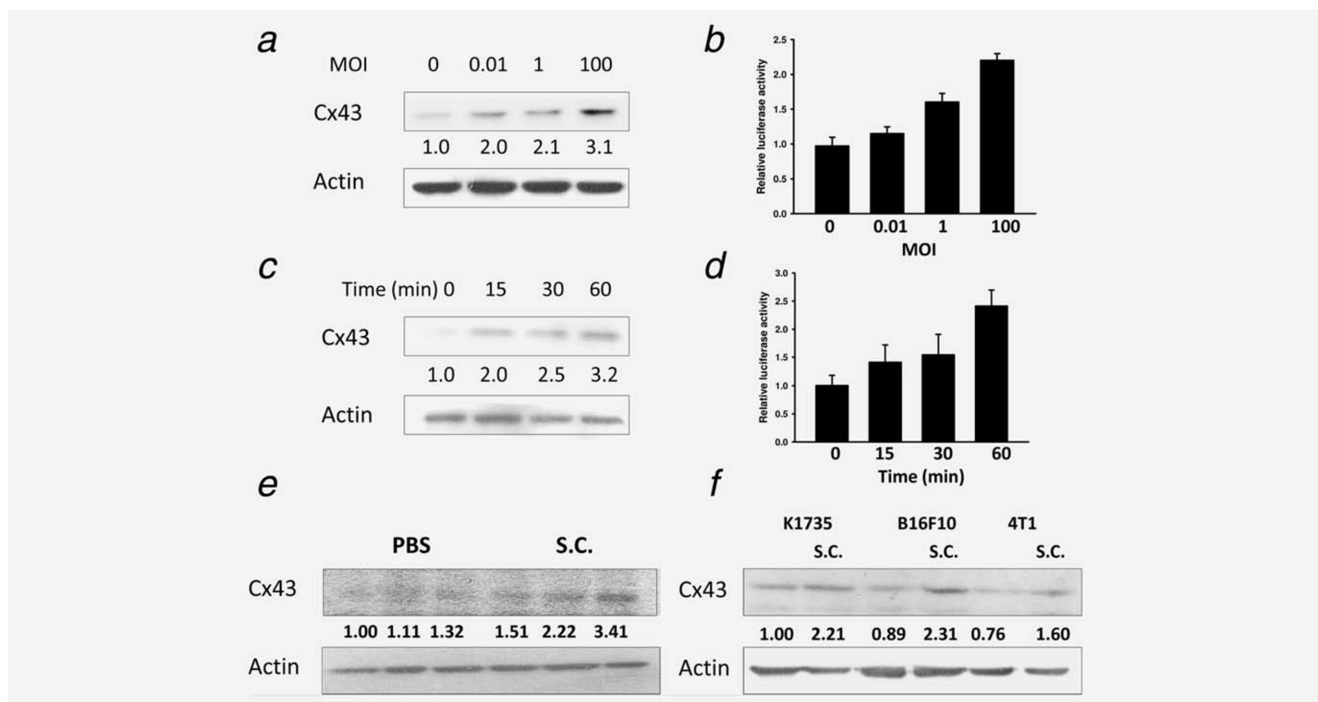


Figure 1. *Salmonella*–induced Cx43 protein expression. (*a*) *Salmonella* induced Cx43 protein expression in K1735 cells in a dose-dependent manner. After exposure to *Salmonella* (MOI: 0–100) for 1 hr, the expression of Cx43 levels in K1735 cells were determined by immunoblot analysis. (*b*) *Salmonella* dose dependently induced Cx43 transcriptional activity in tumor cells. The K1735 cells transfected with luciferase gene under the control of Cx43 promoter were treated with *Salmonella* (MOI: 0–100) for 1 hr. The transcriptional activity of Cx43was determined by the luciferase reporter assay and is expressed as the fold of the relative luciferase activity relative to that in the control tumor cells. (*c*) *Salmonella* induced Cx43 protein expression in K1735 cells in a time-dependent manner. After exposure to *Salmonella* (MOI: 100) at various time courses, the expression of Cx43 levels in K1735 cells were determined by immunoblot analysis. (*d*) *Salmonella* time-dependently induced Cx43 transcriptional activity in tumor cells. The K1735 cells transfected with luciferase gene under the control of Cx43 promoter were treated with *Salmonella* (MOI: 100) at various time courses. The transcriptional activity of Cx43was determined by the luciferase reporter assay and is expressed as the fold of the relative luciferase activity relative to that in the control tumor cells. (*e*) *Salmonella* (S.C.) induced Cx43 protein expression in tumors *in vivo*. K1735-established tumors were treated or not with *Salmonella*. Three days later, mice were killed, tumors were collected and the tumor lyses were analyzed for Cx43 expression by immunoblot analysis. (*f*) Various cells cultured in 6-well plates were infected with *Salmonella* (MOI = 100). Cx43 expression was detected by immunoblot analysis staining 1 hr later. Inserted values indicated relative proteins expression in comparison with β-actin.

for gap junction function showed an increased level of dye transport in K1735 cells, consistent with the presence of Cx43 in these cells. In *Salmonella* treated cells, considerable dye transfer was observed (Fig. 2*a*). Furthermore, our results show that degrees of GJIC in tumor cells were correlated with the expression of Cx43 induced by *Salmonella* (Fig. 2*b*). After incubation of *Salmonella*, a dose-dependent increasing effect of *Salmonella* on GJIC was observed.

