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ORIGINAL ARTICLE

Melatonin-mediated downregulation of thymidylate synthase as a novel mechanism for overcoming 5-fluorouracil associated chemoresistance in colorectal cancer cells

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Abstract

Background: 5-Fluorouracil (5-FU) has been established as the first-line chemotherapy for advanced colorectal cancer (CRC); however, acquired chemoresistance is often the cause of poor therapeutic response. Melatonin is a molecule that is associated with circadian rhythms. Although antitumor effects of melatonin have been shown, the underlying mechanism(s) for its activity and its effect, if any, in chemoresistant CRC has not been studied. We aimed to investigate antitumor effects of melatonin, and more specifically its effect on molecular mechanisms in 5-FU resistant CRC cells.
Methods: The cell growth was assessed in CRC cells, patient-derived organoids and 5-FU resistant CRC cells after treatments with melatonin. In addition, the expression of thymidylate synthase (TYMS) and microRNAs (miRNAs) that are targeting *TYMS* were examined.
Results: We observed that melatonin inhibited the cell growth in 5-FU resistant CRC cells. In addition, we found that melatonin significantly promoted apoptosis. Furthermore, a combination of melatonin and 5-FU markedly enhanced 5-FU-mediated cytotoxicity in 5-FU resistant cells. In addition, melatonin significantly decreased the expression of TYMS. Interestingly, this effect was manifested through the simultaneous increase in the expression of miR-215-5p, for which, *TYMS* serves as the direct downstream target for this miRNA.
Conclusions: Melatonin facilitates overcoming 5-FU resistance through downregulation of TYMS. Melatonin may serve as a potential therapeutic option on its own, or in conjunction with 5-FU, in the treatment of patients with advanced or chemoresistant CRC.

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers and remains a leading cause of cancer-related deaths worldwide (1,2). A significant degree of mortality associated with this malignancy is due to late detection of disease. Nonetheless, due to advances in diagnostic and therapeutic techniques in the recent years, the prognosis for early-stage patients with CRC has improved significantly, but the clinical outcomes in patients with advanced cancers still remain quite poor.

For almost half the century, fluoropyrimidine-based treatments [e.g. 5-fluorouracil (5-FU)] have been the conventional first-line chemotherapy for advanced patients with CRC (3,4). However, almost all patients that receive 5-FU-based chemotherapy eventually develop acquired resistance to this

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Abbreviations

CI	combination index
CRC	colorectal cancer
DMSO	dimethyl sulfoxide
5-FU	5-fluorouracil
mRNA	messenger RNA
miRNA	microRNAs
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
qRT–PCR	quantitative reverse transcriptase polymerase chain reaction
TYMS	thymidylate synthase

treatment. Hence, overcoming such chemoresistance is a pivotal consideration for improving the overall prognosis of patients with advanced CRC. 5-FU is an analog of uracil and is converted intracellularly into 5-fluoro-2-deoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate. The anticancer effects of 5-FU are exerted through inhibition of thymidylate synthase (TYMS), as well as by incorporation of its metabolites into RNA and DNA (5). TYMS is a folate-dependent enzyme that catalyzes the production of an intracellular *de novo* source of thymidylate, which is an essential precursor for DNA biosynthesis (6). Several preclinical studies have shown that the TYMS expression levels are a key determinant for therapeutic responsiveness to 5-FU, because an inverse relationship exists between TYMS expression in cancer cells and 5-FU sensitivity (7–9). In addition, high TYMS expression in tumor tissues indicates lack of responsiveness to 5-FU-based chemotherapy and is predictive of a worse prognosis for patients with CRC (10–12). Considering that TYMS is regarded as the mechanistic influencer of response to 5-FU, it is theorized that suppression of TYMS expression may lead to enhanced responsiveness to 5-FU in CRC.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a small lipophilic molecule secreted by the pineal gland and intimately controlled by the circadian pattern of its production (13). Melatonin is synthesized from tryptophan by a battery of enzymes including tryptophan hydroxylase, arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase (13). It is synthesized in the retina, bone marrow, gastrointestinal tract as well as the pineal gland (14). Commonly prescribed for insomnia, melatonin has many biological actions, including serving as an antioxidant (15) and an anti-inflammatory agent (16). In addition, previous studies proposed that melatonin also has prominent antitumor effects by virtue of its modulation of the signaling pathways related to cell proliferation, cell cycle dynamics and regulation of apoptosis (17–20). Thus far, a limited evidence supports that melatonin may modulate the efficacy of 5-FU in CRC and esophageal squamous cell carcinoma by acting on the NF-κB-inducible nitric oxide synthase, Erk and Akt pathways (21–23). However, several questions remain answered, including the detailed mechanistic insights into the effects of melatonin in modulating 5-FU sensitivity; whether such effects of melatonin are responsible for evading primary resistance to 5-FU, or it has any effect in overcoming chemotherapeutic resistance to 5-FU in cancer; and the nature of relationship between melatonin treatment and TYMS expression, which is a key modulator of its efficacy.

