Melatonin and 5-fluorouracil co-suppress colon cancer stem cells by regulating cellular prion protein-Oct4 axis

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Running title: Melatonin inhibits CSC via the PrP<sup>C</sup>-Oct4 axis

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ABSTRACT

Melatonin suppresses tumor development. However, the exact relationship between melatonin and cancer stem cells (CSCs) is poorly understood. This study found that melatonin inhibits colon CSCs by regulating the PrP<sup>C</sup>-Oct4 axis. In specimens from patients with colorectal cancer, the expressions of cellular prion protein (PrP<sup>C</sup>) and Oct4 were significantly correlated with metastasis and tumor stages. Co-treatment with 5-fluorouracil (5-FU) and melatonin inhibited the stem cell markers Oct4, Nanog, Sox2, and ALDH1A1 by downregulating PrP<sup>C</sup>. In this way, tumor growth, proliferation, and tumor-mediated angiogenesis were suppressed. In colorectal CSCs, PRNP overexpression protects Oct4
against inhibition by 5-FU and melatonin. In contrast, Nanog, Sox2, and ALDH1A1 have no such protection. These results indicate that PrP\textsuperscript{C} directly regulates Oct4, whereas it indirectly regulates Nanog, Sox2, and ALDH1A1. Taken together, our findings suggest that co-treatment with anti-cancer drug and melatonin is a potential therapy for colorectal cancer. Furthermore, PrP\textsuperscript{C} maintains cancer stemness during tumor progression. Therefore, targeting the PrP\textsuperscript{C}-Oct4 axis may prove instrumental in colorectal cancer therapy.

1. INTRODUCTION

Colorectal cancer is a major global health concern. It is the second leading cause of cancer-related death in men and the third leading cause of cancer mortality in women worldwide.\textsuperscript{1} Over time, surgical skills, chemotherapy, and tumor molecule targeting have substantially improved. Nevertheless, therapeutic efficacy is still limited. When colorectal cancer is diagnosed in the early stages, the five-year survival rate is >90%. In contrast, metastasis reduces it to <10%.\textsuperscript{2} A major limiting factor in cancer therapy is drug resistance caused by genetic and biochemical modulation. Furthermore, cancers consist of heterogeneous populations which contribute to therapeutic failure and tumor progression. Cancer stem cells are a subpopulation of self-renewing cells and are also responsible for tumor development and cancer therapy failure.\textsuperscript{3,4} Therefore, the discovery and development of novel therapeutics and molecular targets are essential in the treatment of colorectal cancer.

Octamer-binding transcription factor 4 (Oct4) induces and maintains pluripotency.\textsuperscript{5} Forced expression of Oct4, Sox2, Klf4, and c-Myc reprogram pluripotency. However, only Oct4 cannot be substituted by any other factor in pluripotency induction.\textsuperscript{5} Oct4 gene knockdown suppresses tumor growth by inducing cancer stem cell-like apoptosis.\textsuperscript{6} In addition, Oct4
opposes chemotherapy-induced apoptosis in colon cancer stem cells. It also contributes to epithelial-mesenchymal transition, cell migration, invasion, and metastasis in colorectal cancer cells. Therefore, Oct4 plays a pivotal role in colorectal cancer development including tumor initiation, metastasis, and chemoresistance.

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone produced by the pineal gland and other tissues such as the bone marrow, liver, and gut. It regulates mainly circadian rhythms, sleep, and neuroendocrine activity. Nevertheless, recent studies have shown that it also has anti-tumor effects. It inhibits human colorectal carcinoma cell proliferation by increasing reactive oxygen species (ROS). It also induces apoptosis, autophagy, and senescence in these cells. In addition, melatonin causes cell death in drug-resistant colorectal cancer cells. Furthermore, it acts as an anti-metastasis agent by modulating cell-cell- and cell-matrix interactions, remodeling the extracellular matrix, reorganizing the cytoskeleton, regulating epithelial-mesenchymal transition, and suppressing angiogenesis. These findings suggest that melatonin is a potential therapy for colorectal cancer.

Cellular prion protein (PrP\(^C\)) is a highly ubiquitous glycoprotein expressed in nerve cells and other tissues. Accumulated evidence indicates that PrP\(^C\) participates in tumor behavior, including proliferation, apoptosis, invasion, metastasis, and chemoresistance. In addition, PrP\(^C\) contributes to cancer cell self-renewal. PrP\(^C\) is also a candidate biomarker for colorectal adenoma-to-carcinoma progression. Although PrP\(^C\) is involved in tumor biology, its mechanistic association with colorectal cancer stem cells is largely unexplored. A few
studies have previously shown that in glioblastoma, the expression of Oct4, Sox2, and PrP<sup>C</sup> was significantly increased in neurospheres (stem-like condition), compared with monolayer (non-stem condition).<sup>20</sup> Although the level of Oct4 during differentiation by retinoic acid was negatively correlated with the expression of PrP<sup>C</sup> in mouse embryonic stem cells, the knockdown of PrP<sup>C</sup> did not show the corresponding correlation with Oct4.<sup>21</sup> These findings suggest that the correlation between PrP<sup>C</sup> and Oct4 has not yet been fully defined in stem/cancer stem cell biology and needs to be investigated for addressing tumor development. To determine whether PrP<sup>C</sup> is involved in colorectal cancer development and colon cancer stem cell biology, the expression levels of PrP<sup>C</sup> and Oct4 in human colorectal cancer specimens were evaluated. To investigate whether melatonin inhibits colon cancer stem cells, its effects on PrP<sup>C</sup> and stem cell marker expression in colon cancer stem cells were investigated. Another objective was to examine the synergy between anti-cancer drug and melatonin. In the present study, the effect of co-treatment with 5-fluorouracil (5-FU) and melatonin in colon cancer stem cells was assessed in vitro and in a xenograft model in vivo. This study showed that PrP<sup>C</sup> protects 5-FU- and melatonin-mediated inhibition of Oct4 expression.

2. MATERIALS AND METHODS

2.1. Human colorectal cancer specimens

Forty-four colorectal carcinoma samples in an FFPE block were obtained from patients at Soonchunhyang University Cheonan Hospital in South Korea. Data included age, gender, pathological tumor (pT) stage, pathological lymph node (pN) stage, metastasis, blood- and lymphatic vessels, and TNM stage (Table 1 and 2; Supplemental Tables 1 and 2). All clinicopathological information was obtained by reviewing pathology reports and
hematoxylin-eosin (H&E)-stained sections. The present study and the data obtained from clinical samples were approved by the Ethics Committee of Cheonan Hospital, Soonchunhyang University.

