BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 26(3), 313-321 (2018)

Cellular Prion Protein Enhances Drug Resistance of Colorectal Cancer Cells via Regulation of a Survival Signal Pathway

Jun Hee Lee[1], Chul Won Yun[2] and Sang Hun Lee[2,3,*]

[1]Department of Pharmacology and Toxicology, University of Alabama at Birmingham School of Medicine, Birmingham, AL 35294, USA
[2]Medical Science Research Institute, Soonchunhyang University Seoul Hospital, Seoul 04401,
[3]Department of Medical Bioscience, Soonchunhyang University, Asan 31151, Republic of Korea

Abstract

Anti-cancer drug resistance is a major problem in colorectal cancer (CRC) research. Although several studies have revealed the mechanism of cancer drug resistance, molecular targets for chemotherapeutic combinations remain elusive. To address this issue, we focused on the expression of cellular prion protein (PrP^C) in 5-FU-resistant CRC cells. In 5-FU-resistant CRC cells, PrP^C expression is significantly increased, compared with that in normal CRC cells. In the presence of 5-FU, PrP^C increased CRC cell survival and proliferation by maintaining the activation of the PI3K-Akt signaling pathway and the expression of cell cycle-associated proteins, including cyclin E, CDK2, cyclin D1, and CDK4. In addition, PrP^C inhibited the activation of the stress-associated proteins p38, JNK, and p53. Moreover, after treatment of 5-FU-resistant CRC cells with 5-FU, silencing of PrP^C triggered apoptosis via the activation of caspase-3. These results indicate that PrP^C plays a key role in CRC drug resistance. The novel strategy of combining chemotherapy with PrP^C targeting may yield efficacious treatments of colorectal cancer.

Key Words: Colorectal cancer, Prion protein, Drug resistance, 5-fluorouracil, Anticancer

INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed cancer among males and females, the second leading cause of cancer related death among males, and third leading cause among females (Siegel *et al.,* 2017). Although surgical techniques, chemotherapies, and molecular therapies have improved, clinical outcomes have not kept pace. This lag is mostly because of changes in lifestyle, local recurrence, distal metastasis, and resistance to chemotherapeutics and molecularly targeted therapies (Holohan *et al.,* 2013; Oliphant *et al.,* 2013). Among these hurdles, drug resistance limits therapeutic efficacy, because chemotherapy is one of the principal modes of cancer therapy (Holohan *et al.,* 2013). In particular, chemotherapy failure caused by anti-cancer drug resistance induces most cancer related deaths due to decreases in drug delivery and changes in metabolic enzymes (Hammond *et al.,* 2016). Therefore, understanding the mechanisms underlying drug resistance plays a pivotal role in developing reversal strategies and effective cancer therapeutic combinations.

5-fluorouracil (5-FU) is widely used in the treatment for several cancers, particularly for CRC. It is an antimetabolite drug that inhibits thymidylate synthase and is incorporated into RNA, single DNA, and double DNA helix, leading to cancer cell death (Longley *et al.,* 2003). Several clinical studies have shown that 5-FU-based chemotherapies and chemoradiotherapies increased the survival of patients with several cancers (Pignon *et al.,* 2000; Adelstein *et al.,* 2006; Tsukuda *et al.,* 2010). However, despite the benefits of 5-FU to cancer therapy, acquired resistance to 5-FU is a major clinical problem. Accumulated evidence shows that abnormally high activities of thymidylate synthase, deoxyuridine triphosphatase, Bcl-2, Bcl-XL, and Mcl-1 lead to 5-FU resistance (Zhang *et al.,* 2008). However, the detailed molecular mechanisms for 5-FU chemoresistance in CRC require further investigation.

Cellular prion protein (PrP^C) is highly expressed in a variety of cells, such as lymphocytes, and tissues, including muscle, heart, skin, and nervous tissues (Liang and Kong, 2012). Although studies on PrP^C have initially focused on the nervous system, recent evidence indicates that PrP^C regulates not only self-renewal of stem/progenitor cells and stem cell fate but also proliferation and resistance to apoptosis in cancer cells

Open Access **https://doi.org/10.4062/biomolther.2017.033**

Received Feb 20, 2017 **Revised** Apr 27, 2017 **Accepted** Jun 14, 2017
Published Online Aug 21, 2017

