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Melatonin Promotes Apoptosis of Colorectal Cancer Cells *via* Superoxide-mediated ER Stress by Inhibiting Cellular Prion Protein Expression

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Abstract. *Background/Aim: Melatonin, an endogenously secreted indoleamine hormone that is produced in the pineal gland, is known to possess antitumor effect via various mechanisms including induction of apoptosis and pro-oxidant effects in various cancer cells, including colorectal cancer (CRC). In our study, we hypothesized that melatonin enhances the anticancer effects via suppression of PrP^C and PINK1 levels, thereby increasing superoxide production. Materials and Methods: To investigate the antitumor effects of melatonin in CRC cells, assessing its effects on mitochondrial dysfunction, production of superoxide, induction of endoplasmic reticulum stress, and cellular apoptosis were assessed. Results: Melatonin was found to decrease the expression of PrP^C and PINK1, and increase superoxide accumulation in the mitochondria. In addition, PrP^C-knockdown potentiated the effects of melatonin resulting further in significantly reduced expression of PINK1 and increased superoxide production in CRC. si-PRNP-transfected CRC cells treated with melatonin increased the production of intracellular superoxide and induced endoplasmic reticulum stress associated protein, and apoptosis. Conclusion: Melatonin induces mitochondria-mediated cellular apoptosis in CRC cancer cells via a PrP^C-dependent pathway. PrP^C knockdown combined with melatonin amplifies the effects of melatonin, suggesting a novel therapeutic strategy in targeting CRC cells.*

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Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in the world, placing it as the most common cause of mortality (1). Uncontrolled proliferation of cancer cells is the hallmark of cancer cells and along with this, the ability to metastasis and recurrence makes it excessively hard to treat (2). In response, patients go on extensive chemotherapies inducing apoptosis of target cancer cells in the body, as well as surgery to remove the cancer tumors (3). There has been a proliferation of research in drugs used to control CRC and the progression of the disease. For example, oxaliplatin, a bifunctional alkylating agent, can inhibit DNA replication and transcription by covalently binding to DNA, resulting in induction of apoptosis (4). However, drug resistance of cancer cells is a major limitation in chemotherapy (5). Although the mutation of anticancer drug target protein, defective DNA damage repair, enhanced anticancer drug efflux, and alternative compensating signaling pathways are potential mechanisms of drug resistance, their toxicity could lead to numerous side-effects, even favoring growth of cancer cells by killing the patients' immune system. Thus, natural compounds have been known to be highly desirable clinically due to their minimal side-effects which are more prevalent in chemotherapies and other drugs. Not surprisingly, there exist studies that show natural compounds with anti-tumor effects to be a promising strategy for cancer prevention and therapy (6-9). More studies on novel compounds and therapeutic mechanisms associated with the compounds are desirable for developing efficient therapeutic strategies targeting CRC.

In addition to genetic factors that contribute to the occurrence of the disease, factors such as diet, physical activity, and physiological homeostasis are among the important factors associated with occurrence of CRC (10, 11). Melatonin (N-acetyl-5-methoxytryptamine) is a cytokine secreted by the pineal gland in the body during sleep that has been known to possess multiple physiological homeostatic

functions and offer numerous benefits associated with sleep. More specifically, melatonin plays a role in induction of sleep, regulation of circadian rhythm, immunomodulation, reduction of oxidation (12, 13). Interestingly, studies show that melatonin possesses anti-cancer effects, and in a study related to colorectal cancer, it was shown to induce senescence and apoptosis of the CRC cells (14, 13). Cancer cells treated with melatonin show enhanced production of reactive oxygen species (ROS), resulting in dysfunctional mitochondria and thus decreased cell viability (15-17). Such accumulating evidence instigate further investigations on the effects of melatonin in inducing apoptosis in cancer cells *via* ROS generation and mitochondria mediated apoptosis.

A previous study suggested that melatonin exerts its effects through cellular prion protein (PrP^C)-dependent pathway (18). PrP^C, a cell surface protein tethered to the membrane by glycosylphosphatidylinositol anchor, is highly expressed in cells of various tissues including nervous, muscle and heart tissue (19-21). Although mutated prion proteins are most widely known for their neurodegenerative properties, several studies have indicated that cellular prion proteins possess a crucial role in various physiological functions, such as cell proliferation, apoptosis, invasion, metastasis and drug resistance in various cancers (22, 23). Our previous studies demonstrated that PrP^C is related to cancer proliferation, and that knockdown of PrP^C inhibits colorectal cancer cell growth (7) and induces tumor cell death (24). PrP^C levels in cancer are associated with ROS-mediated endoplasmic reticulum (ER) stress, thereby destroying mitochondria and ER to induce cell death (25). PTEN-induced putative kinase 1 (PINK1), a protein located to the mitochondrial outer membrane, maintains mitochondria homeostasis through mitochondria quality control pathway (26). In damaged mitochondria, PINK1 recruits parkin and autophagy related proteins and results in degradation of damaged mitochondria through autophagy (27). In a recent study, it was shown that PINK1 functions as a regulator of cell cycle progression and has tumor promoting properties (28).

In this study, the effects of melatonin on endoplasmic reticulum stress and apoptosis of colorectal cancer cells was examined. Furthermore, the role of PrP^C in mitochondria-mediated apoptosis by induction of superoxide and deduction of PINK1 expression was studied.

Materials and Methods

Preparation of Melatonin. Melatonin was obtained from Sigma (St. Louis, MO, USA). Melatonin powder was dissolved in 100% ethanol, filter-sterilized through a 0.45 μm pore filter (Sartorius Biotech GmbH, Gottingen, Germany), and aliquots were stored at 4˚C until use.

