



# Colorectal Cancer Therapy Using a *Pediococcus pentosaceus* SL4 Drug Delivery System Secreting Lactic Acid Bacteria-Derived Protein p8

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Despite decades of research into colorectal cancer (CRC), there is an ongoing need for treatments that are more effective and safer than those currently available. Lactic acid bacteria (LAB) show beneficial effects in the context of several diseases, including CRC, and are generally regarded as safe. Here, we isolated a *Lactobacillus rhamnosus* (LR)-derived therapeutic protein, p8, which suppressed CRC proliferation. We found that p8 translocated specifically to the cytosol of DLD-1 cells. Moreover, p8 down-regulated expression of Cyclin B1 and Cdk1, both of which are required for cell cycle progression. We confirmed that p8 exerted strong anti-proliferative activity in a mouse CRC xenograft model. Intraperitoneal injection of recombinant p8 (r-p8) led to a significant reduction (up to 59%) in tumor mass when compared with controls. In recent years, bacterial drug delivery systems (DDSs) have proven to be effective therapeutic agents for acute colitis. Therefore, we aimed to use such systems, particularly LAB, to generate the valuable therapeutic proteins to treat CRC. To this end, we developed a gene expression cassette capable of inducing secretion of large amounts of p8 protein from *Pediococcus pentosaceus* SL4 (PP). We then confirmed that this protein (PP-p8) exerted anti-proliferative activity in a mouse CRC xenograft model. Oral administration of PP-p8 DDS led to a marked reduction in tumor mass (up to 64%) compared with controls. The PP-p8 DDS using LAB described herein has advantages over

other therapeutics; these advantages include improved safety (the protein is a probiotic), cost-free purification, and specific targeting of CRC cells.

**Keywords:** anti-cancer activity, drug delivery system, *Pediococcus pentosaceus* SL4, probiotics, therapeutic protein

## INTRODUCTION

Colorectal cancer (CRC) is a common and possibly fatal disease. Almost 1 million people worldwide develop CRC each year. Of these, around 50% are expected to die of systemic disease within 5 years of diagnosis (Weitz et al., 2005). Despite recent advances in chemotherapeutic treatment, there are 56,000 deaths per year (McWilliams and Erlichman, 2005). Cancer chemoprevention uses natural, synthetic, or biological substances to reverse, suppress, or prevent either the initial phase of carcinogenesis or progression of neoplastic cells to cancer. However, broad application of chemopreventive agents is compromised by their limited efficacy and potential toxicity (Zhang et al., 2010). As the field of molecularly targeted therapies expands, many novel agents have reached the clinical or commercial stages of development. However, even molecular targeted therapies approved for treatment of solid cancers have side effects (Widakowich et al., 2007).

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Clearly, there is an ongoing need for more effective and safer treatments.

To overcome these limitations, we took a novel approach involving screening lactic acid bacteria (LAB), a human intestinal microbe generally regarded as safe, for secreted proteins with anti-CRC effects. If LAB secrete a protein that suppresses CRC, then it would likely have very few adverse side effects. Food-grade bacteria are by definition safe to ingest. Historically, such microbes have not been associated with development of sinister pathologies; indeed, their positive impact on health is very well documented and is monitored systematically in the context of human and animal food production (Steidler and Vandenbroucke, 2006).

Humans evolved as “super organisms”; this is because they have a symbiotic relationship with the microbial community that resides in the gastrointestinal tract and is essential for health (Ley et al., 2008; Quercia et al., 2014). In particular, LAB are beneficial microorganisms that provide health benefits to the host and play a role in therapy (Quigley, 2011). Recent studies of LAB demonstrate that they suppress development of CRC by inhibiting tumor initiation or progression via multiple pathways (Zhong et al., 2014). Several strains belonging to *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium adolescentis*, and *Bifidobacterium breve* species effectively suppress CRC (Sadeghi-Aliabadi et al., 2014). Several mechanisms have been put forward to explain how this occurs. These include boosting the host immune response and exerting anti-proliferative effects by regulating apoptosis, cell differentiation, and tyrosine kinase signaling pathways (Uccello et al., 2012). However, it is assumed that the probiotic activity against CRC likely stems from the microbes’ ability to generate large amounts of significant organic acids (Makarova et al., 2006). The survival rate of LAB in the upper gastrointestinal tract is low because cells appear to be subject to rapid lysis (Drouault et al., 1999). Thus, the cytoplasmic contents may be liberated within the digestive tract (Drouault et al., 1999). It is also reported that *L. casei* lysates reduce intestinal inflammation in a murine model of inflammatory bowel disease (Zakostelska et al., 2011). So, it is possible that immunomodulatory or therapeutic proteins could reside within the cytoplasm of LAB, as well as being secreted. Previously, we showed that a hypothetical protein, p14, isolated from the cytoplasm of *L. casei* selectively down-regulated serum immunoglobulin E and interleukin-4 levels, as well as reducing the atopic dermatitis (AD) index and scratching score, in AD-like NC/Nga mice (Kim et al., 2015). However, despite their importance to human health, few therapeutic proteins have been isolated from LAB and evaluated (Flambard and Juillard, 2000; Hörmannspurger et al., 2013; von Schillde et al., 2012). Therefore, we wondered whether LAB harbor novel proteins that act as CRC suppressors; furthermore, we hypothesized that such proteins would have few side effects.