2.2. Immunohistochemical analysis of human colorectal cancer specimens

Tissues from patients with colorectal cancer were fixed in formalin, embedded in paraffin, and sliced into 4-μm-thick sections. The samples were deparaffinized with xylene and hydrated using a graded ethanol series (100-70%) and distilled water. Endogenous peroxidase was blocked with 3% H₂O₂ (hydrogen peroxide) in methanol for 15 min at 25 °C. Tissues were incubated with anti-mouse PrP<sup>C</sup> (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-rabbit Oct4 (Stemgent, Beltsville, MD, USA) then with Envision HRP-labeled polymer (Dako, Carpinteria, CA, USA) as a secondary antibody. The antibodies were visualized with 3,3’-diaminobenzidine (DAB) counterstained with Mayer’s hematoxylin (Muto Pure Chemical Ltd., Tokyo, Japan). Tissues were mounted in Canada balsam (Sigma-Aldrich Corp., St. Louis, MO, USA). Colorectal tumor slides were evaluated independently by two experienced pathologists.

2.3. Human colon cancer cell line culture

A human colon cancer cell line (SNU-C5/WT), a 5-FU-resistant cell line (SNU-C5/5FUR), and an oxaliplatin-resistant cell line (SNU-C5/Oxal) were obtained from the Chosun University Research Center for Resistant Cells (Gwangju, Korea). The cells were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), L-glutamine (Sigma-Aldrich Corp.,

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seven and identified and measured using a visual inspection microscope (Olympus, Tokyo, Japan).

2.6. shRNA cell infection

PrP\textsuperscript{C} gene (\textit{PRNP})- and scrambled shRNAs were cloned into pGFP-C-shLenti (OriGene Technologies, Rockville, MD, USA). The \textit{PRNP} shRNAs were used to transfect the CSC (S707) lines. The cells were then incubated in a growth medium with puromycin (2 μg mL\textsuperscript{-1}) for 14 d. Resistant cells were then selected. Each individual cell was incubated in puromycin-free growth media. Western blot analysis (WB) was performed to evaluate PrP\textsuperscript{C} protein expression. \textit{PRNP} shRNA No. 1 was the most effective of the four clones at PrP\textsuperscript{C} knockdown and was also more effective than CSCs stably transfected with scrambled shRNA. The knockdown clone was used in subsequent research. The \textit{PRNP} shRNA No. 1 sequence was 5′-GGCCTATTACCAGAGAGGATCGAGCATGG-3′.

2.7. siRNA transfection

In accordance with the manufacturer’s protocols, Lipofectamine\textsuperscript{TM}2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect siRNAs into human CSCs (S707). The cells were first grown to 70% confluence in culture dishes and transfected for 48 h with SMART pool siRNAs (100 nM) specific to Oct4 mRNA.
2.8. PrP\textsuperscript{C} overexpression by plasmid DNA vector

Human CSCs (S707) were seeded in 60-mm plates and grown to 70% confluence. Cells were transfected with \textit{PRNP} pcDNA3.1-C-eGFP in serum-free Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) using Lipofectamine\textsuperscript{TM} 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. CSCs were transfected for 4 h and maintained in DMEM/F12 supplemented with 10% FBS, L-glutamine, EGF, bFGF, B-27, and antibiotics in an incubator at 37 °C under a humidified 5% CO\textsubscript{2} atmosphere. Total proteins were determined by WB. The pcDNA3.1-C-eGFP vector used to delivery \textit{PRNP} and an empty vector were obtained from GeneScript (Piscataway, NJ, USA).

2.9. Western blot analysis

Total cellular protein (20 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for antibody probing. After washing with TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.05% Tween-20), the membranes were blocked with 5% skim milk for 1 h then incubated with primary antibodies specific to PrP\textsuperscript{C}, Oct4, Nanog, Sox2, ALDH1A1, B-cell lymphoma 2 (BCL2), BCL-2-associated X protein (Bax), cleaved caspase-3, cleaved poly(ADP ribose) polymerase-1 (PARP-1), 5’ adenosine monophosphate-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), microtubule-associated proteins 1A/1B light chain 3B (LC3B), p62, autophagy-related protein 7 (ATG7), Beclin 1, α-tubulin, and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). After incubating the membranes with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), the bands were detected using enhanced chemiluminescence (ECL) reagents (Sigma-Aldrich Corp., St. Louis, MO, USA) in a darkroom.

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2.10. Flow cytometry analysis

Flow cytometry with PrPC, Oct4, Nanog, Sox2, and ALDH1A1 was used to identify human CSCs (S707). A two-color flow cytometry system (BD FACS Canto II; BD, Franklin Lakes, NJ, USA) was used to examine the immunostained cells. By comparing the results with the corresponding negative controls, the percentage of stained cells was calculated.

2.11. Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining assay

The hCSCs were washed once in phosphate-buffered saline (PBS) and incubated with 10 μM CFSE (Molecular Probes, Eugene, Oregon, USA) for 10 min at 37 °C. The CSFE-labeled cells were washed twice in PBS and analyzed using a flow cytometer (BD FACS Canto II; BD, Franklin Lakes, NJ, USA).

2.12. Propidium (PI)/Annexin V analysis

To determine the level of apoptosis, human CSCs were stained with PI and annexin V-FITC (De Novo Software, Los Angeles, CA) and evaluated using a Cyflow Cube 8 kit (Partec, Münster, Germany). Data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, CA).

2.13. Autophagy detection assay

Human CSCs were seeded in 60-mm dishes for 24 h and treated with 5-FU (1 μM) and melatonin (500 μM) for 72 h. Cellular autophagy was then analyzed by a FlowCellect™ Autophagy LC3 Antibody-Based Assay Kit (Sigma-Aldrich Corp., St. Louis, MO, USA) and
a flow cytometer (BD FACS Canto II; BD, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions.

2.14. Tumorigenesis in xenograft models

Mice developed tumors when they were subcutaneously injected with human CSCs (S707) either untransfected or transfected with PRNP overexpression. The mice were euthanized when the tumors reached a volume of 10 mm$^3$ 28 d after drug administration. The tumors were measured and histologically confirmed. Two perpendicular tumor dimensions (a = length, b = width) were measured with a Vernier caliper and used to calculate the volume (V; mm$^3$) according to the formula $V = (a \times b^2) / 2$. For the immunohistochemical analysis, the tumor specimens were fixed in 4% formaldehyde (Affymetrix, Santa Clara, CA, USA), embedded in paraffin, sliced into 4-μm-thick sections, stained by immunofluorescence, and viewed.