Cells and cell culture. The human colorectal cancer cell line (SNU-C5/WT) was obtained from the Chosun University Research Center for Resistant Cells (Gwangju, Republic of 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.

Cell viability assay. Exponentially growing colon cancer cells (SNU-C5/WT) were subconfluently incubated in 96-well plates with melatonin (0-1 mM) for 24 h and various periods of time (0-24 h). Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay, which is based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2-tetrazolium to formazan by mitochondrial dehydrogenase. Formazan was quantified by measuring absorbance at 570 nm, using a microplate reader (Tecan, Männedorf, Switzerland).

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-protein kinase R-like endoplasmic reticulum kinase (PERK), PERK, phospho-eukaryotic initiation factor 2-alpha (eIF2α), eIF2α. activating transcription factor 4 (ATF4), phospho-c-JUN N-terminal kinase (JNK), total JNK, phospho-p38, Bcl-2-associated X protein (BAX), cleaved Caspase-3, cleaved poly [ADP-ribose] polymerase 1 (PARP1), β-Actin (Santa Cruz Biotechnology, Dallas, TX, USA), PTEN-induced putative kinase 1 (PINK1), CCAAT-enhancer-binding protein homologous protein (CHOP) (Novus Biologicals, Littleton, CO, USA). After incubation of membranes were incubated with peroxidase conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific). Protein bands were visualized by utilizing enhanced chemiluminescence reagents (Amersham Biosciences, Uppsala, Sweden).

Inhibition of PrP^C expression by small RNA interference. SNU-C5/WT cells were seeded in 60 mm dishes, grown up to 75% confluence, and transfected with siRNA in serum-free Opti-MEM (Gibco BRL) by utilizing Lipofectamine 2000 following the manufacturer's instructions (Thermo Fisher Scientific). At 48 h after transfection, the cells were treated with melatonin for 24 h. Total protein extracts were analyzed by western blot. The siRNA used to target PRNP (The PRNP-siRNA no. 1 sequence: 5'-UCACCGAGACCGACGUUAA-3', no. 2 sequence: 5'-GAUCGAGCAUGGUCCUCUU-3', no. 3 sequence: 5'-AGAUGUGUAUCACCCAGUA-3' and no. 4 sequence: 5'-GACCG UUACUAUCGUGAAA-3') and a scrambled sequence (The scramble-siRNA no. 1 sequence: 5'-UGGUUUACAUGUCGACUAA-3', no. 2 sequence: 5'-UGGUUUACAUGUUGUGUGA-3', no. 3 sequence: 5'-UGGUUUACAUGUUUUCUGA-3', and no. 4 sequence: 5'-UGGUUUACAUGUUUUCCUA-3') was purchased by Dharmacon (Lafayette, CO, USA).

Flow cytometric analysis. To measure mitochondrial superoxide, SNU-C5/WT cells were stained with MitoSOX red (Thermo Fisher Scientific) for 30 min at 37˚C and washed with PBS three times. To confirm apoptosis, the cells were stained with Annexin V-fluorescein isothiocyante (FITC) and propidium iodide (PI) (Sigma). Each sample was quantitively analyzed using CyFlow Cube 8