### Salmonella activate the expression of Cx43 through p38 signaling pathway

We further investigated the potential signaling pathways in *Salmonella*-induced Cx43 expression in tumor cells. *Salmonella* significantly induced the phosphorlyation of extracellular signal-regulated kinase (ERK), c-jun N terminal kinase (JNK) and p38 (Fig. 3*a*). After treatment of the cells with inhibitors for ERK, p38 and JNK, the protein expression of p-ERK, p-p38 and p-JNK was markedly attenuated. However, *Salmonella*-induced Cx43 protein expression was blocked by inhibitor of p38. There was no significant effect of Cx43 after the inhibitors of ERK and JNK (Fig. 3*a*). The similar results were also observed in tumor cells after ERK, JNK and p38 dominant negative plasmids transduction (Fig. 3*b*). In Cx43 overexpressing cells, we found that their expressions of p-p38 were higher than control cells. In contrast, the expression of p-p38 was not significantly different between control cells and Cx43-silenced cells (Supporting Information Fig. S2*a*). The cytoplasmic-tail (C-tail) of Cx43 has been shown to interact with various protein and is essential to regulate cell growth and motility.[21] Previous report demonstrated that an augment effect of the C-tail of Cx43 could affect activation of p38 MAP kinase, which is involved in the control of cell migration.[22] In this study, we also found the p38 phosphorylation increased in K1735 cells overexpressing Cx43. Furthermore, the downregulated p38 in cells did not dramatically influence the cell growth after *Salmonella* infection (Supporting Information Fig. S2*b*). These results suggest that p38 signaling pathway might play a role in the *Salmonella*-induced expression of Cx43.

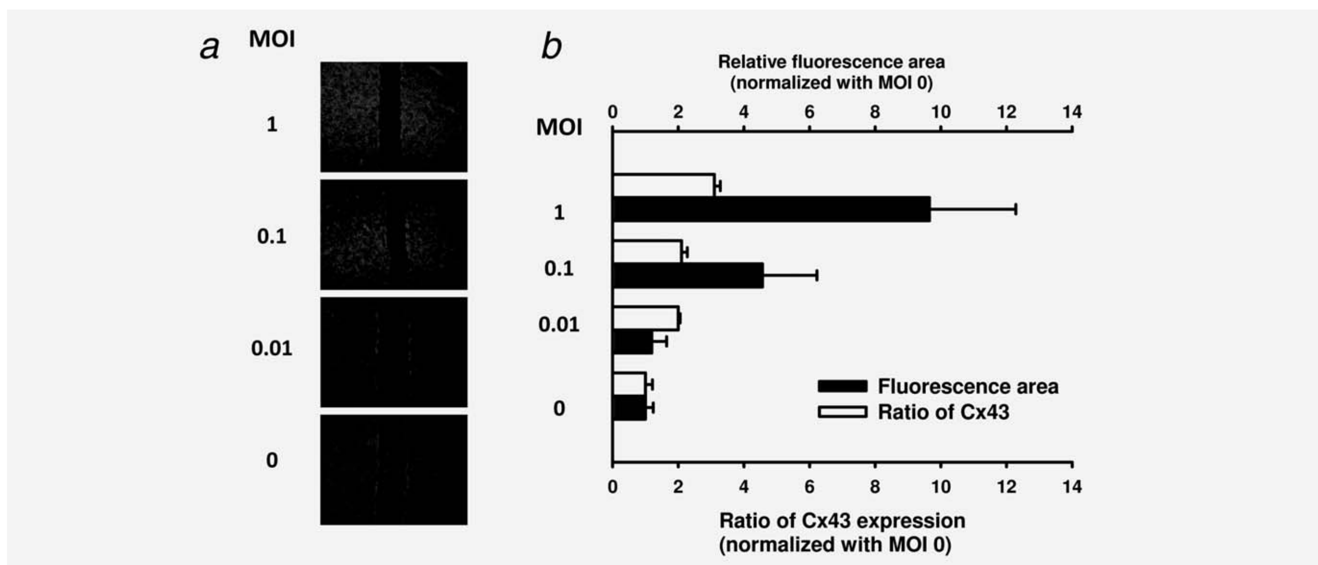


**Figure 2.** *Salmonella* induce a dose-dependent GJIC in tumor cells. (*a*) K1735 cells treated for 1 hr with different MOI of *Salmonella* were determined by scrape loading and dye transfer analysis. (*b*) GJIC was expressed as fold of the control. Ratio of Cx43 expression were determined by densitometric analysis. Data are expressed as mean ± SD of hexaplicate determinations.