2.15. Immunofluorescence staining

For histological analysis, human colon- or mouse tumorigenesis model cancer tissue was fixed in 4% paraformaldehyde (Affymetrix, Santa Clara, CA, USA) and embedded in paraffin. The human colon cancer tissues were incubated with primary antibodies against PrP$^C$, Oct4, Nanog, Sox2, and ALDH1A1 (Santa Cruz Biotechnology, Dallas, TX, USA). Mouse tumorigenesis model cancer tissues were incubated with primary antibodies against proliferating cell nuclear antigen (PCNA), CD31, and α-SMA (Santa Cruz Biotechnology, Dallas, TX, USA). These tissues were incubated with secondary antibodies Alexa-488 and Alexa-594 (Thermo Fisher Scientific, Waltham, MA, USA) as appropriate. Nuclei were

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stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp., St. Louis, MO, USA). Immunostained samples were observed under a confocal microscope (Leica, Wetzlar, Germany).

2.16. Statistical analyses

All data were presented as means ± standard errors of the mean (SEM). All experimental results were evaluated by One-way ANOVA followed by a comparison of the treatment and control groups using the Bonferroni-Dunn test. Differences with $P$ values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Co-treatment with 5-FU and melatonin induces apoptosis of human colon cancer stem cells

To assess the effect of 5-FU and melatonin on colon cancer stem cells, colon cancer cell proliferation was analyzed by CFSE assay after treatment with 5-FU and/or melatonin (Figure 1A). The CFSE assay showed that co-treatment with 5-FU and melatonin significantly inhibited colon cancer stem cell proliferation relative to treatment with either 5-FU or melatonin alone (Figure 1B). The effects of 5-FU and melatonin on human colon cancer stem cell apoptosis were also assessed. The expression of the anti-apoptotic protein BCL2 was significantly decreased in response to co-treatment with 5-FU and melatonin compared with the control and treatment with either 5-FU or melatonin alone. In contrast, the expression levels of pro-apoptotic proteins like BAX, cleaved caspase-3, and cleaved PARP-1 significantly increased (Figures 1C and 1D). A flow cytometry assay for PI and annexin V

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indicated that co-treatment with 5-FU and melatonin significantly increased cancer stem cell apoptosis (Figures 1E and 1F). These findings indicate that co-treatment of colon cancer stem cells with 5-FU and melatonin suppresses proliferation and promotes apoptosis.

3.2. PrP<sup>C</sup> regulates colorectal cancer stem cell markers

To confirm the correlation between PrP<sup>C</sup> and cancer stem cell markers, the expression levels of PrP<sup>C</sup> and the cancer stem cell markers Oct4, Nanog, Sox2, and ALDH1A1 were assessed by WB in both normal- and human colon cancer tissues (Figure 2A). In colorectal cancer tissues, PrP<sup>C</sup> and stem cell marker expression levels were significantly higher than those in normal tissues (Figures 2B-2D). To determine the impact of these expressions in various colon cancer cell lines such as wild type (SNU-C5/WT), 5-FU- and oxaliplatin-resistant (SNU-C5/5FUR and SNU-C5/OxalR) and human cancer stem cells, the expressions of these proteins were confirmed (Figure 2E). Expression levels significantly increased in drug-resistant colorectal cancer cells and cancer stem cells relative to the wild type (Figures 2E-2H). To explore whether PrP<sup>C</sup> regulates cancer stem cell markers, the expressions of Oct4, Nanog, Sox2, and ALDH1A1 were evaluated after PRNP knockdown in human cancer stem cells (Figure 2I). These data indicated that PrP<sup>C</sup> silencing decreased human cancer stem cell markers (Figures 2J-2L). Therefore, PrP<sup>C</sup> may influence colorectal cancer stem cell characteristics.
3.3. Co-treatment with anti-cancer drug and melatonin inhibits the expression of human colon cancer stem cell markers by inhibiting PrP<sup>C</sup> expression

Previous studies showed that PrP<sup>C</sup> promotes drug resistance in colorectal cancer cells and melatonin induces cell death in them by regulating PrP<sup>C</sup>.<sup>14,24</sup> To verify whether co-treatment with anti-cancer drug, 5-FU, and melatonin inhibits colon cancer stem cell markers via PrP<sup>C</sup> expression, human colon cancer stem cells were analyzed by western blot and flow cytometry. PrP<sup>C</sup> expression level was significantly decreased in response to 5-FU/melatonin co-treatment relative to those of the control, treatment with 5-FU alone, or treatment with melatonin alone (Figures 3A-3D). Moreover, the expressions of Oct4, Nanog, Sox2, and ALDH1A1 significant decreased in response to co-treatment with 5-FU and melatonin compared with their expression levels in the control and the treatment with 5-FU alone (Figures 3E-3T). These results indicate that co-treatment with 5-FU and melatonin suppresses cancer stem cell marker expression by inhibiting PrP<sup>C</sup> expression.

3.4. PrP<sup>C</sup> protects against 5-FU- and melatonin-mediated degradation of Oct4 but not Nanog, Sox2, or ALDH1A1

In spheroid formation assay in human colon cancer stem cells, PRNP overexpression protected spheroid formation against 5-FU/melatonin co-treatment (Supplemental Figures 1A and 2A-2C). To verify whether PrP<sup>C</sup> controls the expression of Oct4, Nanog, Sox2, and ALDH1A1 against 5-FU and melatonin, PRNP was overexpressed in human colon cancer stem cells and marker expression levels were assessed by flow cytometry (Supplemental Figure 1A and Figures 4A-4H). PrP<sup>C</sup> overexpression blocked the Oct4 degradation induced by co-treatment with 5-FU and melatonin (Figures 4A and 4B). In contrast, the degradations of Nanog, Sox2, and ALDH1A1 were induced by 5-FU/melatonin co-treatment.