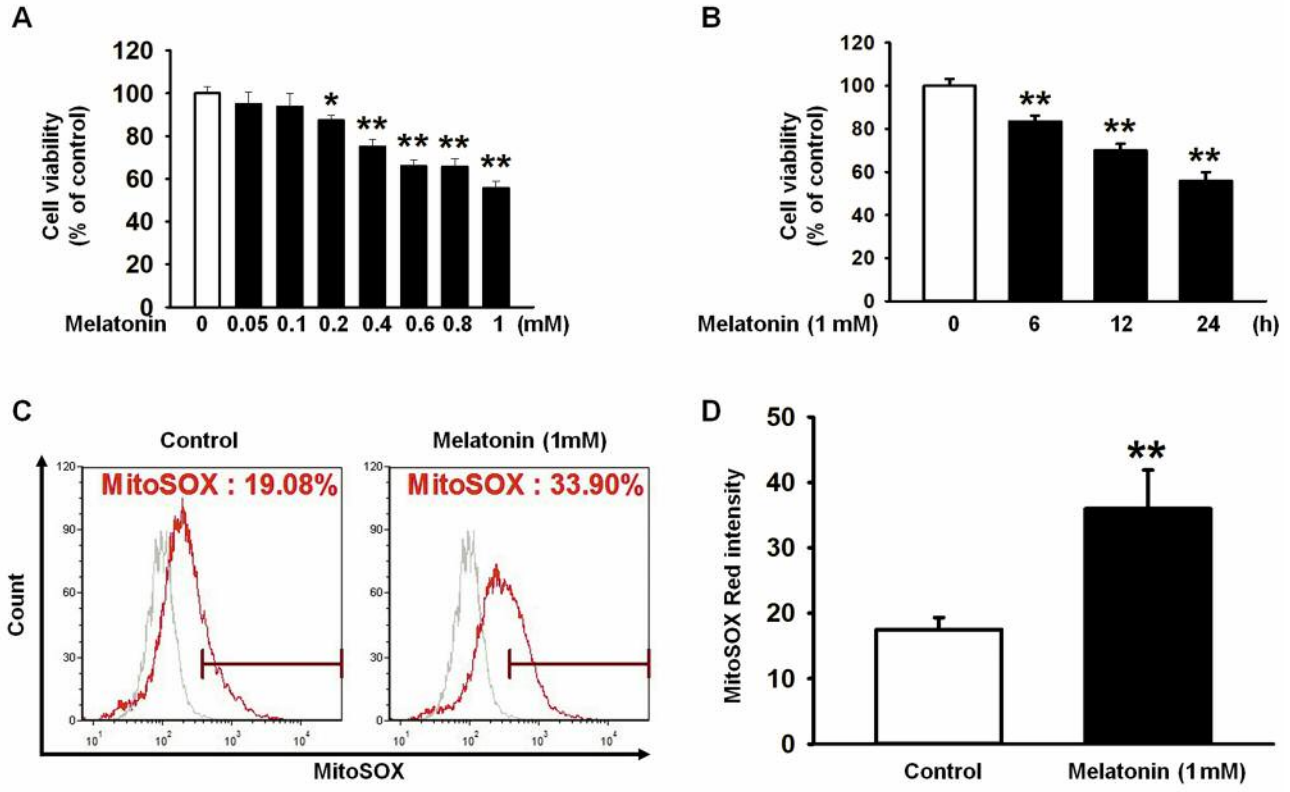

Figure 1. *Melatonin-mediated inhibition of cell viability and induction of mitochondrial superoxide production in SNU-C5/WT colorectal cancer cells. Cell viability was measured using the MTT assay. (A) SNU-C5/WT colon cancer cells were treated with melatonin (0-1 mM) for 24 h (n=3). (B) SNU-C5/WT colon cancer cells were treated with melatonin (1 mM) for various periods of time (0, 6, 12 and 24 h) (n=3). Values represent the means±SEM. *p<0.05 vs. control and **p<0.01 vs. control. (C) SNU-C5/WT colon cancer cells were treated with melatonin (1 mM) for 24 h and superoxide production was assayed using flow cytometric analysis of MitoSOX red staining. (D) The quantitation of the percentage of mitochondrial superoxide levels (n=3). Values represent means±SEM. **p<0.01 vs. control.*

(Sysmex Partec, Münster, Germany). Data analysis was performed using the FCS Express software package (De Novo Software).

Statistical analyses. Results are expressed as the mean±SEM and analyzed by ANOVA. In some experiments, this was followed by a comparison of the treatment mean with the control using a Bonferroni-Dunn test. Data were considered to be significantly different at values of $p<0.05$.

Results

Melatonin decreases cell viability and increases production of superoxide in SNU-C5/WT cells. To evaluate the effect of melatonin on SNU-C5/WT cells, cell viability was measured by utilizing the MTT assay after treatment of SNU-C5/WT cells with melatonin (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM) at various periods of time (0, 6, 12 and 24 h). Melatonin treatment was shown to reduce the cell viability of SNU-C5/WT cells in dose- and time-dependent manner (Figure 1A and B). In order to assess superoxide production in SNU-C5/WT cells treated with Melatonin, the production of mitochondrial superoxide was examined by flow cytometric analysis (Figure 1C and D). The level of mitochondrial superoxide was significantly increased after treatment with melatonin (1 mM). These results suggest that melatonin inhibits cell viability of SNU-C5/WT cells and increases superoxide production.

Melatonin suppresses the expression of PrP^C and PINK1 and increases the production of mitochondrial superoxide in SNU-C5/WT cells. In order to assess the effect of melatonin on the expression of PrP^C and PINK1 in SNU-C5/WT, the cells were treated with various concentrations of melatonin (0, 0.2, 0.6 and 1 mM) at various time periods (0, 6, 12 and 24 h). Western blot analysis was performed to evaluate the expression of PrP^C and PINK1. The results show that PrP^C and PINK1 expression are significantly reduced upon melatonin (1 mM) treatment for 24 h (Figure 2A-D).

Next, to measure the expression of PrP^C and PINK1, SNU-C5/WT cells were transfected with si-PRNP following