## Cx43 increased susceptibility of tumor cells to cisplatin induced cell death

We examined the effects of Cx43 expression on the sensitivity of the tumor cells to killing by cisplatin that induce apoptosis. Cytotoxicity was quantified using a WST-8 assay. K1735 cells were highly resistant to killing by cisplatin (Fig. 4*a*). Treatment with cisplatin, significantly reduced cell viability in *Salmonella* infected cells when compared with uninfected cells. Interestingly, the dead *Salmonella* also induced Cx43 expression in tumor cells to increase susceptibility to cisplatin (Fig. 4*a*). To evaluate the bystander effect of Cx43, we cocultured the heat-killed-*Salmonella*-treated cells with PBS-treated cells at varying ratios in the presence of cisplatin for 48 hr followed by the WST-8 assay to determine the cell survival (Fig. 4*b*). As expected, cisplatin did not induce significant cytotoxicity in PBS-treated cells. Whereas the degree of cytotoxicity was proportional to the percentage of heat-killed-*Salmonella* -treated cells, bystander effect, in which PBS-treated cells were killed by cisplatin treatment, was observed (Fig. 4*b*). To enhance the sensitivity of assessing cytotoxicity induced by the bystander effect, we cocultured the heat-killed-*Salmonella*-treated cells with K1735-Luc cells that expressed luciferase gene under the control of the β-actin promoter at varying ratios with cisplatin treatment for 48 hr followed by measuring their luciferase activity. When the percentage of heat-killed-*Salmonella*-treated cells increased, the luciferase activity of the cell lysate decreased, indicating bystander effect has occurred (Fig. 4*c*). The death signal may transmit through Cx43 to amplify apoptosis in neighboring cells (K1735-Luc). To test the importance of Cx43 function in the increased sensitivity of cells to cisplatin, K1735 cells were overexpressed with Cx43. The viability of cells overexpressed Cx43 significantly reduced than that of control cells after cisplatin treatment (Fig. 5*a*). To obtain more insight into the role of Cx43 in *Salmonella* induced bystander effect, we knocked down Cx43 using siRNA. Initially, K1735 cells were transfected with siRNA for Cx43 and control siRNA. The protein expression of Cx43 was determined by immunoblot analysis after *Salmonella* treatment (Fig. 5*b*). Cx43-siRNA decreased the expression of Cx43 protein induced by *Salmonella* (Fig. 5*b*). The viability of cells infected with *Salmonella* significantly reduced than that of control cells after cisplatin treatment. On the contrary, Cx43 siRNA antagonized the effects of *Salmonella* infection (Fig. 5*c*). These results suggest that *Salmonella*-induced Cx43 expression exerted bystander effects following cisplatin treatment.

## Additive antitumor effects by the combination therapy of Salmonella and cisplatin

Although *Salmonella* monotherapy was effective in retarding the growth of established tumor, complete tumor regression was not observed.[13] Groups of mice that had been inoculated s.c. with K1735 cells ($10^6$) at day 0 were treated i.v. with *Salmonella* ($2 \times 10^6$ cfu) at day 7 followed by cisplatin (2 mg/kg) at days 15, 17 and 19, or with either treatment alone. Vehicle control mice received PBS. Antitumor effects of *Salmonella* alone or combined with low-dose cisplatin were evaluated in terms of tumor growth and survival of the mice bearing K1735 tumors. In mice bearing K1735 tumors, treatment of *Salmonella* alone significantly retarded tumor growth (Fig. 6*a*) and prolonged the survival time (Fig. 6*b*) compared with PBS treatment. Tumor-bearing mice treated with low-dose cisplatin exerted no antitumor effect. The antitumor effects of *Salmonella* combined with cisplatin were evaluated in terms of tumor growth and survival in mice bearing K1735 tumors (Figs. 6*a* and 6*b*). Interestingly, the

Cancer Therapy

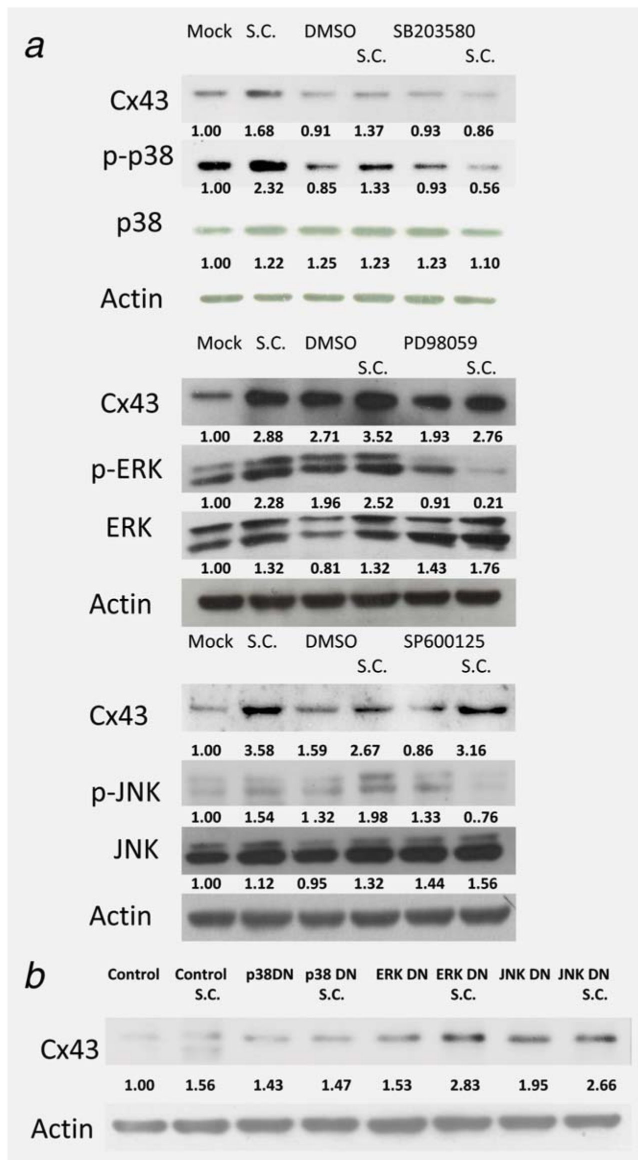


**Figure 3.** p38 inhibitors fail to prevent *Salmonella*-induced Cx43 expression.(*a*) After treatment of cells with inhibitor for ERK (PD98059), p38 (SB203580) and JNK (SP600125), K1735 cells were treated with *Salmonella* (MOI = 100) for 60 min. The cells were lysed and protein expression of p-ERK, p-P38 and p-JNK was examined. (*b*) Cx43 expression was detected in K1735 cells transiently transfected with p38 dominant negative, ERK dominant negative, JNK dominant negative plasmids or with control vector by immunoblot analysis after treatment with *Salmonella* for 1 hr. The expression of β-actin served as the quantitative control. Inserted values indicated relative proteins expression in comparison with β-actin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

combination therapy significantly reduced tumor size compared with *Salmonella* in tumor-bearing mice (Fig. 6*a*). The mean tumor volume for mice in the combination therapy group decreased by 35% compared with those in the *Salmonella*-treated group. The survival of the mice treated with *Salmonella* plus cisplatin was significantly prolonged