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Nevertheless, PrP<sup>C</sup> was overexpressed in cancer stem cells (Figures 4C-4H). In a previous study, heat shock protein family A member 1-like (HSPA1L) stabilized PrP<sup>C</sup> in colorectal cancer cells. The protective mechanism of PrP<sup>C</sup>-mediated Oct4 against 5-FU and melatonin was investigated by cancer stem cell marker immunoprecipitation in wild type and PRNP-overexpressing human colon cancer cells (Figure 4I). HSPA1L expression was significantly increased in PRNP-overexpressing cancer stem cells relative to the wild type (Supplemental Figure 1B). The immunoprecipitation assay showed that HSPA1L binding to Oct4 significantly increased in PRNP-overexpressing cancer stem cells compared with the wild type. On the other hand, HSPA1L binding to Nanog, Sox2, or ALDH1A1 did not significantly differ from the wild type (Figure 4I). These findings suggest that PrP<sup>C</sup> prevents Oct4 degradation by enhancing HSPA1L binding to Oct4.

3.5. PrP<sup>C</sup> and Oct4 regulate 5-FU- and melatonin-mediated autophagy in colon cancer stem cells

To determine whether PrP<sup>C</sup> and Oct4 are associated with autophagy against 5-FU- and melatonin stimulation, autophagy-mediated protein activation was measured. After co-treatment of human colon cancer stem cells with 5-FU and melatonin, AMPK phosphorylation increased and mTOR phosphorylation decreased (Figures 5A and 5B). However, PRNP overexpression blocked these effects (Figures 5A and 5B). Furthermore, Oct4 knockdown significantly increased AMPK phosphorylation relative to co-treatment with 5-FU and melatonin (Figures 5A and 5B). The expressions of autophagy-mediated proteins like LC3B, p62, ATG7, and Beclin 1 were confirmed by western blot analysis (Figures 5C and 5D). The expressions of LC3BII, ATG7, and Beclin 1 were significantly increased in response to co-treatment of colon cancer stem cells with 5-FU and melatonin.
compared with the untreated (Figures 5C and 5D). The expression of p62 was significantly decreased in response to 5-FU/melatonin co-treatment relative to the untreated (Figures 5C and 5D). All of these effects were blocked by PRNP overexpression. Oct4 knockdown significantly increased the expression levels of LC3BII, ATG7, and Beclin 1 relative to co-treatment with 5-FU and melatonin (Figures 5C and 5D). An autophagy detection assay showed that PRNP overexpression significantly blocked 5-FU and melatonin-mediated autophagy. It also revealed that Oct4 knockdown significantly accelerated 5-FU and melatonin-mediated autophagy compared to co-treatment with 5-FU and melatonin (Figures 5E and 5F). These results indicate that PrPC and Oct4 play key roles in 5-FU- and melatonin-mediated autophagy in colon cancer stem cells.

3.6. Co-treatment with 5-FU and melatonin inhibits colon cancer progression by regulating PrPC expression

To determine whether 5-FU/melatonin co-treatment influences colon cancer progression in vivo by regulating PrPC, tumor growth, tumor proliferation, and tumor-mediated angiogenesis were assessed with a xenograft model. Treatment with 5-FU and melatonin significantly decreased tumor volume compared with other groups (Figures 6A-6D). However, PRNP overexpression significantly promoted tumor growth relative to co-treatment with 5-FU and melatonin (Supplemental Figure 3, Figures 6A-6D). In tumor tissues, Oct4 expression decreased in response to co-treatment with 5-FU and melatonin whereas PRNP overexpression blocked the inhibitory effect of Oct4 (Figures 6E-6J). Immunofluorescence staining for PCNA showed that co-treatment with 5-FU and melatonin suppressed tumor cell proliferation in the xenograft model but PRNP overexpression protected tumor cell proliferation from the inhibitory effects of 5-FU and melatonin (Figures 6K and 6L).
Furthermore, 5-FU and melatonin inhibited tumor-mediated angiogenesis in tumor tissues whereas this effect was blocked by PRNP overexpression (Figures 6M and 6N). These findings indicate that co-treatment with 5-FU and melatonin inhibits colon cancer development by reducing PrP<sup>C</sup> expression.

### 3.7. Characterization of colorectal cancer specimens

To determine whether PrP<sup>C</sup> and Oct4 expressions are correlated with patient age, gender, pT stage, pN stage, metastasis, vascular invasion, lymphatic invasion, and stage, PrP<sup>C</sup> and Oct4 expression levels were measured by immunohistochemistry in colorectal cancer specimens. The proteins were either absent or only weakly detected in tumor-adjacent normal tissues (Supplemental Figure 4A). In contrast, they were moderately to strongly detected in tumor tissues (Supplemental Figures 4B and 4C). PrP<sup>C</sup> expression increased in 11/44 (25%) CRC samples. Oct4 expression increased in 14/44 (31.8%). The relationship between the protein levels and the clinicopathological parameter of the 44 CRC patients is presented in Supplemental Table 1 (PrP<sup>C</sup>) and Supplemental Table 2 (Oct4). PrP<sup>C</sup> was not correlated with patient age, gender, pT stage, pN stage, vascular invasion, or lymphatic invasion. However, PrP<sup>C</sup> expression was associated with metastasis and stage ($P = 0.012$ and $P = 0.039$, respectively). Oct4 expression was not correlated with age, gender, pN stage, vascular invasion, or stage. However, it was associated with pT stage ($P = 0.022$), metastasis ($P = 0.034$), and lymphatic invasion ($P = 0.030$). Table 1 and 2 show the relationship between PrP<sup>C</sup> and Oct4 expression and clinicopathologic parameter in the same tissue. As shown in Table 1 and 2, there were 11 (25%) cases in which PrP<sup>C</sup> and Oct4 were expressed simultaneously. In 30 tissues, neither PrP<sup>C</sup> nor Oct4 was expressed (68.2%). Both PrP<sup>C</sup>-positive and Oct4-negative expression (0%) and PrP<sup>C</sup>-negative and Oct4-positive expression
(6.8%) were confirmed. These data suggest that PrP<sup>C</sup> and Oct4 expressions were associated with metastasis in patients with colorectal cancer.

4. DISCUSSION

This study demonstrated that the co-treatment of colorectal cancer cells with 5-FU and melatonin inhibits tumor development by regulating PrP<sup>C</sup> and Oct4 expression. A recent study indicated that melatonin induces apoptosis of oxaliplatin-resistant colorectal cancer cells by suppressing PrP<sup>C</sup>.<sup>14</sup> Our findings indicate that 5-FU and melatonin inhibits PrP<sup>C</sup> expression in colon cancer stem cells. In addition, 5-FU and melatonin decreased the expression of the cancer stem cell markers Oct4, Sox2, Nanog, and ALDH1A1. Therefore, 5-FU and melatonin inhibited colorectal cancer cell development by inducing apoptosis and autophagy. PRNP knockdown in colon cancer stem cells significantly decreased the expressions of Oct4, Sox2, Nanog, and ALDH1A1 whereas PRNP overexpression only blocked 5-FU/melatonin-mediated Oct4 inhibition. Therefore, Oct4 expression is regulated in a PrP<sup>C</sup>-dependent manner. Although 5-FU and melatonin decreased the expressions of Sox2, Nanog, and ALDH1A1, PrP<sup>C</sup> expression also partially regulated them.