***Corresponding Author**
E-mail: ykckss1114@nate.com, jhlee0407@sch.ac.kr
Tel: +82-2-709-9029, Fax: +82-2-792-5812

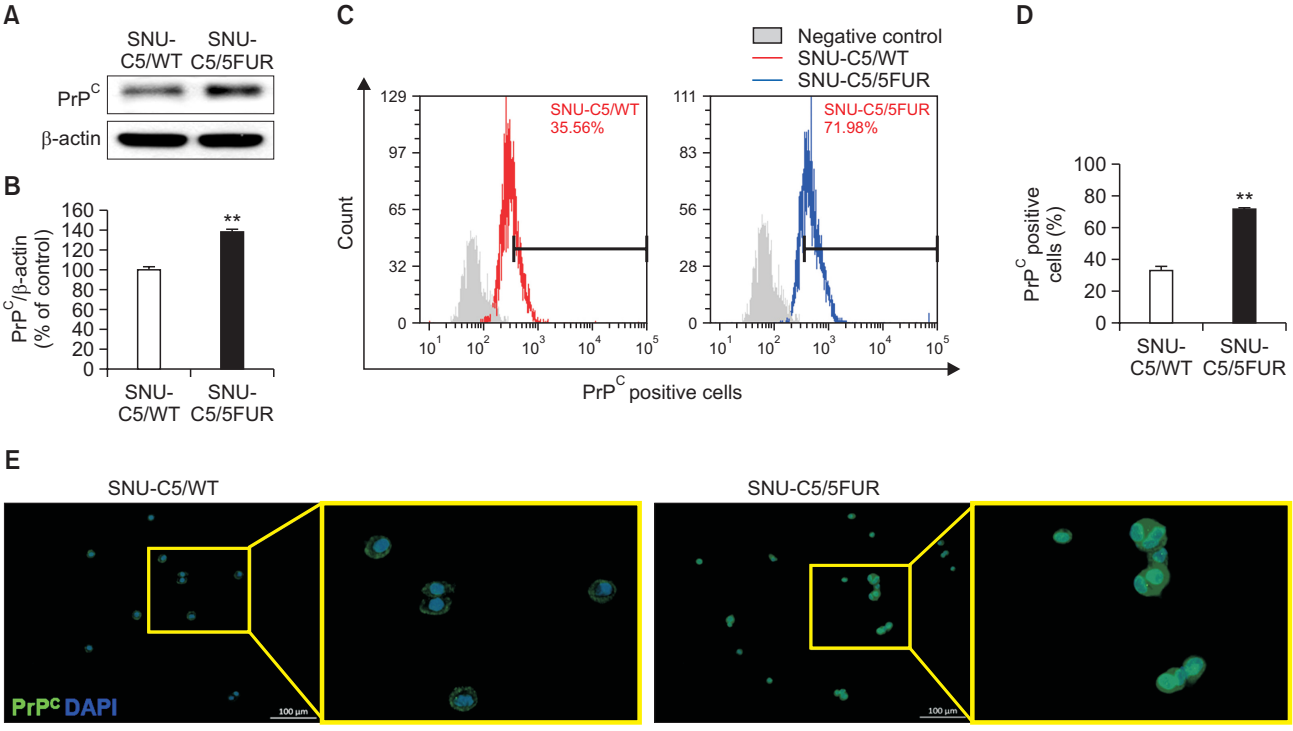

Fig. 1. The levels of cellular prion proteins (PrP^C) in SNU-C5/WT and SNU-C5/5FUR cells. (A) Expression of PrP^C in SNU-C5/WT and SNU-C5/5FUR cells. (B) The expression level of PrP^C was determined by densitometry, relative to β-actin expression. (C) The expression of PrP^C in SNU-C5/WT and SNU-C5/5FUR cells was analyzed by flow cytometry. (D) Standard quantification of the percentage of PrP^C positive cells. (E) Immunocytochemistry for PrP^C (green) in SNU-C5/WT and SNU-C5/5FUR cells. Nuclei were stained by DAPI (blue). Scale bar=100 μm. (B, D) Data are expressed as the mean ± SEM of three independent experiments. $^{**}p<0.01$ vs. SNU-C5/WT.

(Martin-Lanneree *et al.,* 2014). In CRC, PrP^C promotes cancer cell survival by increasing uptake of glucose (Li *et al.,* 2011). PrP^C augmented CRC metastasis through the Fyn-SP1-SATB1 pathway (Wang *et al.,* 2012). Our previous study reveals that silencing PrP^C inhibits colon cancer cell growth (Yun *et al.,* 2016). However, there is little evidence of a relationship between PrP^C and anti-cancer chemoresistance in CRC cells. In this study, we investigated the effect of PrP^C on proliferation and survival in 5-FU resistant CRC cells. Moreover, we explored the underlying mechanism of resistance to 5-FU in CRC cells through regulation of PrP^C expression.

MATERIALS AND METHODS

Preparation of 5-fluorouracil and oxaliplatin

5-Fluorouracil and oxaliplatin were obtained from Sigma (St. Louis, MO, USA). 5-Fluorouracil and oxaliplatin powder were dissolved in dimethyl sulfoxide (DMSO), and aliquots were stored at 4°C until use.

Cell culture

The human colon cancer cell line (SNU-C5/WT), 5-FU resistant cell line (SNU-C5/5FUR) and oxaliplatin-resistant cell line (SNU-C5/Oxal-R) were obtained from the Chosun University Research Center for Resistant Cells (Gwangju, Korea). The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, l-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C with 5% CO_2 in a humidified incubator.

Western blot analysis

Total cell protein was extracted by utilizing RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies against PrP^C, phospho-phosphatidylinositol-3-kinase (PI3K), total PI3K, phospho-AKT, total AKT, cyclin dependent kinase (CDK) 2, CDK4, cyclin D1, cyclin E, phosphor-p38, total p38, phosphor-c-JUN N-terminal kinase (JNK), total JNK, cleaved caspase-3, cleaved poly [ADP-ribose] polymerase 1 (PARP1), β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), phospho-p53, total p53 (Cell Signaling Technology, Danvers, MA, USA), phospho-ataxia-telangiectasia mutated (ATM), and total ATM (Thermo Fisher Scientific). After incubation of membranes with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz biotechnology), bands were detected by utilizing enhanced chemiluminescence reagents (Amersham Biosciences, Uppsala, Sweden).

Flow cytometry

SNU-C5/WT and SNU-C5/5FUR cells were subjected to flow cytometry analysis using anti-PrP^C antibody (Santa Cruz Biotechnology). Flow cytometry was performed using a Cyflow Cube 8 (Partec, Münster, Germany). Data analysis was per-