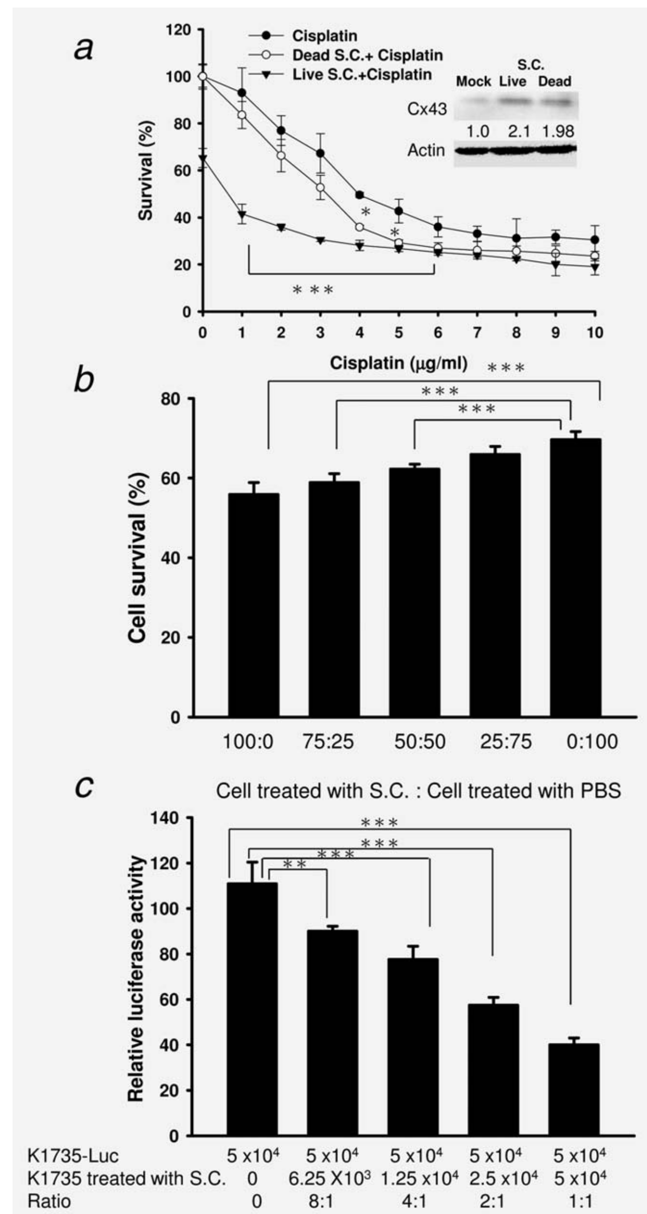


**Figure 4.** *Salmonella* (S.C.)-induced Cx43 expression in conjunction with cisplatin exerted cytotoxic effects on K1735 cells and bystander cytotoxic effects. (*a*) *Salmonella*-treated, heat-killed-*Salmonella* treated or control cells were exposed to cisplatin (0–10 μg/ml) for 48 hr followed by determination of their viability by the WST-8 assay. Data are expressed as mean ± SD of hexaplicate determinations. ($p < 0.05$ for Dead S.C. + Cisplatin *versus* Cisplatin; $p < 0.001$ for Live S.C. + Cisplatin *versus* Cisplatin) (*b*) Heat-killed-*Salmonella* treated and PBS-treated cells were mixed in various ratios to generate 100, 75, 50, 25 and 0% PBS-treated cells. These cells were treated with cisplatin (3 μg/ml) for 48 hr. Cell viability was determined by the WST-8 assay. Data are expressed as mean ± SD of hexaplicate determinations. (*c*) Different cell numbers of *Salmonella* -treated cells were cocultured with K1735-Luc ($5 \times 10^4$) cells in the presence of cisplatin (3 μg/ml) for 48 hr followed by determination of their relative luciferase activity. The decrease of luciferase gene expression was indicative of the bystander effect. The relative luciferase activity is expressed as a ratio of luciferase activity divided by that of K1735-Luc cells without cisplatin treatment. **, $p < 0.01$; ***, $p < 0.001$. Data are expressed as mean ± SD of hexaplicate determinations.