Here, to the best of our knowledge, we describe the first LAB drug delivery system (DDS) with the potential to suppress CRC by delivering therapeutic proteins directly to the human intestine. A major advantage of this system is that using food-grade LAB as a delivery vehicle is unlikely to be

pathogenic. Indeed, LAB have never been shown to pose a risk to health, even when consumed by individuals with an ongoing intestinal disease (Steidler and Vandenbroucke, 2006). Moreover, our approach may lead to development of more cost-effective, intestine-specific, long-term therapies for human CRC.

We screened laboratory strains of LAB (all of which originated from the human intestine) for novel therapeutic proteins against CRC. The screening process identified an 8 kDa protein (p8) with CRC suppressive activity. Next, we characterized the mechanism by which p8 suppressed tumor growth and confirmed its anti-proliferative activity in a DLD-1–derived mouse xenograft model. Moreover, we verified that oral administration of a recombinant *Pediococcus pentosaceus* SL4 (PP) DDS harboring p8 (PP-p8) suppressed tumor growth in the same mouse xenograft model. Taken together, the results presented herein open up new avenues for tumor therapy. Indeed, the findings may be of interest to the medical and pharmaceutical fields, and to the food industry.

## MATERIALS AND METHODS

### Bacterial strains and culture

*L. rhamnosus* (LR) KCTC 12202BP, which was isolated from human intestine, and PP KCTC 10297BP, which was isolated from kimchi, were obtained from the culture collection maintained at Cell Biotech (Korea). Both LR and PP were cultured for 18 to 24 h in MRS broth (Difco, USA) at 37°C. *Escherichia coli* was cultured for 18 to 24 h in M9 broth (Difco) or LB broth (Difco) at 37°C.

### Purification of anti-cancer proteins from LAB

Bacterial cells were harvested from MRS broth by centrifugation for 15 min at 4,000 rpm and then washed twice with phosphate-buffered saline (PBS). To purify soluble proteins from LAB cells under native conditions, cell lysates were prepared by ultrasonication on ice and the supernatant was passed through a 0.2 µm filter. The supernatant was then loaded onto a HiPrep 26/10 size exclusion column (GE Healthcare, USA) equilibrated with buffer A (20 mM Tris-HCl, pH 8.0) and separated using a fast protein liquid chromatography system (GE Healthcare). The column was eluted with buffer A (1 ml/min), and fractions containing proteins were pooled. The proteins were then applied to a Hitrap DEAE FF column (GE Healthcare) and eluted with a linear gradient of 0 to 1 M NaCl in buffer A. Unbound fractions were concentrated using 3 kDa membrane (Merck Millipore, USA) and dialyzed against buffer B (50 mM NaP, pH 6.0). Next, samples were applied to a Hitrap SP FF column (GE Healthcare) equilibrated with buffer B. Bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in buffer B at a flow rate of 1 ml/min. Proteins purified on the cation SP FF column were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were also subjected to N-terminal amino acid sequencing and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry analysis at the Korea Basic Science Institute (Korea).

### Construction and purification of recombinant His-tagged p8 protein from *E. coli*

Recombinant p8 (r-p8) protein isolated from LR was expressed using expression vector pET-28a; the protein had a hexa-histidine (6×His) tag and a TEV protease cleavage site at the N-terminus. The p8 construct was transformed into *E. coli* strain C41(DE3) (Novagen, USA), which was cultured in M9 medium until the optical density value reached 0.6. Over-expression of selenomethionine-substituted (SeMet) p8 was initiated by addition of 0.5 mM IPTG for 4 h. Cells were harvested and resuspended in 20 mM HEPES (pH 7.5)/150 mM NaCl. After sonication, the cell supernatant was obtained by centrifugation. P8 protein was purified by binding to Ni<sup>2+</sup>-NTA agarose (Qiagen, USA), followed by washing with 20 mM HEPES (pH 7.5)/150 mM NaCl/20 mM imidazole, and elution with 20 mM HEPES (pH 7.5)/150 mM NaCl/300 mM imidazole. The 6×His tag was removed by TEV protease in the presence of 1 mM DTT. To check homogeneity of the SeMet-p8 protein, it was applied to a size exclusion column (HiLoad 26/60 Superdex 200 pg; GE Healthcare) equilibrated with 20 mM HEPES (pH 7.5)/150 mM NaCl.