PrP<sup>C</sup> contributes to tumor survival, progression, migration, invasion, metastasis, and chemoresistance.<sup>18, 25-27</sup> Several studies have shown that PrP<sup>C</sup> participates in multicomplex protein formation and the regulation of several different signaling platforms. In this way, it modulates specific cancer cell behaviors.<sup>17, 28</sup> The interaction between PrP<sup>C</sup> and P-glycoprotein is associated with multidrug resistance in breast cancer.<sup>29</sup> PrP<sup>C</sup> interacts with CD44 in drug-resistant breast cancer cells, thereby enhancing both their proliferation and
migration. PrP expression in CD44-positive colon cancer stem cells is associated with metastasis. Cancer stem cell biology is an active and expanding area of cancer research. Cancer stem cells are a subpopulation capable of self-renewal and tumor propagation. Nevertheless, the correlation between PrPC and cancer stem cells remains to be determined. Our previous study has shown that high PrPC expression in colorectal cancer patients was significantly associated with metastasis risk, advanced clinical stages, and patient survival.

We identified the correlation between PrPC and colon cancer stem cells by confirming the association between PrPC and Oct4. The latter marker is at the top of a hierarchy of factors governing pluripotency regulation in cancer stem cells. Others include age, gender, pathological tumor stage, pathological lymph node stage, metastasis, vascular- and lymphatic invasion, and clinical stage (Table 1 and 2). These were evaluated in tissue specimens from 44 patients with colorectal cancer. PrPC and Oct4 co-expression was significantly associated with metastasis and clinical stage. These data suggest that it is through its interaction with Oct4 that PrPC is involved in metastasis.

Oct4 is a master regulator of cancer stem cell self-renewal and pluripotency. Breast cancer cells expressing high levels of Oct4 significantly increased tumorsphere formation relative to cells expressing only low Oct4 levels. Oct4-high cell populations had a higher expression level of cancer stem cell markers including CD133, CD34, and ALDH1. Oct4 is associated with self-renewal and survival of cancer stem cells through the Akt-Oct4 regulatory circuit. Oct4 is also associated with metastasis of cancer. Oct4 and Nanog co-expression promotes tumor migration, invasion, and metastasis via epithelial-mesenchymal transition. Cancer cell drug resistance is also mediated by relative Oct4 expression. Pancreatic cancer cells resistant to 5-FU induce Oct4 expression. Our results showed that co-treatment with 5-FU and melatonin decreased the expression of PrPC and the stem cell markers Oct4, Nanog.
Sox2, and ALDH1A1. In colon cancer stem cells, *PRNP* knockdown decreased stem cell marker expression. In colon cancer stem cells overexpressing *PRNP*, the inhibition of Oct4 expression by 5-FU and melatonin was blocked by PrP<sup>C</sup>. Despite *PRNP* overexpression, Nanog, Sox2, and ALDH1A1 expression levels were decreased by 5-FU/melatonin co-treatment. These findings indicate that PrP<sup>C</sup> directly regulates Oct4 expression but indirectly regulates the expression of Nanog, Sox2, and ALDH1A1. They also suggest that 5-FU and melatonin induce apoptosis and autophagy in colon cancer stem cells by regulating the Oct4-PrP<sup>C</sup> axis. However, the expressions of Nanog, Sox2, and ALDH1A1 are not regulated by the melatonin-PrP<sup>C</sup> axis but by melatonin itself in a PrP<sup>C</sup>-independent manner. Further study is required to elucidate the correlations between melatonin and the levels of Nanog, Sox2, and ALDH1A1.

PrP<sup>C</sup> was correlated with Oct4 expression in colon cancer stem cells. Nevertheless, there was no indication as to how PrP<sup>C</sup> regulated the level of Oct4. Therefore, we focused on the expression of heat shock proteins which act as molecular chaperones. Heat shock proteins are associated with tumor proliferation, metastasis, and the inhibition of apoptosis. A recent study showed that Oct4 suppression either upregulated or downregulated the expression of the HSP60, HSP70, and HSP90 families. PrP<sup>C</sup> and HSP70-HSP90-organizing protein are associated with glioblastoma proliferation and low patient survival rates. Our previous study has shown that HSPA1L stabilizes cellular prion protein. *PRNP* overexpression increased HSPA1L expression. To confirm the interaction between HSPA1L and stem cell markers, immunoprecipitation was performed. Oct4 binding to HSPA1L significantly increased in *PRNP* overexpression relative to the control. However, the binding of Nanog, Sox2, and ALDH1A1 with HSPA1L did not significantly differ between the control and *PRNP* overexpression. These findings suggest that in colon cancer stem cells, PrP<sup>C</sup>-induced...
HSPA1L protects Oct4 from degradation in response to co-treatment with 5-FU and melatonin.

Programmed cell death, including apoptosis, autophagy, and necroptosis play pivotal roles in metastasis.\textsuperscript{41} Under physiological conditions, autophagy inhibits apoptosis by caspase-mediated inhibition of autophagic process; however, excessive autophagy results in ‘autophagic cell death’ under certain circumstances, such as pathophysiological condition.\textsuperscript{41, 42} In cancer cells, multiple oncogene products, including PI3K, Akt, and p53 suppress autophagy, which is an oncogenic event.\textsuperscript{43} In human pancreatic cancer stem cells, rottlerin-induced autophagy leads to apoptotic cell death via the inhibition of PI3K-Akt-mTOR axis.\textsuperscript{44} In anti-cancer drug-treated cells, the formation of autophagosome induces the activation of caspase-8, leading to cell death.\textsuperscript{45} Several studies demonstrated that melatonin exhibits various anti-tumor properties in different cancers. Melatonin promotes apoptosis, autophagy, and senescence in human colorectal adenocarcinoma cells.\textsuperscript{13} It also inhibits cancer proliferation and metastasis by regulating cell-cell interaction, cytoskeleton reorganization, and epithelial-mesenchymal transition.\textsuperscript{11} It sensitizes drug-resistant cancer cells to therapeutic agents.\textsuperscript{14, 46, 47} Furthermore, melatonin suppresses tumor-associated angiogenesis by downregulating of vascular endothelial growth factor and endothelin-1.\textsuperscript{48, 49} Our study shows that co-treatment with 5-FU and melatonin induced apoptosis and autophagy, and suppressed tumor growth and angiogenesis in a xenograft model. These observed effects were blocked by PRNP overexpression. These findings suggest that the co-treatment of colorectal cancer cells with 5-FU and melatonin inhibits tumor development by regulating the PrPC-Oct4 axis.
In the present study, we demonstrated that melatonin influences apoptosis and autophagy in colon cancer stem cells by regulating the PrP\textsuperscript{C}-Oct4-HSPA1L axis (Figure 7). To the best of our knowledge, this is the first study to show the correlations among PrP\textsuperscript{C}, Oct4, metastasis and clinical stage in colorectal cancer patients. Furthermore, we identified a novel mechanism by which PrP\textsuperscript{C} regulates Oct4. In conclusion, co-treatment with an anti-cancer drug and melatonin is a potential therapeutic strategy for colorectal cancer patients. Moreover, PrP\textsuperscript{C} could be an effective therapeutic target for colorectal cancer stem cells.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