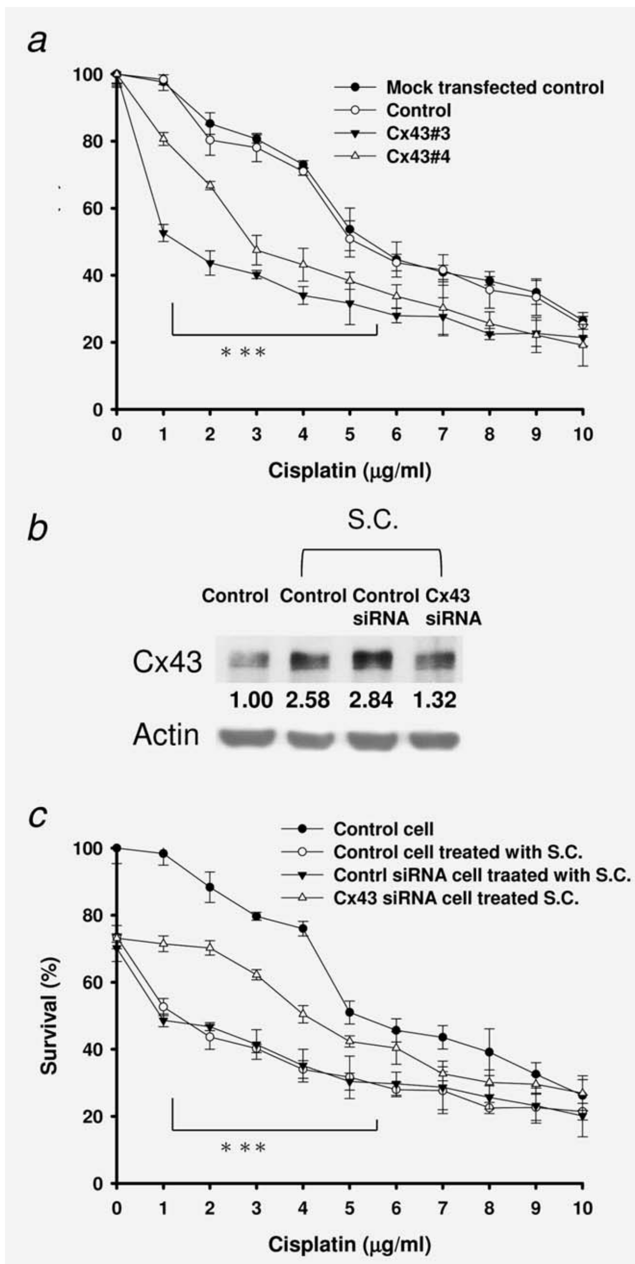


Figure 5. The expression Cx43 enhanced the cytotoxic effect of cisplatin. (*a*) Cx43 overexpressing and control cells were treated with cisplatin (0–10 μg/ml) for 48 hr followed by determination of their viability by the WST-8 assay. ($p < 0.001$ for Cx43#3 or Cx43#4 *versus* control) (*b*) Cx43 siRNA down-regulates *Salmonella* (S.C.)-induced Cx43 expression. *Salmonella*-treated or control cells were transfected with various siRNAs (Control: transfection reagent only; Control siRNA: non-targeting siRNA; CX43 siRNA: specific targeting Cx43). The expression of Cx43 was determined by immunoblot analysis. The expression of β-actin served as the quantitative control. Inserted values indicated relative proteins expression in comparison with β-actin. (c) Cx43 siRNA down-regulates the cytotoxic effect of cisplatin. Cells were treated with cisplatin (0–10 μg/ml) for 48 hr followed by determination of their viability by the WST-8 assay. ($p < 0.001$ for Cx43 siRNA cell treated with S.C. *versus* Control siRNA cell treated with S.C.) Data are expressed as mean ± SD of hexaplicate determinations.

compared with that of the mice treated with *Salmonella* (Fig. 6*b*). Taken together, *Salmonella* as a single-agent therapy could retard tumor growth and enhance survival in murine melanoma tumor models. More strikingly, additive antitumor effects could be achieved with the combination therapy. In order to show that Cx43 is required for the combined activity of cisplatin and *Salmonella*, we generated a tumor cell line stably silenced for Cx43. The combined activity of cisplatin and *Salmonella* was not observed in the cells stably silenced Cx43 (Supporting Information Fig. S3*a*). The survival of the mice treated with *Salmonella* plus cisplatin was not significantly prolonged compared with that of the mice treated with *Salmonella* (Supporting Information Fig. S3*b*). To investigate the relationship between Cx43 and p-p38 *in vivo* after *Salmonella* treatment, mice bearing K1735 tumors were injected with *Salmonella*, and the levels of p-p38 and Cx43 in the tumors were determined by immunofluorescence staining (Fig. 6*c*). The expression of Cx43 and p-p38 were significantly upregulation after *Salmonella* treatment compared with the groups treated with PBS or Cisplatin. In this study, we suggested that *Salmonella* enhanced the expression of Cx43 by inducing p38 signaling pathway *in vitro* and *in vivo*. Furthermore, the tumors from K1735-bearing mice treated with *Salmonella* or cisplatin alone, or in combination were analyzed for apoptotic cells by the TUNEL assay. Representative results for immunohistochemistry are shown in Fig. 6*d*. TUNEL assay shows an increase in the amount of cells undergoing apoptosis in the *Salmonella*-treated tumors compared with cisplatin-treated or PBS-treated tumors (Fig. 6*d* and 6*e*). There was a 1.5-fold increase in the number of apoptotic cells induced by *Salmonella* plus cisplatin compared with that induced by *Salmonella* alone (Fig. 6*e*). Taken together, these results indicate that the combination therapy with *Salmonella* and cisplatin resulted in retarding tumor growth, increasing apoptosis in the tumors.

## Discussion

*Salmonella* have been proposed as anticancer agents.[23,24] Many cancer cells show absence of intercellular communication. Increasing gap junction activity or enhancing GJIC in cancer cells provides the targets to enhance anticancer therapies.[25] Treatment with *Salmonella* results in Cx43 upregulation in tumor cells. We examined the effect of combinational treatment of *Salmonella* and cisplatin in an animal model to show an increase in efficiency of cisplatin via the enhancement of gap junctions. Cisplatin alone did not significantly decrease tumor growth, while combination therapy of *Salmonella* plus cisplatin showed 73% reduction. Histological results also showed an increase in the amount of cells undergoing apoptosis. We have showed for the first time an increase in the efficiency of cisplatin via enhancement of gap junction with *Salmonella*.