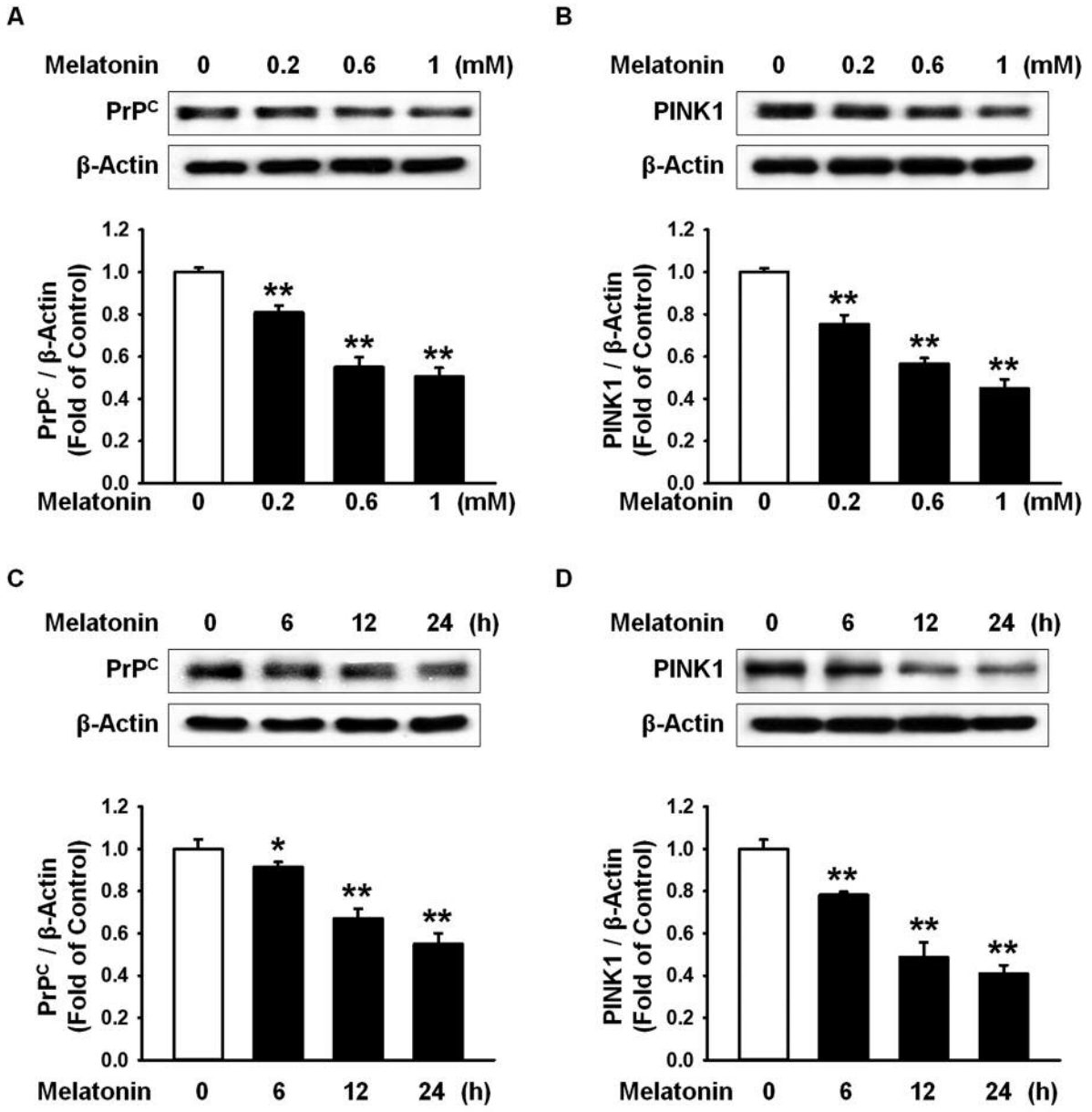

Figure 2. *Melatonin mediated inhibition of cellular prion protein (PrP^C) and PINK1 in SNU-C5/WT colorectal cancer cells. SNU-C5/WT cells were treated with different concentrations of melatonin. PrP^C (A) and PTEN-induced putative kinase 1 (PINK1) (B) was determined by western blot analysis. The bar graph indicates the quantification of expression levels as determined by densitometry relative to β-actin (n=3). Values represent the means±SEM. **p<0.01 vs. control. (C) SNU-C5/WT cells were treated with melatonin for different periods of time. PrP^C (C) and PINK1 (D) were determined by western blot analysis. The bar graph indicates the quantification of expression levels as determined by densitometry relative to β-actin (n=3). Values represent the means±SEM. **p<0.01 vs. control.*

treatment with melatonin (1 mM). In si-PRNP transfected cells, melatonin treatment significantly reduced the expression of PrP^C and PINK1 (Figure 3A and B). In order to evaluate the effect of melatonin on the production of superoxide in SNU-C5/WT flow cytometric analysis was performed. MitoSOX red staining showed that knockdown of PrP^C induced significant production of mitochondrial superoxide upon melatonin treatment compared to control (Figure 3C and D). These results demonstrate that the knockdown of PrP^C significantly potentiated the effects of melatonin.

Melatonin induces endoplasmic reticulum (ER) stress in SNU-C5/WT cells through regulation of PrP^C expression. To assess the effect of melatonin-induced ER stress in si-