J.H.L. conceived and designed the study, acquired, analyzed, and interpreted the data, and drafted the manuscript. C.W.Y., Y.S.H., S.M.K., and H.J.K. acquired, analyzed, and interpreted the data and performed the statistical analysis. D.J.J., H.Y.K., and M.J.B. interpreted the data and drafted the manuscript. S.H.L. conceived and designed the study, acquired, analyzed, and interpreted the data, drafted the manuscript, procured funding, and supervised the study.

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REFERENCES


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FIGURE LEGENDS

Figure 1. Co-treatment with anti-cancer drug and melatonin induces apoptosis of human colon cancer stem cells.

(A) Flow cytometry analysis for CFSE in human colon cancer stem cells (hCSCs) treated with 5-FU and/or melatonin. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. (B) Percentage of CFSE-positive cells in hCSCs treated with 5-FU and/or melatonin. Values represent means ± SEM. **P < 0.01 vs. untreated cells (Control), #P < 0.05 vs. hCSCs treated with 5-FU (5-FU), ¤P < 0.05 vs. hCSCs treated with melatonin (Melatonin; n = 3). (C) Expression levels of BCL2, BAX, cleaved caspase-3, and cleaved PARP-1 in hCSCs treated with 5-FU and/or melatonin. (D) Quantification of expression levels of BCL2, BAX, BCL2/BAX, cleaved caspase-3, and cleaved PARP-1. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. Control, #P < 0.05, ##P < 0.01 vs. 5-FU, $$$P < 0.01 vs. Melatonin (n = 3). (E) Flow cytometry analysis of PI

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and Annexin V in hCSCs treated with 5-FU and/or melatonin. (F) Percentage of apoptotic cells in hCSCs treated with 5-FU and/or melatonin. Values represent means ± SEM. **P < 0.01 vs. Control, *P < 0.05 vs. 5-FU, **P < 0.01 vs. Melatonin (n = 3).

Figure 2. PrP<sup>C</sup> regulates colon cancer cell stemness markers.

(A) Expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in human normal- and colorectal cancer (hCRC) tissues. (B) Quantification of expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in human normal- and hCRC tissues. Values represent means ± SEM. **P < 0.01 vs. normal tissue (n = 3). (C) Immunofluorescence staining of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 (green) in hCRC tissues. Scale bar = 100 μm (n = 3). (D) Immunohistochemistry of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 (brown) in normal and hCRC tissues. Scale bar = 100 μm (n = 3). (E) Expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in wild type colorectal cancer cell line (SNU-C5/WT), 5-FU- and oxaliplatin-resistant colorectal cancer cell lines (SNU-C5/5FUR and SNU-C5/OxalR), and human cancer stem cell line (hCSC; S707). (F) Quantification of expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in human normal- and hCRC tissues. Values represent means ± SEM. *P < 0.05 and **P < 0.01 vs. SNU-C5/WT (n = 3). (G) Flow cytometry analysis for PrP<sup>C</sup> in SNU-C5/WT, SNU-C5/5FUR, SNU-C5/OxalR, and hCSCs. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. (H) Percentages of PrP<sup>C</sup>-positive cells in SNU-C5/WT, SNU-C5/5FUR, SNU-C5/OxalR, and hCSCs. Values represent means ± SEM. **P < 0.01 vs. SNU-C5/WT (n = 3). (I) Expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in hCSC treated with control shRNA (Control) and PRNP shRNA (shPRNP). (J) Quantification of expression levels of PrP<sup>C</sup>, Oct4, Nanog, Sox2, and ALDH1A1 in hCSC treated with control shRNA and shPRNP. Values represent means ± SEM.
SEM. **P < 0.01 vs. Control (n = 3). (K) Flow cytometry analysis for Oct4, Nanog, Sox2, and ALDH1A1 in hCSC treated with control shRNA and shPRNP. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. (L) Percentages of Oct4, Nanog, Sox2, and ALDH1A1-positive cells in hCSC treated with control shRNA and shPRNP. Values represent means ± SEM. **P < 0.01 vs. Control (n = 3).

Figure 3. Melatonin inhibits the levels of PrP<sup>C</sup> and stemness markers in human colon cancer cells.

(A–T) After treatment of human colon cancer cells with 5-FU (1 μM) and/or melatonin (500 μM) for 3 d, expression levels of PrP<sup>C</sup> (A), Oct4 (E), Nanog (I), Sox2 (M), and ALDH1A1 (Q) were assessed by western blot. Expression levels of PrP<sup>C</sup> (B), Oct4 (F), Nanog (J), Sox2 (N), and ALDH1A1 (R) were quantified by normalization relative to β-actin expression. Expression levels of PrP<sup>C</sup> (C), Oct4 (G), Nanog (K), Sox2 (O), and ALDH1A1 (S) were also assessed by flow cytometry. Expression levels of PrP<sup>C</sup> (D), Oct4 (H), Nanog (L), Sox2 (P), and ALDH1A1 (T) were quantified by percentage of indicated marker-positive cells. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. untreated cells (Control), #P < 0.05, ##P < 0.01 vs. hCSCs treated with 5-FU (5-FU), $P < 0.05, $$P < 0.01 vs. hCSCs treated with melatonin (melatonin; n = 3).
Figure 4. PrP<sup>C</sup> protects Oct4 from inhibition by co-treatment with 5-FU and melatonin via HSPA1L binding.