Melanoma is an aggressive cancer with no effective treatment options. Enforced expression of Cx43 increased the sensitivity of melanoma cells to cisplatin. *Salmonella* induced the restoration of Cx43 in several types of tumor cells.[12]

*Salmonella* enhanced cisplatin-induced cytotoxicity in K1735 cells, and downregulation of Cx43 by siRNA treatment abrogated the enhancing effect of *Salmonella*. However, the regulation of Cx43 expression is not clear. Recent studies revealed that angiotension II increase in Cx43 expression is regulated by MAPK-AP-1 signaling pathways. [26] In this study, we investigated the effect of *Salmonella* on Cx43 expression and function. We found that *Salmonella*-induced activation of p38 pathway and are thought to play an important role in the upregulation of Cx43. The dead *Salmonella* had the same effect on upregulation the expression of Cx43 (Fig. 4*a*). Previous report demonstrated that bacterial components, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and flagellin, induced the expression of Cx43 in tumor cells.[12] Gram-positive bacterial cell wall component (peptidoglycan) also can induce the expression of Cx43.[27] The heat-killed *Salmonella* still have the bacterial components. The upregulation of Cx43 is the common mechanism of both live and dead *Salmonella*. We showed that the dead *Salmonella* had the activity to induce the expression of Cx43 in tumor cells (Fig. 4*a*).

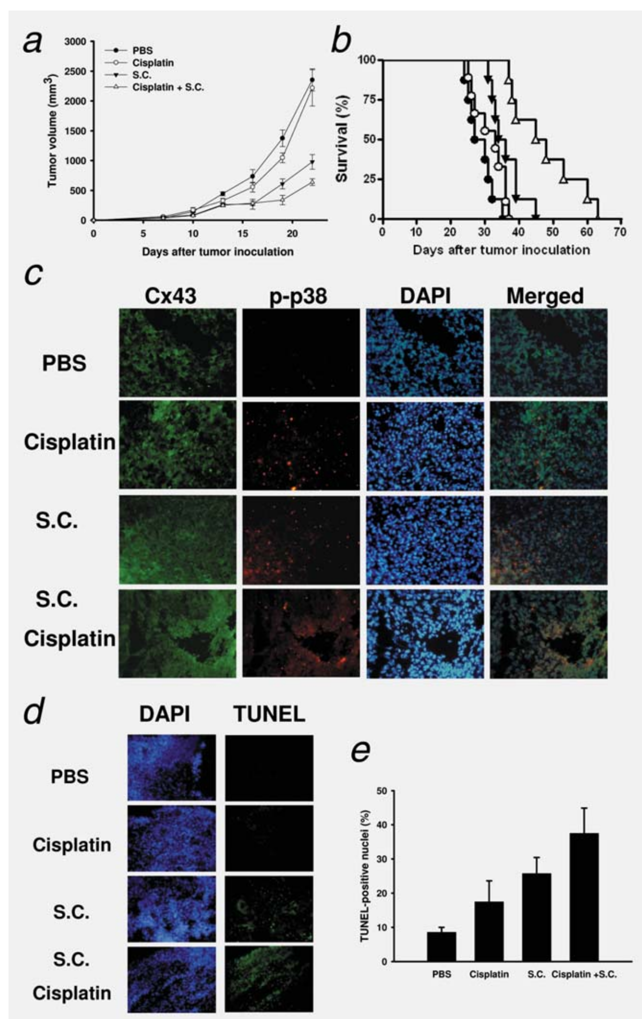


Figure 6.

The use of preferentially replicating bacteria as an oncolytic agent is one of the innovative approaches for the treatment of cancer. *Salmonella* have been employed as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth.[28] Furthermore, *Salmonella* has been demonstrated to grow in the necrotic and relatively hypoxic foci within tumors but not in well-oxygenated tumors at the rim of the growing nodules. The limited ability of *Salmonella* to disperse throughout the tumor may be the most important shortcoming in its use as an anticancer agent. Our previous data suggested that the capability of *Salmonella* to disperse within tumors and hence to delay tumor growth was augmented when combined with low-dose cisplatin.[13] As shown in Supporting Information Fig. S4*b*, the expressions of Cx43 and p-p38 were increased with treatment of *Salmonella* in both normoxic and hypoxic conditions. These results suggest that *Salmonella*-induced Cx43 expression in both normoxic and hypoxic conditions. The death cells can precipitate the death of nearby cells in a process referred to as the bystander effect.[29] We examined the effect of Cx43 expression on the sensitivity of the tumor cells to killing by cisplatin that induces apoptosis. Cx43 is critical for transmission of apoptotic signal, such as cytochrome *c*, DNA fragments reactive oxygen species, to the nontargeted cells.[30] The death signal may transmit through