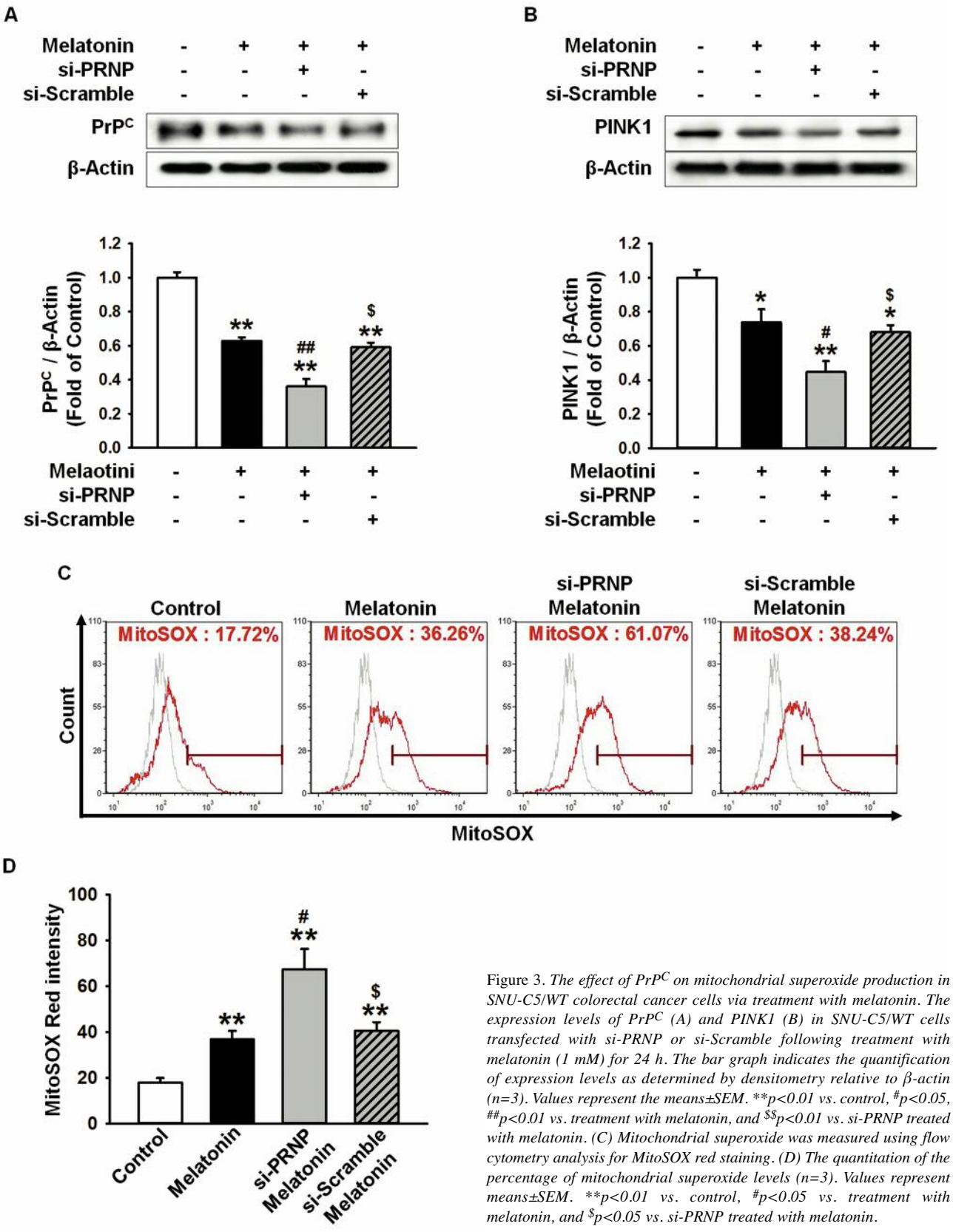

Figure 3. *The effect of PrP^C on mitochondrial superoxide production in SNU-C5/WT colorectal cancer cells via treatment with melatonin. The expression levels of PrP^C (A) and PINK1 (B) in SNU-C5/WT cells transfected with si-PRNP or si-Scramble following treatment with melatonin (1 mM) for 24 h. The bar graph indicates the quantification of expression levels as determined by densitometry relative to β-actin (n=3). Values represent the means±SEM. **p<0.01 vs. control, #p<0.05, ##p<0.01 vs. treatment with melatonin, and $$p<0.01 vs. si-PRNP treated with melatonin. (C) Mitochondrial superoxide was measured using flow cytometry analysis for MitoSOX red staining. (D) The quantitation of the percentage of mitochondrial superoxide levels (n=3). Values represent means±SEM. **p<0.01 vs. control, #p<0.05 vs. treatment with melatonin, and $p<0.05 vs. si-PRNP treated with melatonin.*