(A-H) Flow cytometry analysis of Oct4 (A and B), Nanog (C and D), Sox2 (E and F), and ALDH1A1 (G and H) in PRNP-overexpressing human colon cancer cells (hCSCs) treated with 5-FU and/or melatonin. Expression levels of Oct4 (B), Nanog (D), Sox2 (F), or ALDH1A1 (H) were quantified by percentage of indicated marker-positive cells, respectively. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. untreated cells (Control), #P < 0.05, ##P < 0.01 vs. hCSCs treated with 5-FU (5-FU), $P < 0.05, $$$P < 0.01 vs. hCSCs treated with melatonin (melatonin; n = 3). (I) Co-immunoprecipitation analysis of Oct4, Nanog, Sox2, and ALDH1A1 bound to HSPA1L in empty vector (Control) or PRNP-overexpressing hCSC. Expression level of HSPA1L was normalized by those of Oct4, Nanog, Sox2, and ALDH1A1, respectively. Values represent means ± SEM. **P < 0.01 vs. Control.

Figure 5. Co-treatment with anti-cancer drug and melatonin induces autophagy of human colon cancer stem cells through expression of PrP<sup>C</sup> and Oct4.

(A) After co-treatment with 5-FU and melatonin (5-FU + Mel), expression levels of p-AMPK, AMPK, p-mTOR, and mTOR in PRNP-overexpressing or Oct4 knockdown human colon cancer cells (hCSCs). (B) Quantification of expression levels of p-AMPK and p-mTOR in PRNP-overexpressing or Oct4 knockdown (si-Oct4) hCSCs. Values represent means ± SEM. **P < 0.01 vs. untreated cells, ##P < 0.01 vs. hCSC treated with 5-FU + Mel, $$$P < 0.01 vs. PRNP-overexpressing hCSC treated with 5-FU + Mel (n = 3). (C) After co-treatment with 5-FU and melatonin, expression levels of LC3B, p62, ATG7, and Beclin 1 in PRNP-
overexpressing or Oct4 knockdown human colon cancer cells (hCSCs). (D) Quantification of expression levels of LC3BII, p62, ATG7, and Beclin 1 in PRNP-overexpressing or Oct4 knockdown hCSCs. Values represent means ± SEM. **$P < 0.01$ vs. untreated cells, $^P < 0.05$, $^P < 0.01$ vs. hCSC treated with 5-FU + Mel, $^P < 0.05$, $^$P < 0.01 vs. PRNP-overexpressing hCSC treated with 5-FU + Mel (n = 3). (E) After treatment with 5-FU and/or melatonin, autophagy detection assay with flow cytometry in PRNP-overexpressing or Oct4 knockdown hCSCs. Gray lines indicate cells stained with isotype-matched immunoglobulin G (IgG) as a negative control. (F) Percentage of autophagy-positive cells in PRNP-overexpressing or Oct4 knockdown hCSCs. Values represent means ± SEM. **$P < 0.01$ vs. untreated cells (Control), $^P < 0.05$, $^P < 0.01$ vs. hCSC treated with 5-FU, $^P < 0.05$ vs. hCSC treated with melatonin, $^&^P < 0.01$ vs. PRNP-overexpressing hCSC treated with 5-FU and melatonin (n = 3).

**Figure 6. Co-treatment with anti-cancer drug and melatonin inhibits colorectal cancer development in a xenograft model.**

(A) Representative photographs of tumor growth of normal- or PRNP-overexpressing human cancer stem cells in a murine xenograft model after treatment with 5-FU and/or melatonin. (B and C) Graph shows tumor sizes of individual mice after tumor cell injection (n = 10) and when tumor volume = 10 mm$^3$, 28 d after drug administration. Values represent means ± SEM. $^P < 0.05$, $^P < 0.01$ vs. untreated (Control), $^P < 0.05$, $^P < 0.01$ vs. treatment with 5-FU (5-FU), $^P < 0.05$, $^$P < 0.01 vs. treatment with melatonin (Melatonin), $^P < 0.05$, $^&^P < 0.01$ vs. PRNP-overexpressing group. (D) Individual mouse weights after termination of experiment (n = 10). (E and F) Immunofluorescence staining of Oct4 (E) and PrP$^C$ (F; green) in colorectal cancer tissues of a murine xenograft model. Scale bar = 50 μm (n = 5). (G)
Expression of Oct4 in tumor tissues of a murine xenograft model (n = 3). (H) Quantification of expression levels of Oct4 in tumor tissues. Values represent means ± SEM. **P < 0.01 vs. Control, #P < 0.05, ##P < 0.01 vs. 5-FU, $$$P < 0.01 vs. Melatonin, &&P < 0.01 vs. 5-FU + Melatonin. (I) Expression of PrP\textsuperscript{C} in tumor tissues of a murine xenograft model (n = 3). (J) Quantification of expression levels of PrP\textsuperscript{C} in tumor tissues. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. Control, ##P < 0.01 vs. 5-FU, $P < 0.05, $$$P < 0.01 vs. Melatonin, &&P < 0.01 vs. 5-FU + Melatonin. (K) Immunofluorescence staining of PCNA (green) in colorectal cancer tissues. Scale bar = 50 μm (n = 5). (L) Quantification of PCNA-positive cells in colorectal cancer tissues. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. Control, ##P < 0.01 vs. 5-FU, $$$P < 0.01 vs. Melatonin, &&P < 0.01 vs. 5-FU + Melatonin. (M) Immunofluorescence staining of CD31 and α-SMA (red) in colorectal cancer tissues. Scale bar = 50 μm (n = 5). (N) Quantification of CD31- and α-SMA-positive cells in colorectal cancer tissues. Values represent means ± SEM. *P < 0.05, **P < 0.01 vs. Control, ##P < 0.01 vs. 5-FU, $$$P < 0.01 vs. Melatonin, &&P < 0.01 vs. 5-FU + Melatonin.

Figure 7. Scheme illustrating how PrP\textsuperscript{C} regulates Oct4 expression in colon cancer stem cells.

In patients with metastatic phase colorectal cancer (left panel), the expression levels of PrP\textsuperscript{C} and Oct4 are increased. The expression level of Oct4 is regulated by PrP\textsuperscript{C} upregulation via HSPA1L binding. Melatonin inhibits colon cancer stem cell stemness markers like Oct4, Nanog, Sox2, and ALDH1A1 by inhibiting PrP\textsuperscript{C} expression and inducing colon cancer cell apoptosis (right panel). Oct4 expression is regulated in a melatonin-mediated, PrP\textsuperscript{C}-dependent manner whereas Nanog, Sox2, and ALDH1A1 are indirectly regulated by the
melatonin-PrP<sup>C</sup> signal pathway. This scheme suggests that melatonin potentially inhibits colorectal cancer development and that PrP<sup>C</sup> participates in Oct4 regulation.