Figure 6. Additive antitumor effects of Salmonella (S.C.) in combination with cisplatin on subcutaneous K1735 tumors. (*a*) Groups of 8 mice that had been inoculated s.c. with K1735 cells ($10^6$) at day 0 were treated i.v. with Salmonella ($2 \times 10^6$ cfu) at day 7 followed by cisplatin (2 mg/kg) at days 15, 17 and 19, or with either treatment alone. Vehicle control mice received PBS. Tumor volumes (mean ± SEM, $n = 8$) among different treatment groups were compared in mice bearing K1735 tumors ($p < 0.001$ for S.C.+cisplatin versus cisplatin; $p < 0.01$ for S.C.+cisplatin versus S.C. or PBS; $p < 0.05$ for S.C. versus PBS). (*b*) Kaplan-Meier survival curves of the mice bearing k1735 tumors ($p < 0.01$ for S.C.+cisplatin versus S.C., cisplatin, or PBS; $p < 0.05$ for S.C. versus PBS) with different treatments are shown. (*c*)The expression of Cx43 and p-p38 in tumor treated with Salmonella. Mice that had been inoculated s.c. with K1735 cells ($10^6$) at day 0 were treated i.v. with Salmonella ($2 \times 10^6$ cfu) at day 7 followed by cisplatin (2 mg/kg) at days 15, 17 and 19, or with either treatment alone. Vehicle control mice received PBS. Tumors were excised for double immunofluorescence staining with anti-p-p38 antibody by Texas Red and anti-Cx43 antibody by fluorescein. Nuclei were counterstained with DAPI. (d) Increase in tumor cells undergoing apoptosis in K1735 tumor-bearing mice treated with Salmonella in combination with cisplatin. Groups of 4 mice that had been inoculated s.c. with K1735 cells ($10^6$) at day 0 were treated i.v. with Salmonella ($2 \times 10^6$ cfu) at day 7 followed by cisplatin (2 mg/kg) at days 15, 17 and 19, or with either treatment alone. Vehicle control mice received PBS. Tumors were excised at day 20, and TUNEL assay was used to detect apoptotic cells (× 400). (*e*) TUNEL-positive cells were counted from three fields of highest density of positive-stained cells in each section to determine the percentage of apoptotic cells (mean ± SEM, $n = 4$). ($p < 0.01$ for S.C.+cisplatin versus S.C., cisplatin, or PBS; $p < 0.05$ for S.C. versus PBS). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Cx43 to amplify apoptosis in neighboring cells. Bystander effect to enhance *Salmonella* antitumor efficacy would involve inhibiting the viable rim of tumor growth. Therefore, effective enhancement of apoptosis as seen with expression of Cx43 in tumor cells is a reasonable approach for developing more successful treatment.

We also found that p38 pathway itself did not influence the *Salmonella*-induced cell death (Supporting Information Fig. S2*b*). The signals of Cx43 and p-p38 in tumor treated with *Salmonella* plus cisplatin co-localized compared with untreated tumors. The apoptosis in tumors induced by *Salmonella* and cisplatin may rely on p-p38 signal pathway and Cx43 upregulation. Regulation of Cx43 by stress correlated with the ability of the stressed cells to functionally communicate through gap-junctions with neighboring nonstressed cell. Previous observations suggested that radiation induced Cx43 expression in skin fibroblasts. In contrast, exposure of rat liver epithelial cells to radiation resulted in reduction in expression levels of Cx43. [31]In our study, the downregulation of Cx43 expression was observed in cells exposed to cisplatin and hypoxic condition (Supporting Information Figs. S4*a* and S4b). Such differences suggest that the regulation of Cx43 protein by cell stress may vary.

The colonization of *Salmonella* in tumor sites may destroy the immunosuppressive phenotype of tumor microenvironment. *Salmonella* are potent adjuvants that induce antitumor immunity by activating immune cells in host. Bacteria within tumors may induce inflammatory responses, leading to the recruitment of immune cells, such as macrophages, neutrophils and lymphocytes to the tumor site.[28] As *Salmonella* replication and lysis of tumor cells may induce cell-mediated immune responses to tumor cells, higher oncolysis could account, in part, for an increased infiltrate of $CD8^+$ T cells in bacteria-treated tumors. The cytotoxic T cell response against tumor cells may enhance the antitumor efficacy of bacteria.[32–36] *Salmonella* induced antimicrobial response in tumors to activate cytotoxic $CD8^+$ T cells via Cx43. *Salmonella* act both locally, by recruiting T cells that inhibit tumor growth, and systemically, where bacteria provide the development of immune response via the cross-presentation of tumor antigen.[12]

By taking advantages of the tumoricidal effect of *Salmonella* and pleiotropic activities of cisplatin, we conclude that *Salmonella* in combination with cisplatin appears to hold promise for the treatment of solid tumors. Meanwhile, our work also implicated the underlying mechanism of antitumor effects by the combination therapy of *Salmonella* and cisplatin.