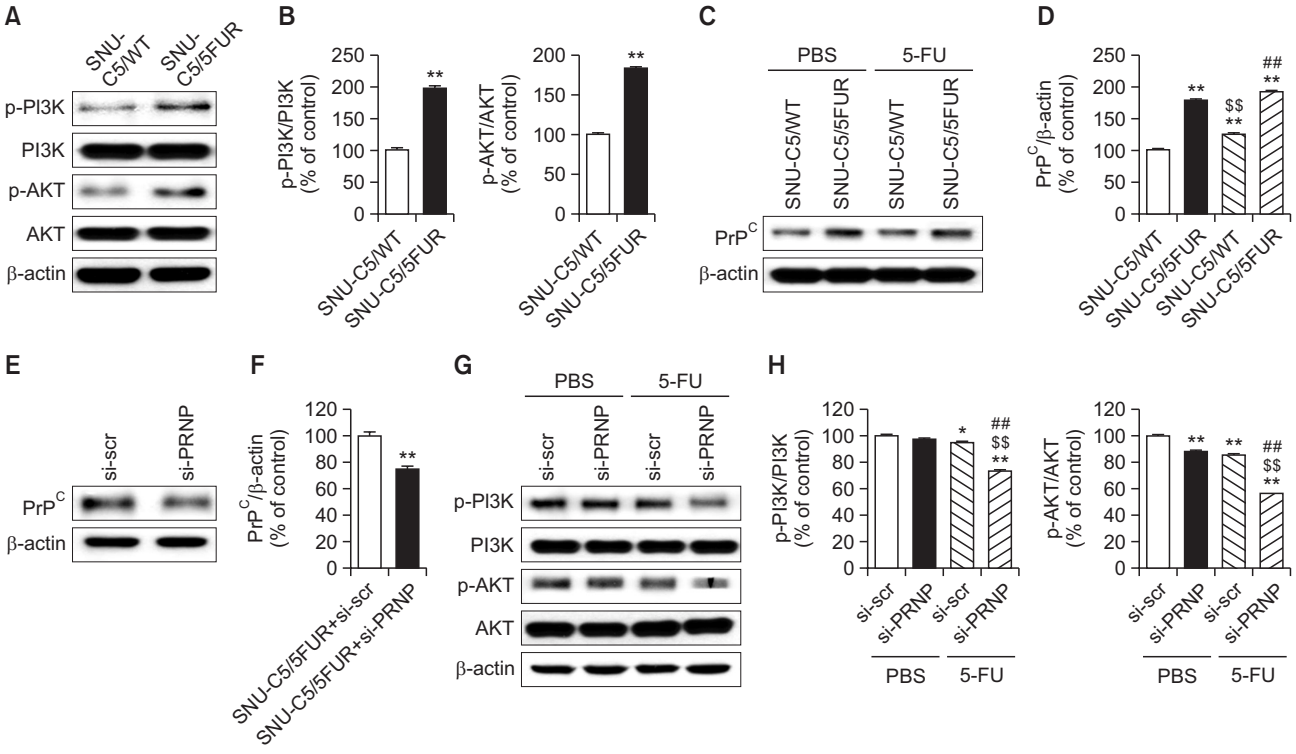

Fig. 2. The effect of PrP^C on cell survival-associated signaling pathways in colorectal cancer cells. (A) The level of phosphor-PI3K (p-PI3K) and phospho-AKT (p-AKT) in SNU-C5/WT and SNU-C5/5FUR cells. (B) The expression level of p-PI3K and p-AKT was determined by densitometry, relative to total PI3K and AKT expression, respectively. Measurements are reported as means ± SEM. ***p*<0.01 vs. SNU-C5/WT. (C) The level of PrP^C in SNU-C5/WT cells and SNU-C5/5FUR cells after treatment with 5-FU (140 μM). (D) The expression level of PrP^C was determined by densitometry, relative to β-actin expression. Measurements are reported as means ± SEM. ***p*<0.01 vs. SNU-C5/WT treated with PBS, $^{\$\$}p$<0.01 vs. SNU-C5/5FUR treated with PBS, and $^{\#\#}p$<0.01 vs. SNU-C5/WT treated with 5-FU. (E) The expression of PrP^C after transfection of SNU-C5/5FUR cells with *PRNP* siRNA (si-*PRNP*). (F) The expression level of PrP^C was determined by densitometry, relative to β-actin expression. Measurements are reported as means ± SEM. ***p*<0.01 vs. SNU-C5/5FUR transfected with scrambled siRNA (si-scr). (G) The level of phosphor-PI3K (p-PI3K) and phospho-AKT (p-AKT) in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (H) The expression level of p-PI3K and p-AKT was determined by densitometry, relative to total PI3K and AKT expression, respectively. Measurements are reported as means ± SEM. **p*<0.05, ***p*<0.01 vs. si-scr treated with PBS, $^{\$\$}p$<0.01 vs. si-*PRNP* treated with PBS, and $^{\#\#}p$<0.01 vs. si-scr treated with 5-FU.

formed using standard FSC Express (De Novo software; Los Angeles, USA).

Immunocytochemistry

SNU-C5/WT and SNU-C5/5FUR cells on the cover glass slide were fixed in 4% paraformaldehyde solution for 10 min. After washing three times in PBS, cells were blocked with 10% goat serum for 1 h, and then incubated with primary antibodies against PrP^C (Santa Cruz Biotechnology) at 4°C for 24 h. After washing three times in PBS, cells were incubated with secondary antibodies conjugated with Alexa-488 (Thermo Fisher Scientific) for 1 h. Immunostained slides were imaged by fluorescent microscope (ZEISS, Oberkochen, Germany).

Silencing of PrP^C expression by small interfering RNA

SNU-C5/5FUR and SNU-C5/Oxal-R cells were seeded in 60 mm plates and grown up to 75% confluence. Cells were transfected with siRNA in serum-free Opti-MEM (Thermo Fisher Scientific), using Lipofectamine 2000 (Thermo Fisher Scientific) by following the manufacturer's protocols. At 48 h after transfection, the level of PrP^C was analyzed by western blot. The siRNA used to target *PRNP* and a scrambled sequence were synthesized by Dharmacon (Lafayette, CO, USA).

Kinase assays

The cells were lysed using RIPA lysis buffer (Thermo Fisher Scientific). Cdk4 kinase assays were performed using a CKD4 Kinase Assay Kit (Cusabio, Baltimore, USA). Briefly, 10 mM ATP was added to 1.25 ml of 6 μM substrate peptide. The mixture was diluted with dH_2O to 2.5 ml to yield a 2× ATP/substrate cocktail. Then 1 ml of 10× kinase buffer was added to 1.5 ml of dH_2O to yield a 2.5 ml 4× reaction buffer and the enzyme was diluted in reaction buffer to give the reaction cocktail. The reaction cocktail was added to 12.5 μl/well of prediluted compound of interest (usually approximately 10 μM) and incubated for 5 min at room temperature. ATP/substrate cocktail was added to 25 μl/well preincubated reaction cocktail/compound. The final assay conditions for a 50-μl reaction were therefore 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM b-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 200 μM ATP, 1.5 μM peptide, and 50 ng CDK4 kinase. The reaction was incubated at room temperature for 30 min, then the stop buffer was added. From each reaction, 25 μl was transferred to a 96-well streptavidin-coated plate and incubated at room temperature for 1 h. Plates were washed three times with PBS. Phospho-Rb (Ser780) antibody was added at 100 μl/well and incubated at room temperature for 2 h. Plates were