In this study, we undertook a series of experiments by establishing 5-FU resistant CRC cells, to address several of these questions pertaining to the antitumor effects of melatonin. Herein, we report multiple lines of data supporting the anticancer effects of melatonin, including a novel finding that melatonin treatment leads to upregulation of the expression of miR-215-5p, which is known to downregulate *TYMS* messenger RNA (mRNA) (24); hence facilitating sensitization of chemoresistance CRC cells to 5-FU.

Materials and methods

Cell culture and reagents

Human colon cancer cell lines HCT116, SW480, COLO320, DLD-1, HT29, RKO, CaCO2 and SW620 were purchased from ATCC (Manassas, VA). All cells were cultured in Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (Thermo Fisher Scientific), 1% penicillin and 1% streptomycin (Sigma–Aldrich, St. Louis, MO). 5-FU resistant cells (HCT116-5FU and SW480-5FU) were established by a previously described method (25), by culturing cell lines with increasing concentrations of 5-FU over a duration of >9 months. 5-FU resistant cells were maintained in culture medium containing 10 μM 5-FU. The 5-FU (Sigma–Aldrich) and melatonin (Sigma–Aldrich) were dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich). All cell lines were obtained from the ATCC during the past 4–6 years, were periodically authenticated every 4–6 months using a panel of short tandem repeat markers and a panel of genes with known genetic and epigenetic signatures, and the last authentication was performed in July 2018.

MTT assay

Cell viability was determined by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay as described previously (26). Briefly, colon cancer cell lines were seeded into 96-well plates (10 000 cells/well) and incubated for 24 h. The cells were thereafter treated with 100 μL of fresh serum-free medium containing melatonin and 5-FU for 72 h. Optical density was measured using Infinite® 200 PRO (Tecan, Männedorf, Switzerland). Cell viability was calculated as a percentage of the negative controls treated with the same concentration of DMSO.

Apoptosis assay

At 24 h after seeding in 6-well plates (5×10^5 cells/well), cells were treated with 1 mM melatonin for 48 h. The apoptotic cell fraction was measured using Muse® Annexin V and Dead Cell Assay Kit (MilliporeSigma, Burlington, MA) according to the manufacturer's instructions.

Colony formation assay

Twenty-four hours after seeding in 6-well plates (500 cells/well), cells were treated with 1 mM melatonin. Cells were incubated for a total of 10 days and then stained with crystal violet as described previously (27). The number of colonies (>50 cells) was counted using the Image J program, ver. 1.51 (National Institutes of Health, Bethesda, MD) (28).

Patient-derived organoids

Cancer tissues were collected from two patients undergoing surgery for CRC at the Baylor University Medical Center, Baylor Scott and White Health, Dallas, TX. Cells from cancer tissues were grown into organoids as described previously (29), with minor modifications (Supplementary Method, available at *Carcinogenesis* Online). For the treatment of patient-derived organoids, 2 mM melatonin was added to the culture medium and each organoid was cultured for 10 days. The human protocol was approved by the institutional review board at Baylor Scott and White Research Institute (Dallas, TX). Written informed consents from all patients providing tissue samples were obtained in accordance with the Declaration of Helsinki.

Wound healing assay

HCT116-5FU cells were seeded in 6-well plates and cultured to near confluence (>90%). A sterile 200 μL pipette tip was used to make a scratch in the cell monolayer. The culture medium was thereafter removed and replaced with fresh medium containing 0, 200 μM or 1 mM of melatonin. The wounded monolayer was re-incubated for 48 h. The rates of wound closure were calculated by measuring the distance between the cells at the edges.