### Cell culture

Human CRC cell lines DLD-1 and HT-29 were purchased from the Korean Cell Line Bank and maintained under 5% CO<sub>2</sub>/37°C in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco).

### Cell proliferation assay

DLD-1 and HT-29 cells were seeded in 96-well plates at a density of  $1 \times 10^3$  cells per well and incubated at 37°C. After 24 h, various concentrations of r-p8 protein (0-40 μM) were added to each well and incubated for a further 72 h. Cell viability was determined using Cell Counting Kit-8 (Dojindo Laboratories, Japan), according to the manufacturer's protocol. Absorbance was measured using a multifunctional microplate reader (SpectraMax M5; Molecular Devices, USA). For cell staining, cells were fixed for 30 min with 4% paraformaldehyde (PFA) and then stained with crystal violet for 30 min prior to visualization.

### Western blotting

Soluble proteins isolated from DLD-1 cells were extracted in RIPA buffer (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (Roche, Germany). Following centrifugation, the supernatant was passed through a 0.2 μm filter. To extract total protein from mouse xenograft tissues (DLD-1-derived), the ground tissue powder was lysed in RIPA buffer containing a protease inhibitor cocktail (Roche). Next, proteins (40 μg total) were separated by SDS-PAGE and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Bioscience, USA). Blotted membranes were blocked in 5% skimmed milk diluted in T-TBS and then incubated overnight at 4°C with appropriate primary antibodies (Cell Signaling Technology, USA); all antibodies were diluted 1:1,000. The membranes were washed three times (each for 15 min) with T-TBS and then blocked in 5% skimmed milk diluted in T-TBS. The membranes were

then incubated for 1 h with HRP-linked secondary antibody (Cell Signaling Technology) at 4°C. GAPDH was used as an internal control. Protein bands were detected using an enhanced chemiluminescence kit (Millipore, USA), followed by autoradiography using a Chemi-doc™ Touch Imaging System (Bio-Rad Laboratories, USA).

### Immunocytochemistry using ImageXpress® Micro Confocal microscopy

CRC cells were seeded onto coverslips placed in 6-well plates. After 24 h, r-p8 protein (0-40 μM) was added to each well and incubated for a further 72 h. Cells were fixed for 15 min at room temperature in 3% PFA and then washed three times in PBS. For permeabilization, cells were incubated for 2 min with 0.2% Triton X-100 in PBS and then washed. To reduce background signals, cells were blocked for 30 min with 4% bovine serum albumin in PBS. Next, cells were incubated overnight at 4°C with a rabbit polyclonal anti-p8 antibody (Young In Frontier, Korea) or for 2 h at 4°C. Protein localization was visualized using FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, USA). For nuclear staining, cells were incubated for 1 h at room temperature with 5 μg/ml Hoechst 33258 (Sigma, USA), rinsed three times in PBS, and mounted. For live/dead staining, cells were incubated for 30 min with LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, USA). Images were obtained using ImageXpress® Micro Confocal (Molecular Devices).

### Animals

Male athymic nude mice (BALB/cAnN.Cg-Foxn1nu/CrI(Nar); 5 weeks of age, Study I: 35 in total, Study II: 60) were purchased from the SR Bio (Korea). Around a specific pathogen-free animal facility at constant temperature (20 ± 3°C) and humidity (40 ± 20%) and 12 h light cycle (Laboratory Animal Center of Cell Biotech). The animals had free access to irradiation sterilized dry pellet-type feeds and water during the study period. In accordance with the study schedule, the mice were sacrificed by inhaling CO<sub>2</sub> at the end of administering test substance. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) board in the Cell Biotech (approval No. CBT-2019-04) based on guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

### Transplantation of tumor cell and tumor inoculation

DLD-1 CRC cell were maintained *in vitro* in RPMI1640 (Gibco) medium supplemented with 10% FBS (Gibco) and 0.1 mM NEAA (Gibco). The cells growing in exponential growth phase were harvested and counted for tumor inoculation. Each mouse was inoculated subcutaneously at the rear right flank with DLD-1 tumor cells ( $2 \times 10^6$  cells) in 0.1 ml of PBS for tumor development. After 7 days of tumor inoculation, the animals were weighed and measured for tumor volume and randomly divided into 5 groups of 7 animals each based on the randomized block design method for homogeneous group formation when the mean tumor size reached approximately 100-150 mm<sup>3</sup> (5 days).

### Drug treatment

When tumors reached the average size of 100-150 mm<sup>3</sup>, the mice of Study I were randomized into five treatment groups (n = 7, total n = 35). The treatment was started intraperitoneal (i.p.) injection on the 7th day post tumor inoculation with 0.9% saline (Group 1), 40 mg/kg 5-Fluorouracil (5-Fu; Group 2), 1 mg/kg r-p8 (Group 3), 10 mg/kg r-p8 (Group 4), and 20 mg/kg r-p8 (Group 5), respectively. The treatment was continued for 4 weeks with a regimen of twice per week (i.p.) for mono therapy r-p8 and 5-Fu.