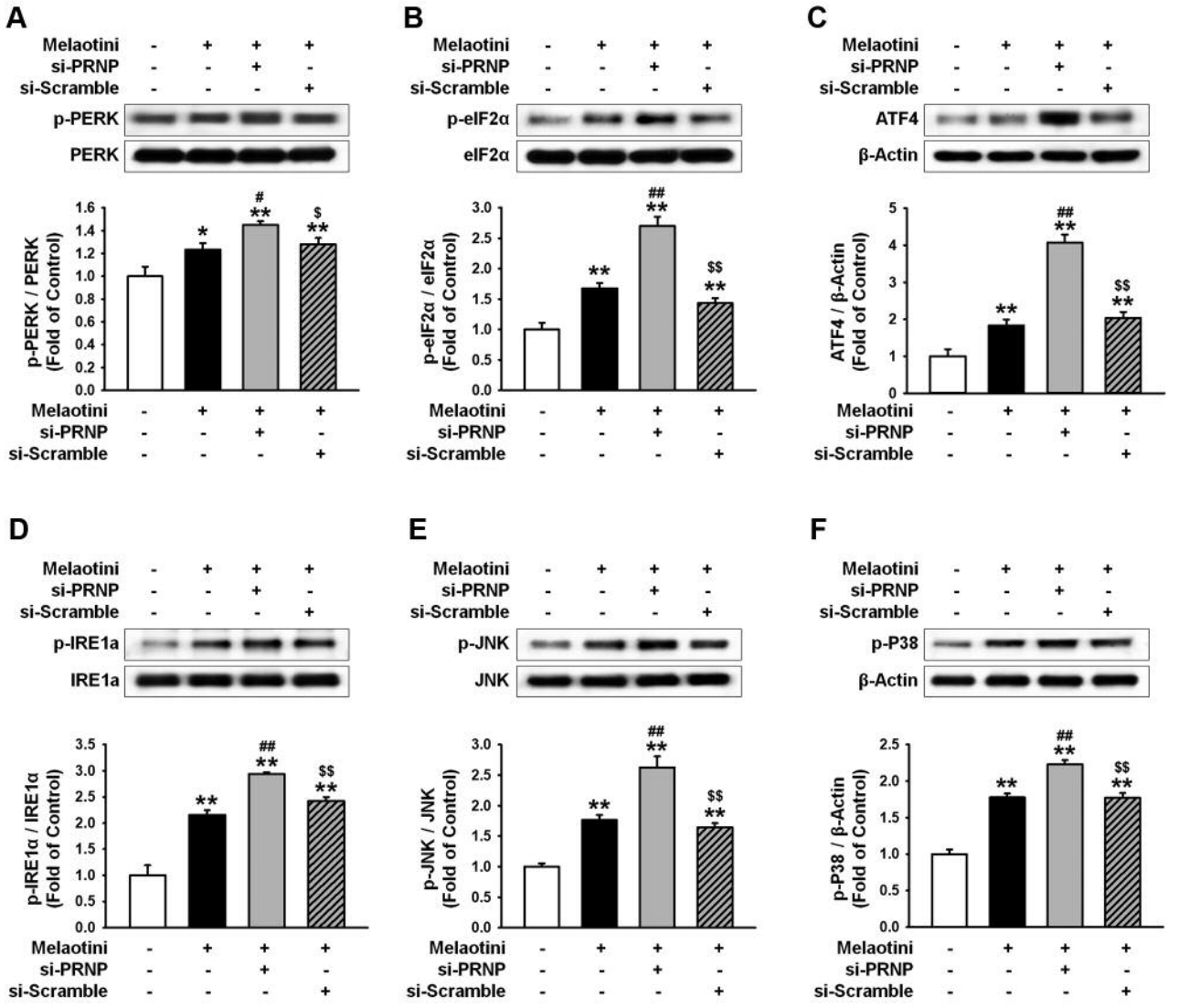

Figure 4. *The effect of PrP^C on melatonin-induced endoplasmic reticulum (ER) stress in SNU-C5/WT colorectal cancer cells. The expression levels of protein kinase R-like endoplasmic reticulum kinase (PERK) (A), eukaryotic initiation factor 2-alpha (eIF2α) (B), activating transcription factor 4 (ATF4) (C), inositol-requiring protein 1 alpha (IRE1α) (D), c-Jun N-terminal kinase (JNK) (E), and p38 (F) were determined by western blot analysis in SNU-C5/WT cells transfected with si-PRNP or si-Scramble following treatment with melatonin (1 mM) for 24 h. The bar graph indicates the quantification of expression levels as determined by densitometry relative to β-actin (n=3). Values represent the means±SEM. *p<0.05 vs. control, **p<0.01 vs. control, #p<0.05 vs. treatment with melatonin, ##p<0.01 vs. treatment with melatonin, $p<0.05 vs. si-PRNP treated with melatonin and $$p<0.01 vs. si-PRNP treated with melatonin.*

PRNP transfected SNU-C5/WT cells, the expression and activation of ER-stress associated proteins, such as PERK, eIF2α, ATF4, IRE1α, JNK, and p38 was examined by western blot analysis (Figure 4A-F). si-PRNP transfected SNU-C5/WT treated with melatonin (1 mM) showed significantly higher levels of expression of the ER stress marker (ATF4) and of the phosphorylation of ER stress regulators (PERK, eIF2α, IRE1α, JNK and p38) compared to control. These findings indicate that melatonin induces ER stress and knockdown of PrP^C enhances melatonin-mediated induction of ER stress.

Inhibition of PrP^C expression enhances melatonin-mediated effect of apoptosis in SNU-C5/WT cells. In order to investigate melatonin-mediated effect on apoptosis in si-PRNP transfected SNU-C5/WT cells, ER stress marker (CHOP) and apoptosis associated proteins, including BAX, cleaved caspase3, and cleaved PARP1 were determined by