Supplemental Figure Legend

Supplemental Figure 1. Expression of PrP<sup>C</sup> and HSPA1L in PRNP-overexpressing colon cancer stem cells.

(A and B) Expression levels of PrP<sup>C</sup> (A) and HSPA1L (B) in PRNP-overexpressing human colon cancer stem cells (hCSCs). Quantification of PrP<sup>C</sup> (A) and HSPA1L were normalized by β-actin expression. Values represent means ± SEM. **<i>P</i> < 0.01 vs. hCSCs treated with empty vector (Control).

Supplemental Figure 2. Effect of co-treatment with anti-cancer drug and melatonin on spheroid formation in human colorectal cancer stem cells.

(A) Images of spheroid formation of normal or PRNP-overexpressing human colorectal cancer stem cells (hCSCs) after treatment with 5-FU and/or melatonin. (B) Quantification of spheroid number in hCSCs. Values represent means ± SEM. **<i>P</i> < 0.01 vs. Control, ##<i>P</i> < 0.01 vs. 5-FU, $$$<i>P</i> < 0.01 vs. Melatonin. (C) Quantification of spheroid size in hCSCs. Values represent means ± SEM. **<i>P</i> < 0.01 vs. Control, ## <i>P</i> < 0.01 vs. 5-FU.
Supplemental Figure 3. Co-treatment with anti-cancer drug and melatonin in a xenograft model.

Representative photograph of tumor growth of normal or PRNP-overexpressing human colorectal cancer stem cells in a murine xenograft model, after treatment with 5-FU and/or melatonin.

Supplemental Figure 4. Expression of PrPC and Oct4 in normal- and tumor tissues isolated from colorectal cancer patients.

Immunohistochemistry for PrPC and Oct4 in normal (A, PrPC-/Oct4-) and tumor tissues (B, PrPC-/Oct4+; C, PrPC+/Oct4+) from patients with colorectal cancer.
Table 1. Association of clinicopathological features and PrP<sup>C</sup>- and Oct4-matched expression

<table>
<thead>
<tr>
<th>Clinicopathological Factor</th>
<th>PrP&lt;sup&gt;C&lt;/sup&gt;- and Oct4-Matched</th>
<th>Total (n = 44)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n = 33)</td>
<td>Positive (n = 11)</td>
<td></td>
</tr>
<tr>
<td>Age, Mean (SD)</td>
<td>64.0 (10.2)</td>
<td>56.1 (15.5)</td>
<td>62.8 (11.8)</td>
</tr>
<tr>
<td>Gender, N(%)</td>
<td>0.296</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>15 (45.5)</td>
<td>7 (63.6)</td>
<td>22 (50.0)</td>
</tr>
<tr>
<td>M</td>
<td>18 (54.5)</td>
<td>4 (36.4)</td>
<td>22 (50.0)</td>
</tr>
<tr>
<td>pT stage, N (%)</td>
<td>0.071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>2</td>
<td>9 (27.3)</td>
<td>1 (9.1)</td>
<td>10 (22.7)</td>
</tr>
<tr>
<td>3</td>
<td>23 (69.7)</td>
<td>7 (62.6)</td>
<td>30 (68.2)</td>
</tr>
<tr>
<td>4</td>
<td>1 (3.0)</td>
<td>2 (18.2)</td>
<td>3 (6.8)</td>
</tr>
<tr>
<td>pN stage, N (%)</td>
<td>0.322</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>16 (48.5)</td>
<td>4 (36.4)</td>
<td>20 (45.5)</td>
</tr>
<tr>
<td>1</td>
<td>10 (30.3)</td>
<td>6 (54.5)</td>
<td>16 (36.4)</td>
</tr>
<tr>
<td>2</td>
<td>7 (21.2)</td>
<td>1 (9.1)</td>
<td>8 (18.2)</td>
</tr>
<tr>
<td>Metastasis, N(%)</td>
<td>0.012</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>33 (100.0)</td>
<td>9 (81.8)</td>
<td>42 (95.5)</td>
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<tr>
<td>Positive</td>
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<td>2 (18.2)</td>
<td>2 (4.5)</td>
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<td>Vascular invasion, N (%)</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>28 (84.8)</td>
<td>9 (81.8)</td>
<td>37 (84.1)</td>
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<tr>
<td>Positive</td>
<td>5 (15.2)</td>
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<td>7 (15.9)</td>
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<td>Lymphatic invasion, N (%)</td>
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<td>7 (63.6)</td>
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<tr>
<td>Positive</td>
<td>6 (18.2)</td>
<td>4 (36.4)</td>
<td>10 (22.7)</td>
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<tr>
<td>Stage, N (%)</td>
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<td>4 (9.1)</td>
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<td>II</td>
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<td>16 (36.4)</td>
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<tr>
<td>III</td>
<td>15 (45.5)</td>
<td>4 (36.4)</td>
<td>19 (43.3)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0.0)</td>
<td>2 (18.2)</td>
<td>2 (4.5)</td>
</tr>
</tbody>
</table>

pT: pathological tumor stage according to the American Joint Committee on Cancer TNM classification system; pN: pathological lymph node stage according to the American Joint Committee on Cancer TNM classification system.
Table 2. The percentage of PrP<sup>C</sup> and Oct4 expression in tissue specimens from patients from colorectal cancer

<table>
<thead>
<tr>
<th>PrP&lt;sup&gt;C&lt;/sup&gt; expression</th>
<th>Oct4 expression</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>11/44 (25.0)</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>0/44 (0.0)</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>3/44 (6.8)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>30/44 (68.2)</td>
</tr>
</tbody>
</table>

+: Positive, -: Negative
Figure 4

A

B

C

D

E

F

G

H

I

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**Figure 7**

**Metastatic phase colorectal cancer**

- Increase in stemness markers
- Augmentation of survival
- Inhibition of autophagy

**Co-treatment with anti-cancer drug and melatonin**

- Decrease in stemness markers
- Inhibition of Oct4 expression via PrP<sup>C</sup> directly
- Inhibition of Nanog, Sox2, ALDH1 via melatonin
- Suppression of survival and tumor progression