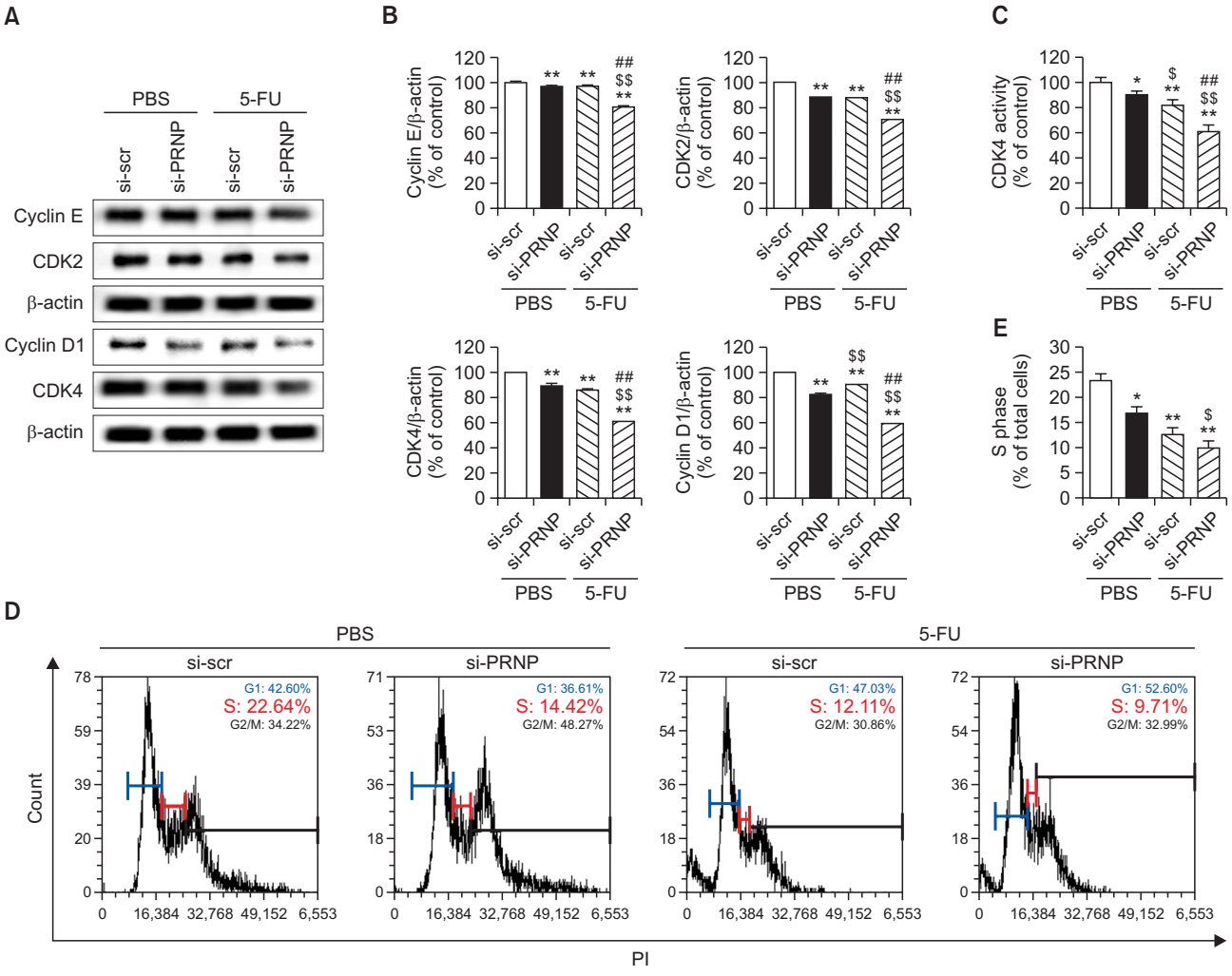

Fig. 3. The effect of PrP^C on proliferation in colorectal cancer cells. (A) The level of cyclin E, CDK2, cyclin D1, and CDK4 in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (B) The expression level of cyclin E, CDK2, cyclin D1, and CDK4 was determined by densitometry, relative to β-actin expression. (C) The kinase activity of CDK4 in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (D) The percentage of G1, S, and G2/M phase in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU was determined by flow cytometry analysis of PI-stained cells. (E) The quantitation of the percentage of cells in S phase. (B, C, E) Data are expressed as the mean ± SEM of three independent experiments. *p<0.05, **p<0.01 vs. scrambled siRNA-transfected SNUC5/5FUR (si-scr) treated with PBS, $^{\$}p$<0.05, $^{\$\$}p$<0.01 vs. si-PRNP treated with PBS, and $^{\#\#}p$<0.01 vs. si-scr treated with 5-FU.

washed three times with PBS. HRP-avidin was added to each well, and incubated at 37°C for 1 h. Plates were washed five times with PBS. TMB substrate and stop solution were added. The absorbance was read at 450 nm with a microtiter plate reader (Tecan Group AG, Mänedorf, Switzerland).

Cell cycle analysis

SNU-C5/5FUR cells were harvested and fixed with 70% ethanol at -20°C for 2 h. After washing twice with cold PBS, cells were subsequently incubated with RNase and propidium iodide (PI; Sigma). The cell cycle histograms were assessed using a Cyflow Cube 8 (Partec). Results were analyzed using the FCS Express software package (De Novo Software).

Propidium iodide/Annexin V flow cytometric analysis

Apoptosis of SNU-C5/5FUR and SNU-C5/Oxal-R cells was evaluated with a Cyflow Cube 8 (Partec) following staining of the cells with Annexin V- FITC and PI (Sigma). Data analysis was performed using the FCS Express software package (De Novo Software).

Statistical analyses

Data are expressed as means ± SEM. Statistical significance was assessed using the Student's *t* test, where p<0.05 was considered significant.

RESULTS

Expression of PrP^C in drug resistant CRC cells

Several studies have shown that the expression of PrP^C is increased in gastric, breast, and CRC cells (Yang *et al.*, 2014).

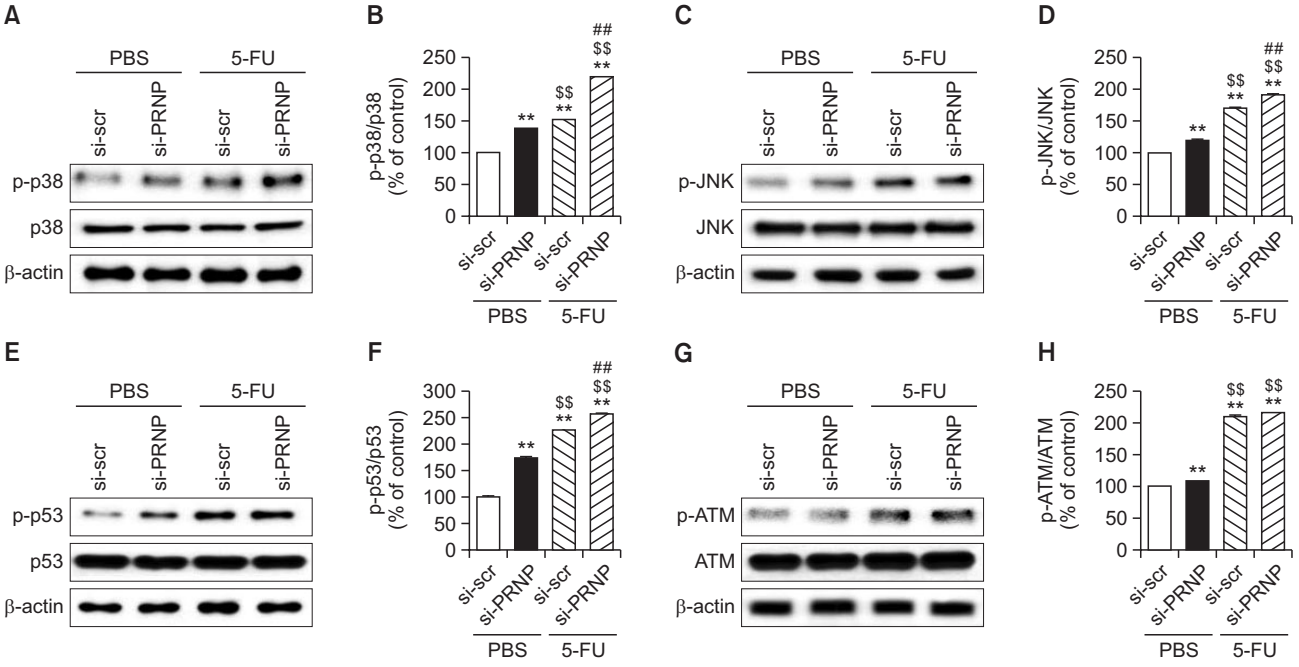

Fig. 4. The effect of PrP^C on cell stress-associated protein in colorectal cancer cells. (A) The phosphorylation of p38 in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (B) The level of p-p38 was determined by densitometry, relative to total p38 expression. (C) The phosphorylation of JNK in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (D) The level of p-JNK was determined by densitometry, relative to total JNK expression. (E) The phosphorylation of p53 in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (F) The level of p-p53 was determined by densitometry, relative to total p53 expression. (G) The phosphorylation of ATM in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (H) The level of p-ATM was determined by densitometry, relative to total ATM expression. (B, D, F, H) Data are expressed as the mean ± SEM of three independent experiments. $^{**}p<0.01$ vs. scrambled siRNA-transfected SNU-C5/5FUR (si-scr) treated with PBS, $^{\$\$}p<0.01$ vs. si-*PRNP* treated with PBS, and $^{\#\#}p<0.01$ vs. si-scr treated with 5-FU.

In addition, PrP^C is associated with multidrug resistance in gastric and breast cancer cells (Liang *et al.,* 2009; Cheng *et al.,* 2014). To explore whether PrP^C is upregulated in drug resistant CRC cells, we analyzed the expression of PrP^C in wild type (SNU-C5/WT) and 5-FU resistant colorectal cancer cell lines (SNU-C5/5FUR) by western blot assay (Fig. 1A). The expression level of PrP^C was significantly increased in SNU-C5/5FUR, compared with that in SNU-C5/WT (Fig. 1B). In addition, flow cytometry analysis showed that the ratio of PrP^C positive cells was significantly increased in SNU-C5/5FUR (Fig. 1C, 1D). Immunofluorescent staining also revealed an increased level of PrP^C in SNU-C5/5FUR (Fig. 1E). These results indicate that 5-FU-induced drug resistant CRC cells express high levels of PrP^C.