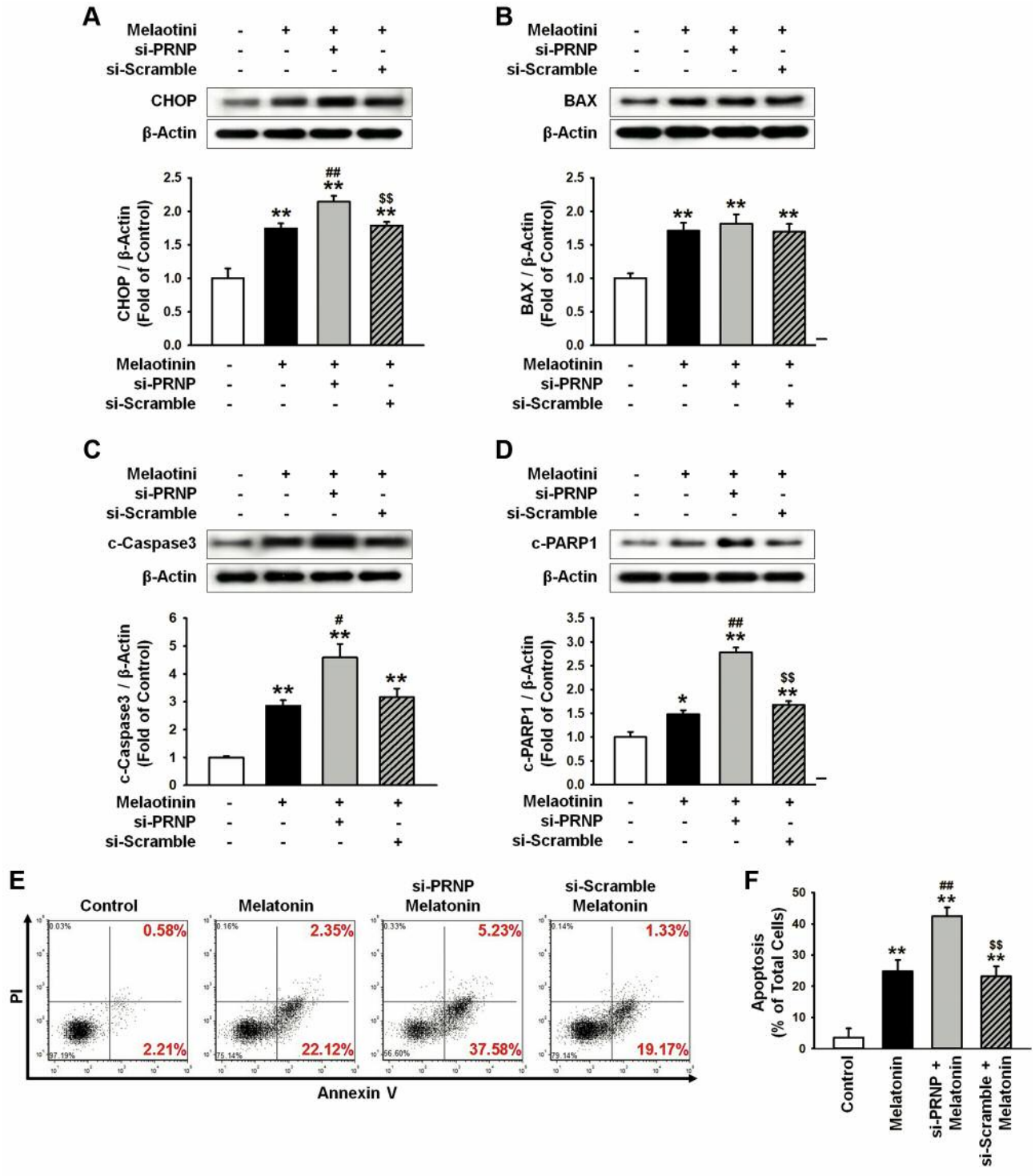

Figure 5. *The effect of PrP^C on melatonin-enhanced ER stress-mediated apoptosis in SNU-C5/WT colorectal cancer cells. The expression levels of ER stress marker (CCAAT-enhancer-binding protein homologous protein (CHOP)) (A) and apoptosis associated proteins, including Bcl-2-associated X protein (Bax) (B), cleaved caspase3 (C), and cleaved poly [ADP-ribose] polymerase 1 (PARP1) (D), were determined by western blot analysis in SNU-C5/WT cells transfected with si-PRNP or si-Scramble following treatment with melatonin (1 mM) for 24 h. The bar graph indicates the quantification of expression levels as determined by densitometry relative to β-actin (n=3). Values represent the means±SEM. **p<0.01 vs. control, #p<0.05 vs. treatment with melatonin, ##p<0.01 vs. treatment with melatonin and $$p<0.01 vs. si-PRNP treated with melatonin. (E) Apoptosis of cells was measured utilizing propidium iodide (PI)/annexin V staining and flow cytometric analysis. (F) Standard quantification of PI/annexin V-positive apoptotic cells (n=3). Values represent the mean±SEM. **p<0.01 vs. control, ##p<0.01 vs. treatment with melatonin and $$p<0.01 vs. si-PRNP treated with melatonin.*

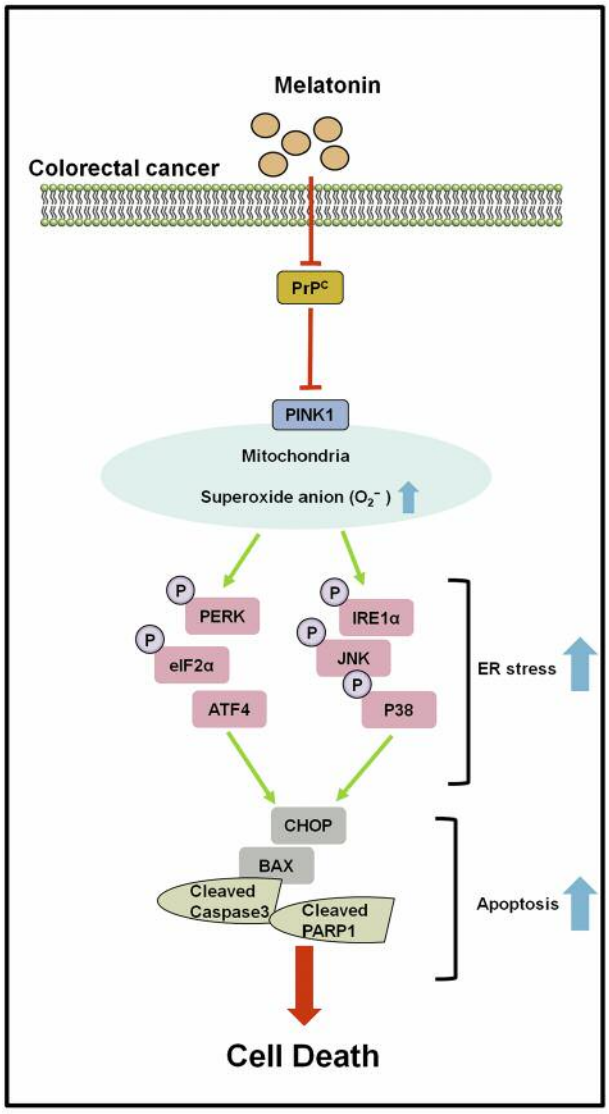

Figure 6. *Schematic illustrating the mechanism of melatonin-mediated anticancer effects in SNU-C5/WT colorectal cancer cells. In colorectal cancer cells, melatonin induces the production of superoxide and decreases the levels of PrP^C. Silencing PrPC expression enhanced the anticancer effects of melatonin via the production of mitochondrial superoxide, activation of ER stress- and apoptosis- mediated proteins.*

western blot analysis. Under melatonin-mediated induction of ER stress, knockdown of PrP^C significantly enhanced CHOP, Bax, cleaved caspase3 and cleaved PARP1, compared to untreated cells (Figure 5A-D). Melatonin significantly increased the percentage of early and late apoptotic cells to 24.47% compared to control (2.79%). In addition, si-PRNP transfected SNU-C5/WT treated with melatonin show significant increase in early and late apoptotic cells to 42.81% (Figure 5E and F). These findings indicate that combination of si-PRNP and melatonin induces apoptosis of SNU-C5/WT *via* ER stress induction.