In Study II, mice were randomized into seven treatment groups (n = 10, total n = 70), treatment started by oral administration on the 7th day post tumor inoculation; with 0.9% saline (Group 1), 1 × 10<sup>10</sup> cfu/head wild-type PP (WT-PP; Group 2), 1 × 10<sup>10</sup> cfu/head PP empty vector DDS (PP-EV; Group 3) and 1 × 10<sup>10</sup> cfu/head of PP-p8 DDS (Group 4) and i.p. injection of 10 mg/kg r-p8 (Group 5) and 40 mg/kg 5-Fu (Group 6) as specified in Study I. Also the treatment continued for 4 weeks, for the r-p8 and 5-Fu, respectively.

### Tumor measurement and survival

The animals were survival monitored for once/week for body weight (gain/loss) and tumor size. Tumor volumes were calculated using formula: Vol. = (width/2)<sup>2</sup> × length where length and width are the long and short tumor diameters respectively and euthanized when the tumor volume reached a predetermined size of approximately 2,000 mm<sup>3</sup>. This endpoint tumor size was chosen to maximize the number of tumor doublings within the exponential growth phase in the untreated group.

### Statistical analysis

Statistical analysis of data was performed using one-way ANOVA on the Prism 4 (GraphPad Software, USA). The animal data results are presented as mean ± SD. Multiple comparisons were made by using Tukey's multiple comparison tests to determine which groups significantly differed from

each other. A value of *P* < 0.05 was regarded as statistically significant.

### Construction of the plasmid harboring DDS-p8 for use in the *P. pentosaceus* SL4 system

P8 was cloned into the plasmid pCBT24-2 (KCCM12182P). The dual promoter system selected for maximum expression of p8 was ligated to an usp45 secretion signal peptide, thereby enabling synthesis of DNA fragments (Cosmogenetech, Korea). A portion of each promoter ligated to the signal peptide was digested with *NdeI/PstI* and *BamHI/PstI* restriction enzymes, respectively. DNA fragments encoding PK-usp45-p8 were inserted into the pCBT24-2 expression vector. Finally, the pCBT24-2-PK-p8-PK-p8 plasmid (accession No. KCCM12181P) was transformed into PP cells.

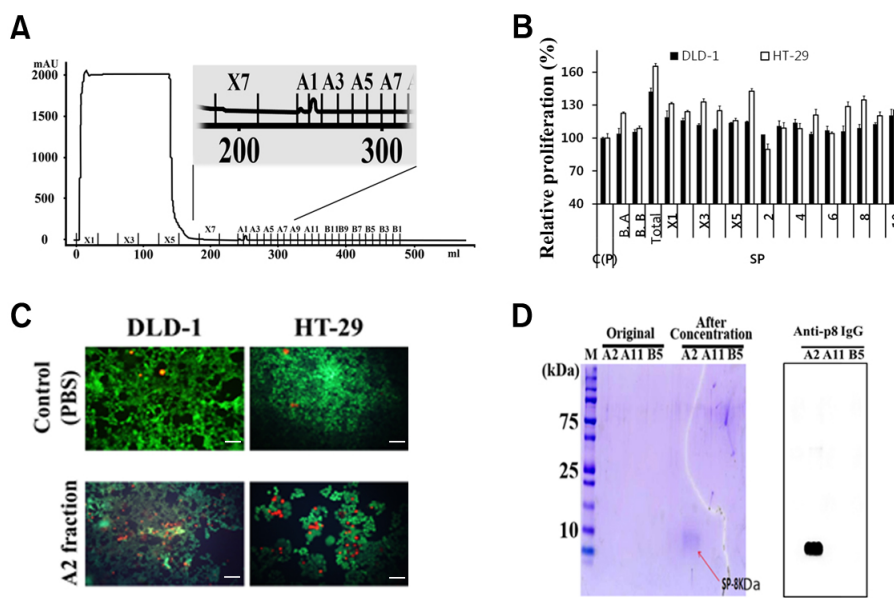
### Transformation of *P. pentosaceus* SL4 and detection of p8 in the culture supernatant

Transformants grown on MRS agar plates were inoculated into 10 ml of MRS broth containing 10 mg/ml erythromycin and cultured at 37°C for 15 h (no shaking). Next, 1 ml of pre-culture was inoculated into 10 ml of M9 minimal medium containing 10 mg/ml erythromycin and cultured at 37°C for 48 h (no shaking). Next, 5 ml of culture was centrifuged and the supernatant was collected. The supernatant was concentrated by trichloroacetic acid (TCA) precipitation to isolate total protein. Finally, p8 protein was detected by western blotting.