PrP^C regulates CRC cell survival via the PI3K-Akt axis

The PI3K-Akt signaling pathway is a pivotal regulator and central node in cell survival signaling downstream of growth factor, cytokines, and several cellular stimuli (Luo *et al.,* 2003; Manning and Cantley, 2007). To investigate whether PrP^C regulates the PI3K-Akt signaling pathway, we evaluated the phosphorylation of PI3K and Akt in SNU-C5/WT and SNU-C5/5FUR (Fig. 2A). The phosphorylation of PI3K and Akt were significantly enhanced in SNU-C5/5FUR, compared with that in SNU-C5/WT (Fig. 2B). To investigate whether the expression of PrP^C is associated with regulation of the PI3K-Akt signaling pathway in 5-FU-resistant CRC cells, we confirmed activation of PI3K-Akt in the presence or absence of 5-FU (140 μM). Treatment of SNU-C5/WT with 5-FU increased the level of PrP^C (Fig. 2C, 2D). In addition, the level of PrP^C was significantly increased in SNU-C5/5FUR in the presence and absence of 5-FU, compared with in SNU-C5/WT (Fig. 2C, 2D). The expression of PrP^C was suppressed using *PRNP* siRNA (si-*PRNP*) in SNU-C5/5FUR (Fig. 2E, 2F). After treatment of SNU-C5/5FUR with 5-FU, the suppression of PrP^C induced a decrease in phosphorylation of PI3K and Akt (Fig. 2G, 2H). These data suggest that PrP^C regulates the PI3K-Akt signaling pathway in 5-FU resistant CRC cells.

PrP^C is involved in cancer cell proliferation through the regulation of cell cycle-associated proteins

To confirm the effect of PrP^C on cell proliferation in 5-FU resistant CRC cells, the expression of cell cycle-associated proteins, such as cyclin E, CDK2, cyclin D1, and CDK4, was analyzed by western blot assay after treatment of SNU-C5/5FUR with 5-FU (Fig. 3A). SNU-C5/5FUR transfected with *PRNP* siRNA had a significant decrease in the levels of cyclin E, CDK2, cyclin D1, and CDK4 after treatment with 5-FU (Fig. 3B). In addition, SNU-C5/5FUR transfected with *PRNP* siRNA showed a significant decrease in CDK4 kinase activity after treatment with 5-FU (Fig. 3C). Furthermore, flow cytometry analysis for PI staining showed that PrP^C knockdown SNU-C5/5FUR exhibited a significant decrease in the ratio of S phase (Fig. 3D, 3E) after treatment with 5-FU. These findings suggest that PrP^C regulates proliferation of 5-FU resistant

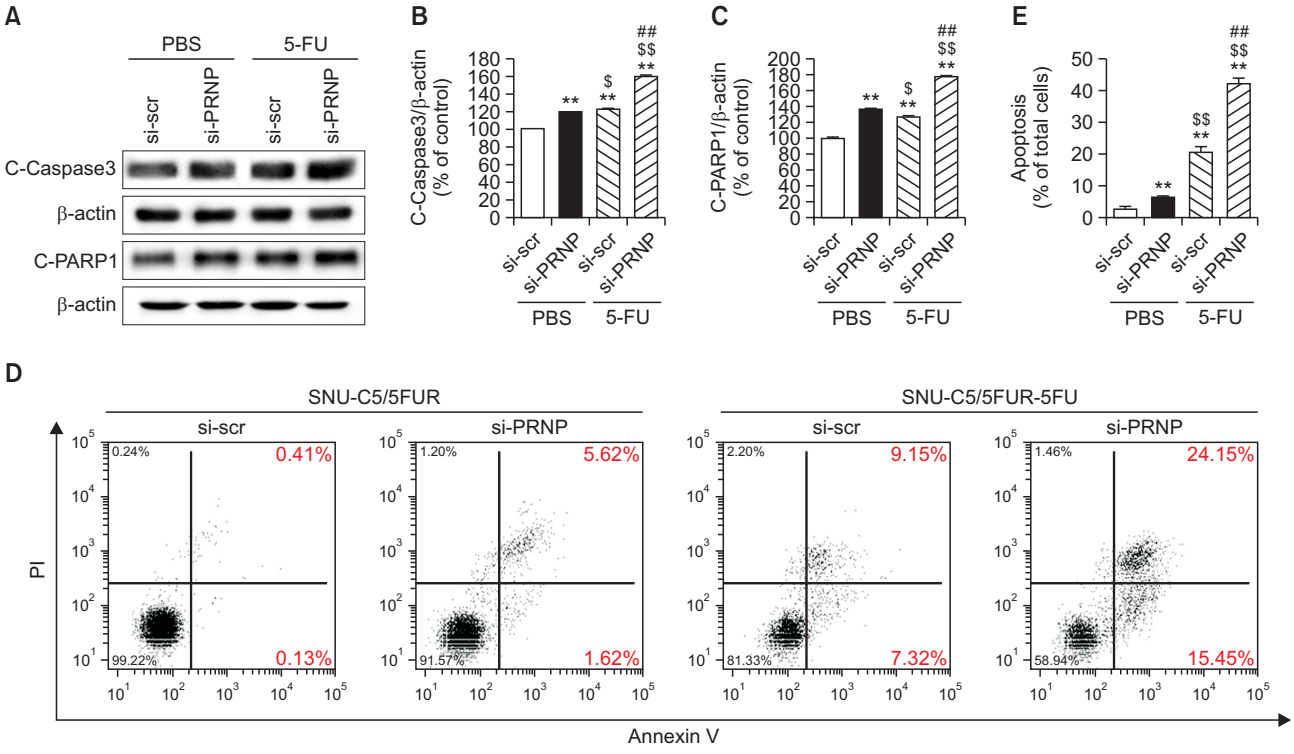

Fig. 5. The effect of PrP^C on apoptosis in colorectal cancer cells through inhibition of caspase-3 activation and PARP1 cleavage. (A) The level of cleaved caspase-3 (C-Caspase3) and cleaved PARP1 (C-PARP1) in SNU-C5/5FUR cells transfected with si-scr or si-*PRNP* after treatment with PBS or 5-FU (140 μM) for 48 h. (B) The level of C-Caspase3 was determined by densitometry, relative to β-actin expression. (C) The level of C-PARP1 was determined by densitometry, relative to β-actin expression. (D) Apoptosis was measured using flow cytometry analysis for PI/Annexin V staining. (E) The quantitation of the percentage of apoptotic cells. (B, C, E) Data are expressed as the mean ± SEM of three independent experiments. $^{**}p<0.01$ vs. scrambled siRNA-transfected SNU-C5/5FUR (siscr) treated with PBS, $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs. si-*PRNP* treated with PBS, and $^{\#\#}p<0.01$ vs. si-scr treated with 5-FU.

CRC cells via regulation of cell cycle-associated proteins in the presence of this anti-cancer drug.