## References


1. Liu F, Zhang L, Hoffman RM, et al. Vessel destruction by tumor-targeting Salmonella typhimurium A1-R is enhanced by high tumor vascularity. *Cell Cycle* 2010;9:4518–24.
2. Nagakura C, Hayashi K, Zhao M, et al. Efficacy of a genetically-modified Salmonella typhimurium in an orthotopic human pancreatic cancer in nude mice. *Anticancer Res* 2009;29:1873–78.
3. Yam C, Zhao M, Hayashi K, et al. Monotherapy with a tumor-targeting mutant of S. typhimurium inhibits liver metastasis in a mouse model of pancreatic cancer. *J Surg Res* 2010;164:248–255.
4. Hayashi K, Zhao M, Yamauchi K, et al. Systemic targeting of primary bone tumor and lung metastasis of high-grade osteosarcoma in nude mice with a tumor-selective strain of Salmonella typhimurium. *Cell Cycle* 2009;8:870–75.
5. Kimura H, Zhang L, Zhao M, et al. Targeted therapy of spinal cord glioma with a genetically modified Salmonella typhimurium. *Cell Prolif* 2010;43:41–8.
6. Hayashi K, Zhao M, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Hoffman RM. Cancer metastasis directly eradicated by targeted therapy with a modified Salmonella typhimurium. *J Cell Biochem* 2009;106:992–8.
7. Zhao M, Geller J, Ma H, Yang M, Penman S, Hoffman R. Monotherapy with a tumor-targeting mutant of *Salmonella typhimurium* cures orthotopic metastatic mouse models of human prostate cancer. *Proc Natl Acad Sci USA* 2007;104:10170–74.
8. Zhao M, Yang M, Ma H, et al. Targeted therapy with a Salmonella typhimurium leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice. *Cancer Res* 2006;66:7647–52.
9. Sharrow AC, Li Y, Micsenyi A, et al. Modulation of osteoblast gap junction connectivity by serum, TNF-$\alpha$, and TRAIL. *Exp Cell Res* 2008;314:297–308.
10. Wang M, Berthoud VM, Beyer EC. Connexin43 increases the sensitivity of prostate cancer cells to TNF-$\alpha$-induced apoptosis. *J Cell Sci* 2007;120:320–29.
11. Mancuso M, Pasquali E, Leonardi S, et al. Role of connexin43 and ATP in long-range bystander radiation damage and oncogenesis in vivo. *Oncogene* 2011;30:4601–08.
12. Saccheri F, Pozzi C, Avogadri F, et al. Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity. *Sci Transl Med* 2010;2:44ra57.
13. Lee CH, Wu CL, Tai YS, et al. Systemic administration of attenuated Salmonella choleraesuis in combination with cisplatin for cancer therapy. *Mol Ther* 2005;11:707–16.
14. Lee CH, Wu CL, Shiau AL. Endostatin gene therapy delivered by Salmonella choleraesuis in murine tumor models. *J Gene Med* 2004;6:1382–93.
15. Wang SW, Wu HH, Liu SC, et al. CCL5 and CCR5 interaction promotes cell motility in human osteosarcoma. *PLoS One* 2012;7:e35101.
16. Lin HY, Tang CH, Chen JH, et al. Peptidoglycan induces interleukin-6 expression through the TLR2 receptor, JNK, c-Jun, and AP-1 pathways in microglia. *J Cell Physiol* 2011;226:1573–82.
17. Tang CH, Yang RS, Chien MY, et al. Enhancement of bone morphogenetic protein-2 expression and bone formation by coumarin derivatives via p38 and ERK-dependent pathway in osteoblasts. *Eur J Pharmacol* 2008;579:40–9.
18. Lee CH, Wu CL, Shiau AL. Toll-like receptor 4 signaling promotes tumor growth. *J Immunother* 2010;33:73–82.
19. Lee CH, Wu CL, Shiau AL. Salmonella choleraesuis as an anticancer agent in a syngeneic model of orthotopic hepatocellular carcinoma. *Int J Cancer* 2008;122:930–5.
20. Bhattacharjee R, Kaneda M, Nakahama K, et al. The steady-state expression of connexin43 is maintained by the PI3K/Akt in osteoblasts. *Biochem Biophys Res Commun* 2009;382:440–4.
21. Crespin S, Bechberger J, Mesnil M, et al. The carboxy-terminal tail of connexin 43 gap junction protein is sufficient to mediate cytoskeleton changes in human glioma cells. *J Cell Biochem* 2010;110:589–97.
22. Behrens J, Kameritsch P, Wallner S, et al. The carboxyl tail of Cx43 augments p38 mediated cell migration in a gap junction-independent manner. *Eur J Cell Biol* 2010;89:828–38.
23. Pawelek JM, Low KB, Bermudes D. Bacteria as tumour-targeting vectors. *Lancet Oncol* 2003;4:548–56.
24. Pawelek JM, Low KB, Bermudes D. Tumor-targeted Salmonella as a novel anticancer vector. *Cancer Res* 1997;57:4537–44.
25. Cottin S, Gould PV, Cantin L, et al. Gap junctions in human glioblastomas: implications for suicide gene therapy. *Cancer Gene Ther* 2011;18:674–81.
26. Jia G, Cheng G, Gangahar DM, et al. (2008) Involvement of connexin 43 in angiotensin II-induced migration and proliferation of saphenous vein smooth muscle cells via the MAPK-AP-1 signaling pathway. *J Mol Cell Cardiol* 2008;44:882–90.
27. Robertson J, Lang S, Lambert PA, et al. Peptidoglycan derived from Staphyococcus epidermidis induces connexin 43 hemichannel activity with consequences on the innate immune response in endothelial cells. *Biochem J* 2010;432:133–43.
28. Lee CH. Engineering bacteria toward tumor targeting for cancer treatment: current state and


Cancer Therapy

perspectives. *Appl Microbiol Biotechnol* 2012;93:517–23.
29. Lee CH, Wu CL, Shiau AL. Hypoxia-induced cytosine deaminase gene expression for cancer therapy. *Hum Gene Ther* 2007;18:27–38.
30. Peixoto PM, Ryu SY, Pruzansky DP, et al. Mitochondrial apoptosis is amplified through gap junctions. *Biochem Biophys Res Commun* 2009;390:38–43.
31. Azzam EI, de Toledo AM, Little JB. Expression of connexin 43 is highly sensitive to ionizing radiation and other environmental stresses. *Cancer Res* 2003;63:7128–35.
32. Lee CH, Hsieh JL, Wu CL, et al. T cell augments the antitumor activity of tumor-targeting Salmonella. *Appl Microbiol Biotechnol* 2011;90:1381–88.
33. Avogadri F, Martinoli C, Petrovska L, et al. Cancer immunotherapy based on killing of Salmonella-infected tumor cells. *Cancer Res* 2005;65:3920–7.
34. Lee CH, Wu CL, Shiau AL. (2008) Toll-like receptor 4 mediates an antitumor host response induced by Salmonella choleraesuis. *Clin Cancer Res* 2008;14:1905–12.
35. Lee CH, Hsieh JL, Wu CL, et al. B cells are required for tumor-targeting Salmonella in host. *Appl Microbiol Biotechnol* 2011;92:1251–60.
36. Lee CH, Lin YH, Hsieh JL, et al. A polymer coating applied to Salmonella prevents the binding of Salmonella-specific antibodies. *Int J Cancer* 2013;132:717–25.

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