## RESULTS

### Screening of anti-cancer proteins derived from LR

Among LAB, LR inhibits cytokine-mediated apoptosis of mouse and human intestinal epithelial cells by regulating signaling pathways (Yan et al., 2007). Adriana et al. (2018) suggested that the LR secretes proteins that suppress CRC. Therefore, we decided to attempt to isolate anti-cancer



**Fig. 1. Isolation of anti-cancer proteins from *L. rhamnosus*.** (A) LR lysates were fractionated using a three-step chromatography approach. (B) To determine the anti-cancer activity of each fraction, each was tested in a cell proliferation assay based on DLD-1 and HT-29 cells. (C) The anti-cancer properties of fraction A2 were confirmed by fluorescence microscopy at ×200 magnification. Green, live cells; Red, dead cells. Scale bars = 200 μm. (D) Each fraction was separated by SDS-PAGE and stained with Coomassie blue-250. P8 protein of each fraction was determined by western blotting.

proteins from LR lysates. To separate and isolate functional proteins, we adopted a three-step process comprising size exclusion (data not shown), anion-exchange (data not shown), and cation-exchange (Fig. 1A) chromatography. Figure 1A shows the peaks corresponding to the separated proteins. To test the anti-cancer activity of each fraction, all were examined in a cell proliferation assay using two CRC cell lines (DLD-1 and HT-29). The results revealed that fraction A2 showed the strongest anti-proliferative effects against both cell lines (Fig. 1B); the anti-cancer effects were confirmed by fluorescence microscopy (Fig. 1C). Both results of SDS-PAGE and western blotting revealed that fraction A2 contained a single abundant protein with a molecular mass (MW) of approximately 8 kDa (named p8) (Fig. 1D).

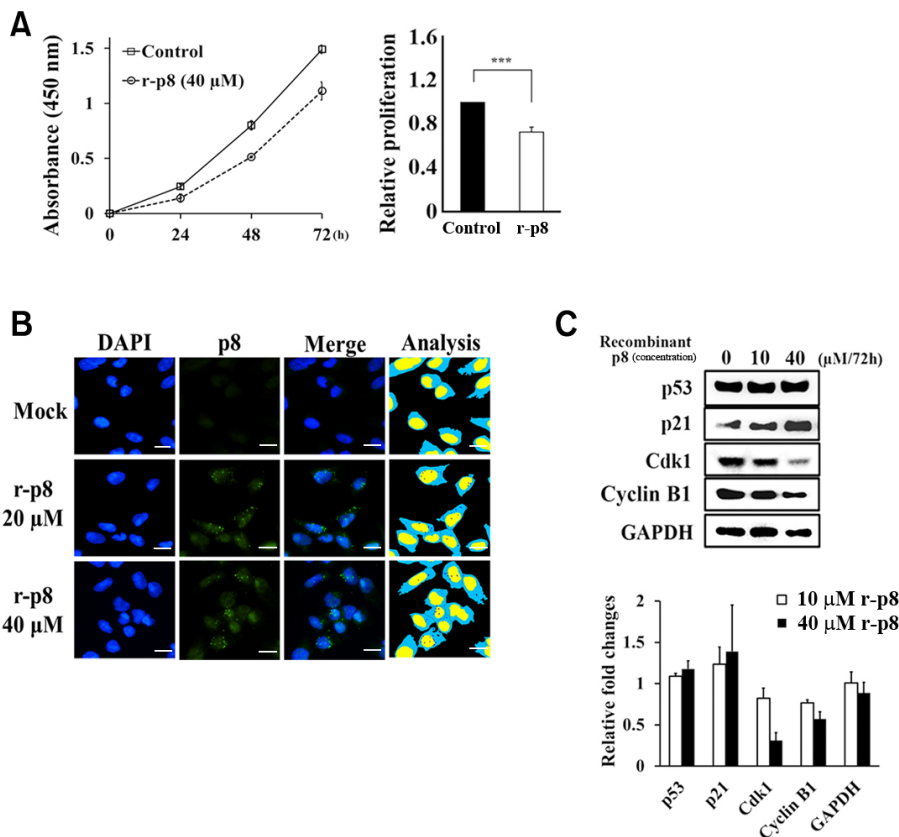
Next, we subjected p8 to Edman degradation analysis. The N-terminal 10 amino acid sequence was identified as A-T-V-D-P-E-K-T-L-F. In addition, MALDI-TOF mass spectrometry analysis revealed that p8 shared 94% amino acid identity with a hypothetical protein from *L. rhamnosus* LGG\_02452 (Supplementary Fig. S1). As a first step to characterizing p8, we performed a BLAST search of the National Center for Biotechnology Information microbial genome database and found that p8 demonstrated greatest conservation (in terms of sequence and domains) with a hypothetical protein predicted to be encoded by the genomes of *L. rhamnosus* sp., *L. casei* sp., *Lactobacillus paracasei*, and *Lactobacillus zeae*. However, there was no evidence to suggest that p8 had any anti-cancer effects.