PrP^C regulates anti-cancer drug-induced stress-associated signaling

The main mechanism of 5-FU is the inhibition of DNA synthesis and mRNA translation (Alvarez *et al.,* 2012). To explore whether PrP^C regulates stress-associated proteins in the presence of this drug, we assessed the phosphorylation of stress-associated proteins, including p38, JNK, p53, and ATM, after treatment of SNU-C5/5FUR with 5-FU. SNU-C5/5FUR transfected with *PRNP* siRNA exhibited a significant increase in the phosphorylation of p38, JNK, and p53 (Fig. 4A-4F) after treatment with 5-FU. However, although knockdown of PrP^C induced the phosphorylation of ATM in the PBS-treated group, there was no significant increase in the phosphorylation of ATM after treatment with 5-FU, implying that PrP^C is not involved in ATM signaling in the presence of 5-FU (Fig. 4G, 4H). These results indicate that PrP^C inhibits the activation of stress-associated signaling against an anti-cancer drug through suppression of p38, JNK, and p53 phosphorylation.

PrP^C inhibits apoptosis of anti-cancer drug resistant CRC cells via suppression of caspase-3 activation and PARP1 cleavage

To investigate whether PrP^C is involved in apoptosis of 5-FU resistant CRC cells, activation of caspase-3 and cleavage of PARP1 were assessed using a western blot after treatment of SNU-C5/5FUR with 5-FU (Fig. 5A). The levels of cleaved caspase-3 (C-Caspase-3) and PARP1 (C-PARP1) were significantly increased in treatment with 5-FU, compared with those in treatment with PBS (Fig. 5B, 5C). SNU-C5/5FUR transfected with *PRNP* siRNA had a significant increase in the levels of C-Caspase-3 and C-PARP1 after treatment with 5-FU (Fig. 5B, 5C). In addition, flow cytometry for PI/Annexin V staining showed that silencing of PrP^C significantly increased in the percentage of early and late apoptotic cells after treatment with 5-FU (Fig. 5D, 5E). These data indicate that PrP^C inhibits apoptosis of CRC cells in anti-cancer drug conditions via suppression of caspase-3 activation and PARP1 cleavage. In addition, SNU-C5/5FUR transfected with *PRNP* siRNA showed a significant decrease in the kinase activity of CDK4 after treatment with 5-FU (Fig. 3C).

siRRNP enhanced apoptosis in oxaliplatin-resistant CRC cells

We also confirmed apoptosis inhibition by the level of PrP^C expression in other drug-resistant CRC cells. We analyzed the expression of PrP^C in wild-type (SNU-C5/WT) and oxaliplatin-resistant colorectal cancer cell lines (SNU-C5/Oxal-R) by western blot assay (Fig. 6A, 6B). SNU-C5/Oxal-R transfected with *PRNP* siRNA showed a significant decrease in CDK4 kinase activity after treatment with oxaliplatin (Fig. 6C). In addition, flow cytometry for PI/Annexin V staining showed that

silencing of PrP^C significantly increased the percentage of early and late apoptotic cells after treatment with oxaliplatin (Fig. 6D).

DISCUSSION

In this study, we demonstrated that the level of PrP^C is increased in 5-FU resistant CRC cells, implying that PrP^C increased cell survival in anti-cancer drug conditions by regulating survival and apoptosis signaling pathways. After 5-FU treatment of SNU-C5/5FUR, a 5-FU resistant colorectal cancer cell line, silencing of PrP^C yielded the opposite effects, including inhibition of proliferation and augmentation of apoptosis. Previous studies have shown that PrP^C contributes to protection from oxidative stress, regulation of copper metabolism, cell cycle control, and cell adhesion (Brown *et al.,* 1997; McLennan *et al.,* 2004; Liang *et al.,* 2007; Malaga-Trillo *et al.,* 2009). In addition, PrP^C promotes pro-proliferative and anti-apoptotic effects in stem cells, such as hematopoietic stem cells, neural stem cells, mesenchymal stem cells, and human embryonic stem cells (Martin-Lanneree *et al.,* 2014). In cancer cell biology, PrP^C accelerates cell proliferation, invasion, metastasis, and resistance to cytotoxic drugs (Liang *et al.,* 2007; Mehrpour and Codogno, 2010; Wang *et al.,* 2012; Du *et al.,* 2013). Our results revealed for the first time that 5-FU resistant CRC cells express high levels of PrP^C. In gastric cancer cells, PrP^C expression enhanced drug resistance via an increase in Bcl-2 expression (Wang *et al.,* 2011). Moreover, treatment of HCT116 CRC cells with PrP-specific antibodies inhibited cancer cell growth and promoted the effects of anti-cancer drugs such as 5-FU, cisplatin, and doxorubicin (McEwan *et al.,* 2009). We also confirmed that the level of PrP^C expression was significantly increased in oxaliplatin-resistant SNU-C5 cells, compared with wild-type cells (Fig. 6A, 6B). Silencing of PrP^C decreased the kinase activity of CDK4 (Fig. 6C) and induced cell death in oxaliplatin-resistant CRC cells after treatment with oxaliplatin (Fig. 6D). These findings suggest that PrP^C could be a key protein for resistance to anti-cancer drugs in CRC cells.

The PI3K signaling pathway is involved in various cellular processes, including cell survival, metabolism, inflammation, motility, and cancer progression (Vanhaesebroeck *et al.,* 2010). In particular, the PI3K-Akt signal axis regulates survival and proliferation in cancer cells (Yuan and Cantley, 2008). In human lung cancer cells, Akt amplification plays a pivotal role in acquiring cisplatinum resistance (Liu *et al.,* 2007). Our results indicated that the phosphorylation of PI3K and Akt are significantly increased in 5-FU resistant CRC cells, and inhibition of PrP^C expression reduced the activation of PI3K and Akt in the presence of 5-FU. Activation of Akt also promotes cell proliferation through multiple downstream targets for cell cycle regulation (Manning and Cantley, 2007). We also revealed that downregulation of PrP^C inhibited proliferation via suppression of cell cycle-associated proteins, such as cyclin E, CDK2, cyclin D1, and CDK4 after 5-FU treatment of 5-FU resistant cells. PrP^C accelerated the G1/S transition via the PI3K-Akt-cyclin D1 axis (Liang *et al.,* 2007). These findings suggest that PrP^C plays pivotal roles in cell survival and proliferation in drug resistant CRC cells via regulation of the PI3K-Akt signaling pathway.