Discussion

In this study, the effects of melatonin, a hormone secreted by the pineal gland, on the viability of cancer cells was investigated. Melatonin treatment of SNU-C5/WT induced superoxide levels and decreased cell viability. Moreover, melatonin inhibited PrP^C expression and subsequently reduced PINK1 expression ultimately destroying mitochondrial homeostasis. In combination with melatonin, knockdown of PrP^C induced further inhibition of PINK1 expression and production of superoxide, melatonin-mediated ER stress, and ultimately cellular apoptosis. Together, melatonin treatment and PrP^C knockdown led to increased apoptosis of SNU-C5/WT *via* PrP^C-dependent mitochondria/ER signaling. Our study suggests melatonin as a novel anti-cancer agent.

Melatonin, a hormone that is most widely known for its sleep inducing abilities has the potential to regulate CRC cell growth by inducing apoptosis. Several studies demonstrated that melatonin causes cancer cell death *via* mitochondrial reactive oxygen species production (15, 16) and inhibits tumor growth (8, 29). In addition, another study suggested that melatonin regulates Endothelin 1 (ET-1) levels and suppresses tumor progression in CRC (30), suggesting that melatonin has a clear potential in cancer therapy. In accordance with our previous study, SNU-C5/WT cells, when treated with melatonin, show decreased cell viability and increased mitochondrial dysfunction in dose and time dependent manner. Many cancer cells show elevated ROS production compared to normal cells, because of oncogenic mutation, increased metabolic activity, and cancer microenvironment. Specifically, superoxide has been known as one of the most potent mitochondrial deregulators and excess superoxide is associated with apoptosis in many cancer cells (31-35). Our results also suggested that melatonin caused an increase in superoxide production in cancer cells, leading to the apoptotic potentials of the cells after the treatment.

Previous studies suggested that PrP^C plays a role in the protection of cells against oxidative stress by clearing up toxic superoxide dismutase (SOD) activities (36). In addition, studies have shown that when the gene for PrP^C, PRNP, is silenced, the activities of antioxidant enzymes including catalase and glutathione reductase are reduced (37, 38). Thus, in this study the effects of melatonin in the induction of ROS and mitochondrial dysfunction through PrP^C dependent pathway was examined. It was found that PINK1 expression is decreased even further when PrP^C was silenced *via* si-PRNP transfection compared to treatment with melatonin alone, and that knockdown of PrP^C increased superoxide production in CRC cells even further than melatonin treatment alone. These results are supported by previous studies showing that melatonininduced ROS production of

CRC cells lines (16, 15), and this effect was mediated by decrease of PINK1 expression (39). Thus, our results suggest that melatonin plays a critical role in superoxide production and PINK1 expression in SNU-C5/WT cells *via* PrP^C dependent pathway. Further study is required to investigate the precise mechanism of the effects of melatonin-mediated PINK and PrP^C expression in CRC cells.

A previous study has suggested that PrP^C function may be related to antioxidant activities against ROS production in cancer (40). Other studies on PrP^C and cancer indicated that PrP^C expression levels associate with ROS, ER stress (25) and induction of tumor cell death (24, 7). The results of this study support the hypothesis that ER stress is involved in melatonin/PrP^C, mediated apoptosis of cancer cells. Our results from western blotting analysis suggest that melatonin increased ROS mediated ER stress associated proteins resulting in alterations in the levels of oxidative stress related proteins, including p-PERK, p-eIF2α, ATF4, p-IRE1α, p-JNK, p-P38, and CHOP and subsequent apoptosis associated proteins, such as BAX, c-Caspase3, and c-PARP1, finally leading to cancer cell death (41, 42). In addition, our western blot analysis results show that silencing of PrP^C magnifies the effects of melatonin on ER and apoptosis, highlighting the importance of PrP^C in melatonin mediated cancer therapy. These results signify the importance of combination of melatonin and silencing PrP^C in CRC cell treatment which regulates superoxide production and ER stress. However, further mechanistic studies are required to investigate whether combination of melatonin and anticancer drugs have synergistic effects in CRC cancer.

In summary, our findings reveal that melatonin inhibits cell viability *via* excessive production of superoxide and increased PrP^C expression. In addition, silencing PrP^C enhances melatonin mediated accumulation of superoxide and activates ROS mediated ER stress. Finally, apoptosis of CRC cancer cells is activated (Figure 6). Further studies are necessary to understand how melatonin regulates PrP^C expression in CRC cancer. This study demonstrated that combination of melatonin and silencing PrP^C in SNU-C5/WT cells results in increased anticancer effects. These findings indicate that understanding the effect of melatonin and PrP^C in colorectal cancer may help design a novel therapeutic strategy in cancer therapy.

Conflicts of Interest

The Authors have no conflicts of interest to declare with regards to this study.

Acknowledgements

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