### P8 suppresses proliferation by entering the cytoplasm of DLD-1 cells

To undertake functional studies of p8, we generated a recombinant 6×His tag-TEV-p8 protein using an *E. coli* expression system. Recombinant p8 lacking a 6×His tag was purified (Supplementary Fig. S2) as described in the Materials and Methods. Next, we generated an anti-p8 polyclonal antibody for use in immunoblotting and confocal microscopy experiments. To evaluate the anti-cancer activity of p8 in DLD-1 cells, we first assessed its effects on cell proliferation. The number of viable cells detected after exposure to 40 μM p8 treated cells fell by 0.72-fold when compared with the control (Fig. 2A). This suppressive effect was detectable at 24 h post-p8 treatment and lasted for 48 to 72 h. The detailed molecular mechanism by which p8 exerts these effects is unclear. Therefore, we examined functional translocation of p8 using an ImageXpress® Micro Confocal microscope. P8 was detected in the cytosol of DLD-1 cell; the intensity of the signal was dose-dependent (Fig. 2B). Taken together, these data indicate that p8 enters CRC cells and displays anti-cancer activity.

### Anti-cancer properties of p8 in DLD-1 cells

Next, we examined the effects of p8 on apoptosis and cell cycle arrest in DLD-1 cells. First, we determined the effects of p8 on the cell cycle using western blotting to detect cell cycle-related proteins (Fig. 2C). The results showed that the total amount of Cyclin B1 and its partner protein Cdk1 in DLD-1 cells fell significantly in a p8 dose-dependent manner.



**Fig. 2. Anti-cancer properties of recombinant p8 in DLD-1 cells.**

(A) Effect of r-p8 (40 μM) on proliferation of DLD-1 cells for 72 h (left panel). Relative anti-proliferation activity of r-p8 after 72 h was represented (right panel). (B) Functional translocation of r-p8 examined using ImageXpress® Micro Confocal microscopy at ×60 magnification. Green, p8 protein; Blue, DAPI. Scale bars = 20 μm. (C) Effect of p8 on expression of proteins involved in G<sub>2</sub> cell cycle arrest of DLD-1 cells. Protein expression was determined by western blotting. A value of \*\*\*P < 0.001 was regarded as statistically significant.

Moreover, expression of p21, which suppresses Cyclin B1/Cdk1, increased in a p8 dose-dependent manner. However, p8 had no effect on expression of p53 by DLD-1 cells. These data suggest that p8 might put a brake on the p53-p21 signaling pathway, resulting in G<sub>2</sub> arrest of DLD-1 cells. P8 did not affect signaling pathways related to apoptosis (Supplementary Fig. S3).

### P8 exhibits anti-cancer effects in a mouse xenograft model of CRC

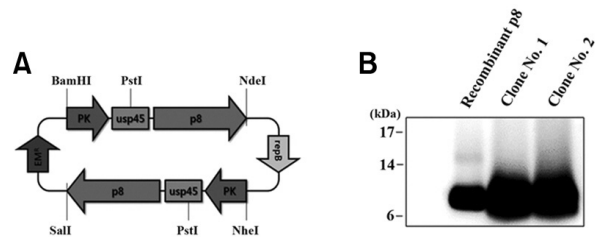
Next, we examined the efficacy of p8 against DLD-1 cell-derived xenografts in athymic nude mice. Figures 3A and 3B shows tumor growth curves after treatment with p8. Compared with controls, mice treated for 4 weeks (twice per week) with 1, 5, or 10 mg/kg p8 showed a marked reduction in tumor size. This result was presented that the weight of tumor tissue by p8 compared with the controls, it was reduced in a dose-dependent manner (Fig. 3C). This result compared well with that showing that 5-Fu monotherapy inhibited cancer growth at a maximum tolerated dose of 40 mg/kg.