Activation of PI3K-Akt pathway by PrP^C expression promotes anti-apoptotic effect (Vassallo *et al.,* 2005). Conversely, downregulation of PrP^C promotes caspase-3 activation by reducing Akt activation (Weise *et al.,* 2006). In hypoxic conditions, increased PrP^C inhibits TRAIL-mediated apoptosis

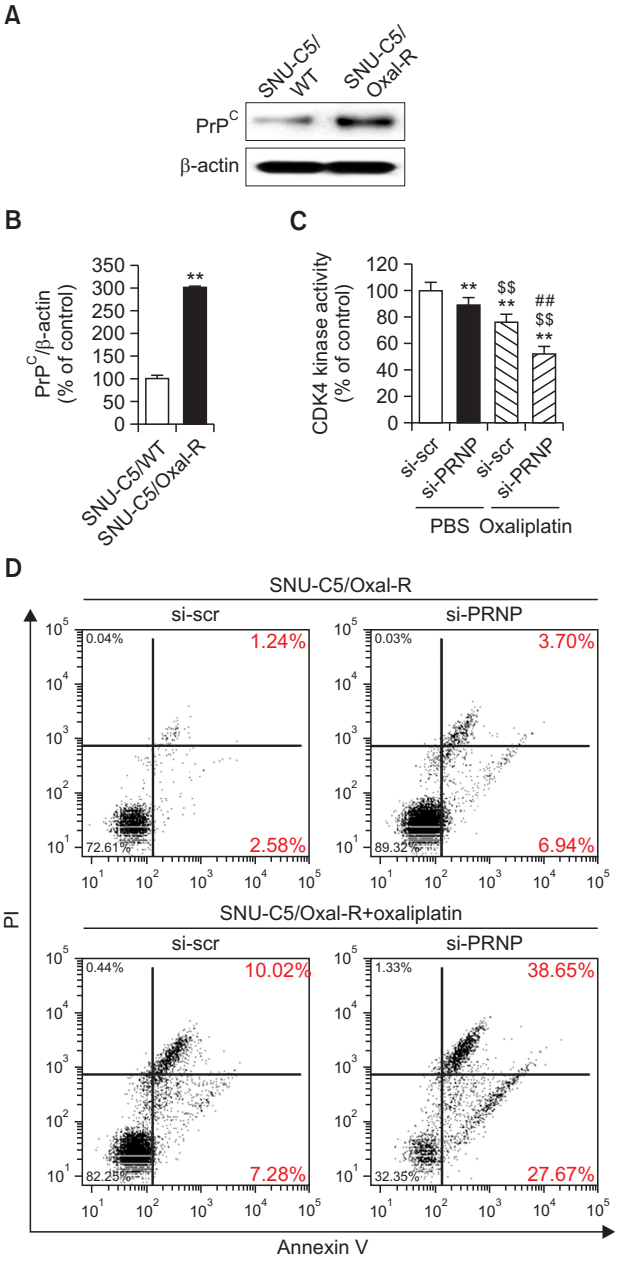

Fig. 6. The effect of PrP^C on proliferation and survival in oxaliplatin-resistant colorectal cancer cells. (A) Expression of PrP^C in SNU-C5/WT and oxaliplatin-resistant SNU-C5 (SNU-C5/Oxal-R) cells. (B) The expression level of PrP^C was determined by densitometry, relative to β-actin expression. (C) The kinase activity of CDK4 in SNU-C5/Oxal-R cells transfected with si-scr or si-*PRNP* after treatment with PBS or oxaliplatin (10 μM) for 48 h. (D) Apoptosis was measured using flow cytometry analysis for PI/Annexin V staining. (B, C) Data are expressed as the mean ± SEM of three independent experiments. $^{**}p<0.01$ vs. si-scr treated with PBS, $^{\$\$}p<0.01$ vs. si-*PRNP* treated with PBS, and $^{\#\#}p<0.01$ vs. si-scr treated with oxaliplatin.

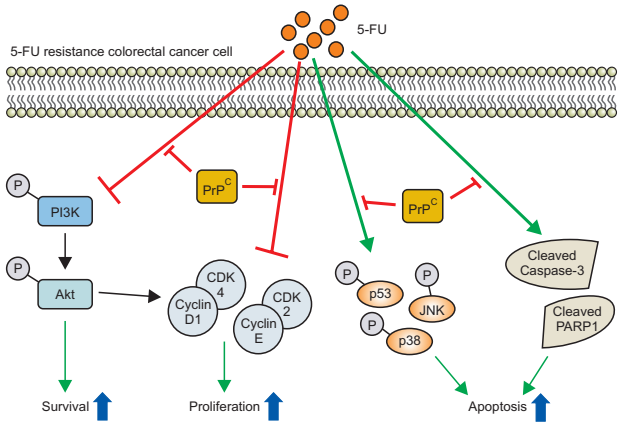

Fig. 7. Schematic illustrating the protective mechanism of PrP^C on 5-FU in drug resistant cancer cells. In 5-FU resistant cancer cells, the expression level of PrP^C is increased. In the presence of 5-FU, PrP^C promotes cell survival, proliferation, and apoptosis via activation of the PI3K-Akt signaling pathway, suppression of cell cycle-associated protein inhibition, and blockage of stress- and apoptosis- mediated protein activation.

(Park *et al.,* 2015). Our previous study revealed that silencing of PrP^C increased apoptosis of CRC cells. To explore the relationship between PrP^C and anti-cancer drug resistance, we showed that treatment with 5-FU induced the activation of stress-associated proteins, such as p38, JNK, and p53, and suppression of PrP^C triggered additional activation of these proteins. This additional activation implies that PrP^C inhibits the activation of stress-associated proteins in drug resistant CRC cells. Furthermore, PrP^C suppressed the activation of caspase-3 after 5-FU treatment of drug-resistant CRC cells. These results suggest that the level of PrP^C plays an important role in the development of chemoresistance by CRC cells.

Our findings reveal for the first time that 5-FU resistant colorectal cancer cells express high levels of PrP^C, and that PrP^C facilitates anti-cancer drug resistance by regulating survival, proliferation, and apoptosis signaling pathways (Fig. 7). Further studies are required to understand how the expression of PrP^C is induced during the acquisition of anti-cancer drug resistance. This study may help clarify the physiological function of PrP^C in drug resistant colorectal cancer cells. The strategy of targeting PrP^C may enable novel molecular target combination chemotherapies.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by the Soonchunhyang University Research Fund, a National Research Foundation grant funded by the Korean government (NRF-2016R1D-1A3B01007727), and a grant from the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (HI14C2253). The funders had no role in the study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

REFERENCES

Adelstein, D. J., Saxton, J. P., Rybicki, L. A., Esclamado, R. M., Wood, B. G., Strome, M., Lavertu, P., Lorenz, R. R. and Carroll, M. A. (2006) Multiagent concurrent chemoradiotherapy for locoregionally advanced squamous cell head and neck cancer: mature results from a single institution. *J. Clin. Oncol.* **24**, 1064-1071.

Alvarez, P., Marchal, J. A., Boulaiz, H., Carrillo, E., Velez, C., Rodriguez-Serrano, F., Melguizo, C., Prados, J., Madeddu, R. and Aranega, A. (2012) 5-Fluorouracil derivatives: a patent review. *Expert Opin. Ther. Pat.* **22**, 107-123.

Brown, D. R., Schulz-Schaeffer, W. J., Schmidt, B. and Kretzschmar, H. A. (1997) Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp. Neurol.* **146**, 104-112.

Cheng, Y., Tao, L., Xu, J., Li, Q., Yu, J., Jin, Y., Chen, Q., Xu, Z., Zou, Q. and Liu, X. (2014) CD44/cellular prion protein interact in multidrug resistant breast cancer cells and correlate with responses to neoadjuvant chemotherapy in breast cancer patients. *Mol. Carcinog.* **53**, 686-697.