### Anti-cancer activity of PP-p8 DDS in a mouse xenograft model

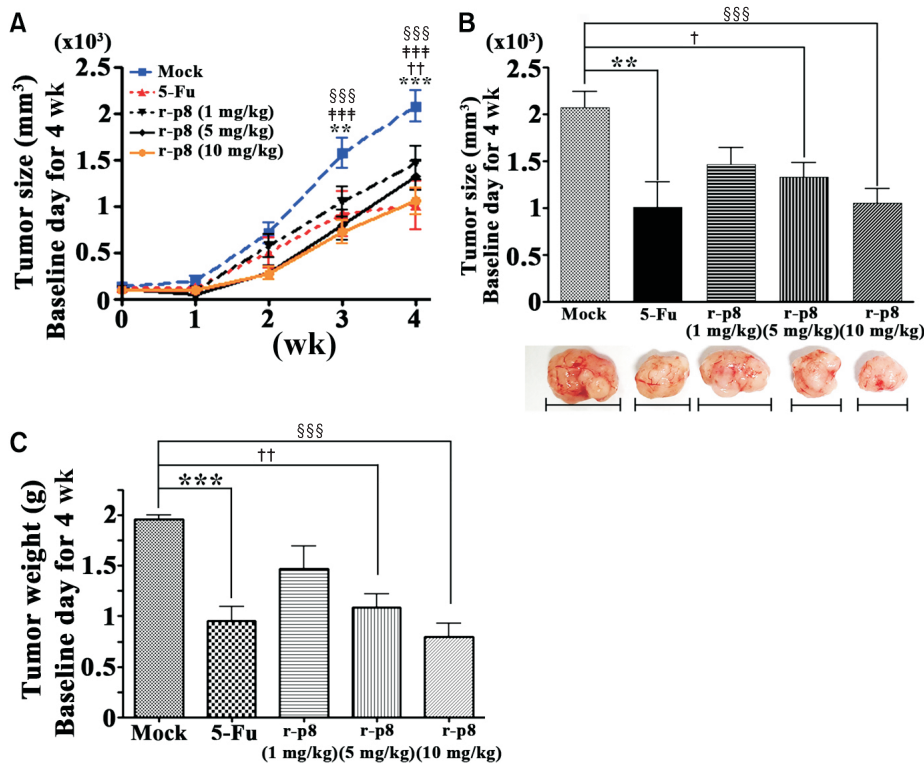
P8 is a protein drug that is likely to be susceptible to digestion by proteases present in the human intestine. Therefore, an efficient delivery method is required. To develop a DDS for p8, we first constructed an expression vector (Fig. 4A) and then measured the amount of p8 secreted into PP-p8 bacterial culture supernatants (Fig. 4B).

Next, to determine whether orally administered PP-p8

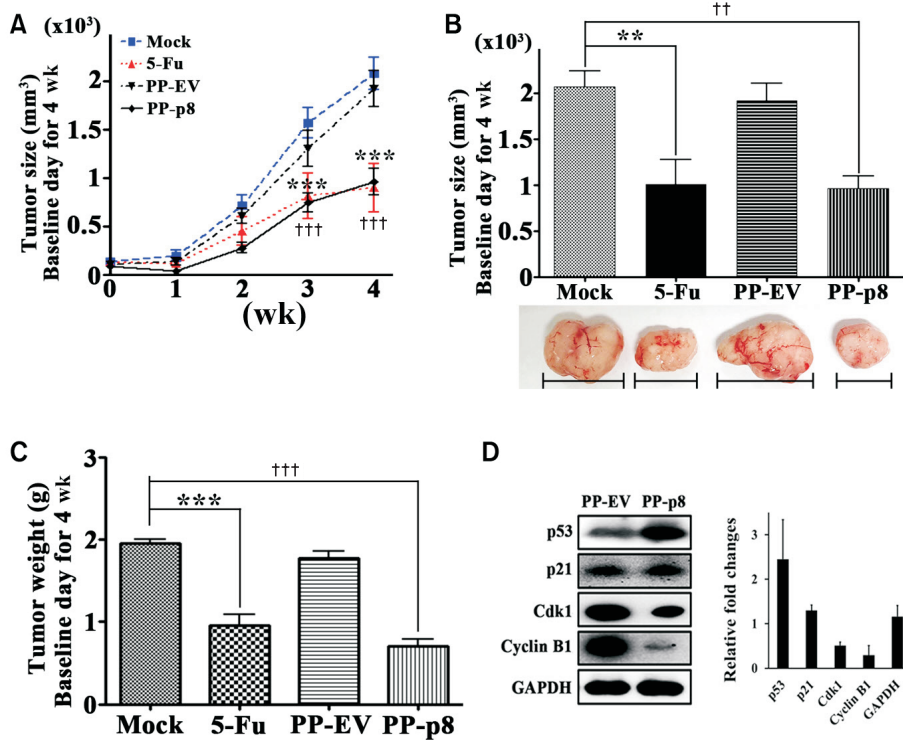
DDS shows anti-cancer activity similar to that of injected r-p8, we examined its efficacy *in vivo* under the experimental conditions shown in Figure 3. Figures 5A to 5C shows tumor growth rates after oral administration of each drug. PP-p8 DDS, r-p8, or 5-Fu led to a marked reduction in growth rate (64%, 59%, and 52%, respectively) when compared with the controls. However, WT-PP and PP-EV DDS were less effective. Next, we asked whether cell cycle arrest induced by PP-p8 inhibits the growth of CRC xenografts. Western blot analysis revealed that expression of cell cycle regulatory factors Cyclin B1 and Cdk1 in tumor tissue fell significantly in response to treatment with PP-p8 DDS (Fig. 5D). Moreover, expression of p21, which suppresses Cyclin B1/Cdk1, increased after PP-p8 DDS administration. In addition, expression of p53 increased significantly. Again, these data suggest that p8 might act as a brake on the p53-p21 signaling pathway,



**Fig. 4. Development of the PP-p8 DDS.** (A) Schematic showing the expression vector for PP-p8. (B) The amount of p8 secreted into the culture supernatant was assessed by western blotting. Control = 40 ng of r-p8.



**Fig. 3. Anti-tumor efficacy of p8 in a DLD-1-derived xenograft model.** (A) Recombinant p8 treatment led to a marked reduction in the size of DLD-1-derived xenografts in athymic mice. Mice received 1, 5, or 10 mg/kg r-p8 (intraperitoneal injection twice weekly) and/or 40 mg/kg 5-fluorouracil (intraperitoneal injection once/week). (B) Mean  $\pm$  SEM tumor volume on day 28 post-treatment. (C) Mean  $\pm$  SEM tumor weight on day 28 post-treatment. A value of  $P < 0.05$  was regarded as statistically significant. Mock vs 5-Fu: \*, Mock vs r-p8 (1 mg/kg): +, Mock vs r-p8 (5 mg/kg): †, Mock vs r-p8 (10 mg/kg): § (\*, †, ‡, §  $P < 0.05$ ; \*\*, ††, ‡‡, §§  $P < 0.01$ ; \*\*\*, †††, ‡‡‡, §§§  $P < 0.001$ ).



**Fig. 5. The anti-tumor efficacy of PP-p8 DDS was measured in a DLD-1-derived xenograft model.** (A) Oral administration of PP-p8 DDS led to a marked reduction in growth of DLD-1 tumors. Mice received  $1 \times 10^{10}$  cfu/head PP-EV DDS (five oral administrations/week),  $1 \times 10^{10}$  cfu/head PP-p8 DDS (five oral administrations/week), and/or 40 mg/kg 5-fluorouracil (intraperitoneal injection once/week). (B) Mean  $\pm$  SEM tumor volume on day 28 post-treatment. (C) Mean  $\pm$  SEM tumor weight on day 28 post-treatment. (D) Western blot showing effect of PP-p8 on expression of molecules associated with G<sub>2</sub> arrest in DLD-1 xenografts. EV, empty vector. A value of  $P < 0.05$  was regarded as statistically significant. Mock vs 5-Fu: \*, Mock vs PP-p8: + (\*, +  $P < 0.05$ ; \*\*, ++  $P < 0.01$ ; \*\*\*, +++  $P < 0.001$ ).

resulting in G<sub>2</sub> arrest of DLD-1 cells.

## DISCUSSION

The results presented two advantages in this work suggest a novel therapeutic approach for CRC therapy. First advantage, we used *P. pentosaceus* SL4, as a suitable vehicle, for production and delivery of therapeutic molecular. *P. pentosaceus* SL4 belongs to food-grade LAB which strain is a Gram-positive, non-motile, facultative anaerobe and is acid tolerant (Dantoft et al., 2013). Strain SL4 is frequently used as an indigenous starter in natural and controlled fermentations such as a kimchi (Papagianni and Anastasiadou, 2009). Moreover, SL4 strain secretes bacteriocin which specifically inhibits the growth of *Listeria monocytogenes* and *Staphylococcus aureus* (Shin et al., 2008). Second advantages, we used LAB derived p8 protein, as a therapeutic anti-cancer molecular, for CRC therapy. Because of LAB-derived proteins are thought to be both beneficial and safe for humans (Steidler and Vandenbroucke, 2006). In addition, many studies show that colonic LAB inhibit development of CRC (Cousin et al., 2016; Hendler and Zhang, 2018) and Orlando et al. (2012) have demonstrated that the tyndalized LAB exhibited anti-proliferative and pro-apoptotic effects to gastric cancer and CRC cell lines. However, most mechanisms put forward to explain the anti-cancer characteristics of LAB involve alteration of the metabolic activity of gut micro-flora and the colonic environment, removal of carcinogens, production of anti-tumor-origenic or anti-mutagenic substances, and strengthening of host immunity (Sadeghi-Aliabadi et al., 2014). Furthermore, no study has shown that proteins secreted by gut bacteria

suppress CRC. Here, we developed a novel therapeutic approach, *P. pentosaceus* SL4 based DDS installing p8 secretion system, for CRC therapy and this approach by which p8 could be delivered orally to CRC patients. CRC cells are located on the inner surface of the intestine; therefore, p8 would have to pass through the digestive tract, exposing it to multiple digestive enzymes. Therefore, to deliver active p8 to CRC cells in the intestine.

Taken together, the results presented herein open up new avenues to developing therapeutic options for CRC. In particular, p8 protein could be delivered as a probiotic or via food products designed specifically for CRC patients. This could deliver p8 to the intestine and increase the efficacy of CRC therapies, particularly those that trigger cell cycle arrest.

Note: Supplementary information is available on the Molecules and Cells website ([www.molcells.org](http://www.molcells.org)).

## Disclosure

The authors have no potential conflicts of interest to disclose.

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