Du, L., Rao, G., Wang, H., Li, B., Tian, W., Cui, J., He, L., Laffin, B., Tian, X., Hao, C., Liu, H., Sun, X., Zhu, Y., Tang, D. G., Mehrpour, M., Lu, Y. and Chen, Q. (2013) CD44-positive cancer stem cells expressing cellular prion protein contribute to metastatic capacity in colorectal cancer. *Cancer Res.* **73**, 2682-2694.

Hammond, W. A., Swaika, A. and Mody, K. (2016) Pharmacologic resistance in colorectal cancer: a review. *Ther. Adv. Med. Oncol.* **8**, 57-84.

Holohan, C., Van Schaeybroeck, S., Longley, D. B. and Johnston, P. G. (2013) Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* **13**, 714-726.

Li, Q. Q., Sun, Y. P., Ruan, C. P., Xu, X. Y., Ge, J. H., He, J., Xu, Z. D., Wang, Q. and Gao, W. C. (2011) Cellular prion protein promotes glucose uptake through the Fyn-HIF-2α-Glut1 pathway to support colorectal cancer cell survival. *Cancer Sci.* **102**, 400-406.

Liang, J., Ge, F., Guo, C., Luo, G., Wang, X., Han, G., Zhang, D., Wang, J., Li, K., Pan, Y., Yao, L., Yin, Z., Guo, X., Wu, K., Ding, J. and Fan, D. (2009) Inhibition of PI3K/Akt partially leads to the inhibition of PrP^C-induced drug resistance in gastric cancer cells. *FEBS J.* **276**, 685-694.

Liang, J. and Kong, Q. (2012) α-Cleavage of cellular prion protein. *Prion* **6**, 453-460.

Liang, J., Pan, Y., Zhang, D., Guo, C., Shi, Y., Wang, J., Chen, Y., Wang, X., Liu, J., Guo, X., Chen, Z., Qiao, T. and Fan, D. (2007) Cellular prion protein promotes proliferation and G1/S transition of human gastric cancer cells SGC7901 and AGS. *FASEB J.* **21**, 2247-2256.

Liu, L. Z., Zhou, X. D., Qian, G., Shi, X., Fang, J. and Jiang, B. H. (2007) AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. *Cancer Res.* **67**, 6325-6332.

Longley, D. B., Harkin, D. P. and Johnston, P. G. (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer* **3**, 330-338.

Luo, J., Manning, B. D. and Cantley, L. C. (2003) Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* **4**, 257-262.

Malaga-Trillo, E., Solis, G. P., Schrock, Y., Geiss, C., Luncz, L., Thomanetz, V. and Stuermer, C. A. (2009) Regulation of embryonic cell adhesion by the prion protein. *PLoS Biol.* **7**, e55.

Manning, B. D. and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274.

Martin-Lanneree, S., Hirsch, T. Z., Hernandez-Rapp, J., Halliez, S., Vilotte, J. L., Launay, J. M. and Mouillet-Richard, S. (2014) PrP^C from stem cells to cancer. *Front Cell Dev Biol* **2**, 55.

McEwan, J. F., Windsor, M. L. and Cullis-Hill, S. D. (2009) Antibodies to prion protein inhibit human colon cancer cell growth. *Tumour Biol.* **30**, 141-147.

McLennan, N. F., Brennan, P. M., McNeill, A., Davies, I., Fotheringham, A., Rennison, K. A., Ritchie, D., Brannan, F., Head, M. W., Ironside, J. W., Williams, A. and Bell, J. E. (2004) Prion protein accumulation and neuroprotection in hypoxic brain damage. *Am. J. Pathol.* **165**, 227-235.

Mehrpour, M. and Codogno, P. (2010) Prion protein: From physiology to cancer biology. *Cancer Lett.* **290**, 1-23.

Oliphant, R., Nicholson, G. A., Horgan, P. G., Molloy, R. G., McMillan, D. C. and Morrison, D. S. (2013) Deprivation and colorectal cancer surgery: longer-term survival inequalities are due to differential postoperative mortality between socioeconomic groups. *Ann. Surg. Oncol.* **20**, 2132-2139.

Park, J. Y., Jeong, J. K., Lee, J. H., Moon, J. H., Kim, S. W., Lee, Y. J. and Park, S. Y. (2015) Induction of cellular prion protein (PrP^C) under hypoxia inhibits apoptosis caused by TRAIL treatment. *Oncotarget* **6**, 5342-5353.

Pignon, J. P., Bourhis, J., Domenge, C. and Designe, L. (2000) Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. *Lancet* **355**, 949-955.

Siegel, R. L., Miller, K. D. and Jemal, A. (2017) Cancer Statistics, 2017. *CA Cancer J. Clin.* **67**, 7-30.

Tsukuda, M., Ishitoya, J., Matsuda, H., Horiuchi, C., Taguchi, T., Takahashi, M., Nishimura, G., Kawakami, M., Watanabe, M., Niho, T., Kawano, T., Ikeda, Y., Sakuma, Y., Shiono, O. and Komatsu, M. (2010) Randomized controlled phase II comparison study of concurrent chemoradiotherapy with docetaxel, cisplatin, and 5-fluorouracil versus CCRT with cisplatin, 5-fluorouracil, methotrexate and leucovorin in patients with locally advanced squamous cell carcinoma of the head and neck. *Cancer Chemother. Pharmacol.* **66**, 729-736.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M. and Bilanges, B. (2010) The emerging mechanisms of isoform-specific PI3K signalling. *Nat. Rev. Mol. Cell Biol.* **11**, 329-341.

Vassallo, N., Herms, J., Behrens, C., Krebs, B., Saeki, K., Onodera, T., Windl, O. and Kretzschmar, H. A. (2005) Activation of phosphatidylinositol 3-kinase by cellular prion protein and its role in cell survival. *Biochem. Biophys. Res. Commun.* **332**, 75-82.

Wang, J. H., Du, J. P., Zhang, Y. H., Zhao, X. J., Fan, R. Y., Wang, Z. H., Wu, Z. T. and Han, Y. (2011) Dynamic changes and surveillance function of prion protein expression in gastric cancer drug resistance. *World J. Gastroenterol.* **17**, 3986-3993.

Wang, Q., Qian, J., Wang, F. and Ma, Z. (2012) Cellular prion protein accelerates colorectal cancer metastasis via the Fyn-SP1-SATB1 axis. *Oncol. Rep.* **28**, 2029-2034.

Weise, J., Sandau, R., Schwarting, S., Crome, O., Wrede, A., Schulz-Schaeffer, W., Zerr, I. and Bahr, M. (2006) Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic caspase-3 activation, and exacerbation of ischemic brain injury. *Stroke* **37**, 1296-1300.

Yang, X., Zhang, Y., Zhang, L., He, T., Zhang, J. and Li, C. (2014) Prion protein and cancers. *Acta Biochim. Biophys. Sin. (Shanghai)* **46**, 431-440.

Yuan, T. L. and Cantley, L. C. (2008) PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510.

Yun, C. W., Yun, S., Lee, J. H., Han, Y. S., Yoon, Y. M., An, D. and Lee, S. H. (2016) Silencing Prion Protein in HT29 Human Colorectal Cancer Cells Enhances Anticancer Response to Fucoidan. *Anticancer Res.* **36**, 4449-4458.

Zhang, N., Yin, Y., Xu, S. J. and Chen, W. S. (2008) 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules* **13**, 1551-1569.