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Western blotting

Proteins were extracted from cells using a RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing Protease Inhibitor Cocktail (Thermo Fisher Scientific). Twenty microgram of each total protein sample was resolved on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Anti-poly ADP-ribose polymerase-1/2 (Santa Cruz Biotechnology, Dallas, TX, Cat# sc-7150, RRID:AB_2160738), anti-Caspase 3 (Santa Cruz Biotechnology, Cat# sc-7148, RRID:AB_637828), anti-TYMS (Cell Signaling Technology, Cat# 9045), anti-β actin (Sigma–Aldrich, Cat# A5441, RRID:AB_476744) and species-appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Cat# sc-2004, RRID:AB_631746; Cat# sc-2005, RRID:AB_631736) were used for detection. Immunoblots were visualized using Super-Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Band intensity was quantified using the Image J program (28) and expressed as a ratio to β-actin band intensity.

Quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from cells using QIAzol Lysis Reagent and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For the analysis of mRNA expression, the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to convert RNA to complementary DNA. Quantitative reverse transcriptase polymerase chain reaction (qRT–PCR) was performed using a SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK) and the QuantiStudio 7 Flex Real Time PCR System (Thermo Fisher Scientific). Cycle conditions were as follows: polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 5 s and annealing/extension at 60°C for 20 s. The relative expression of *TYMS* was normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase. The primer sequences are listed in Supplementary Table, available at *Carcinogenesis* Online.

For the analysis of microRNA (miRNA) levels, the TaqMan MicroRNA reverse Transcription Kit (Thermo Fisher Scientific) and TaqMan™ MicroRNA Assays hsa-miR-215 (Thermo Fisher Scientific) were used, and qRT–PCR performed using a SensiFAST Probe Lo-ROX Kit (Bioline) and the QuantiStudio 7 Flex Real Time PCR System. Cycle conditions were as follows: polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 10 s and annealing/extension at 60°C for 20 s. The expression of miRNAs was normalized against miR-16 (TaqMan™ MicroRNA Assays hsa-miR-16, Thermo Fisher Scientific).

qRT–PCR was performed in duplicate for each sample and the mean value was used to calculate the expression level and all results were expressed as $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$.

Analysis of combination chemotherapy with 5-FU and melatonin

The effect of combining melatonin and 5-FU on cell growth inhibition was analyzed using isobologram and combination index (CI) (30–32). Briefly, based on the results of the MTT assays, dose–effect curves were plotted. From these curves, the doses of melatonin and 5-FU that induced 50% inhibition (IC_{50} doses) both as single agents and in combination were determined and plotted graphically. A line was drawn between the melatonin IC_{50} point on the *y*-axis and the 5-FU IC_{50} point on the *x*-axis, denoting the theoretical alignment of each agent, which would give rise to an additive effect. CI was calculated using the Chou–Talalay equation at IC_{50} (30,32). The resulting CI offers a quantitative definition for an additive effect (CI = 1), synergism (CI < 1) and antagonism (CI > 1) in drug combinations.

Prediction of miRNAs targeting *TYMS* expression

Putative miRNAs targeting *TYMS* were identified by the combined use of TargetScan, (www.targetscan.org), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) and microRNA.org (http://www.microrna.org/) target prediction programs.

Statistical analysis

Student's two-tailed *t*-test was used for the statistical analyses, and *P* values < 0.05 were considered statistically significant. Error bars denote standard deviation for the columns in the figures.

Results

Melatonin inhibits cell viability and colony formation and induces apoptosis in CRC cells

To elucidate the antitumor effects of melatonin in CRC, we initially assessed the growth inhibitory effects of melatonin on cell viability and colony formation. The MTT assay revealed that melatonin led to the inhibition of cell growth and proliferation in all eight CRC cell lines in a dose-dependent manner (Figure 1A). To determine whether inhibition of cell growth and proliferation by melatonin was due to induction of cell death, apoptosis assays were performed. The numbers of apoptotic cells were significantly increased in CRC cells treated with melatonin in comparison with the DMSO-treated cells ($P < 0.01$; Figure 1B). Furthermore, in the colony formation assay, melatonin treatment caused a significant reduction in the number of surviving colonies ($P < 0.01$; Figure 1C).

Melatonin inhibits growth of patient-derived organoids

To validate that melatonin inhibits cell growth and proliferation, tumor organoids derived from two independent patients with CRC were used. Interestingly, in line with our earlier findings, the growth of both the patient-derived CRC organoids treated with 2 mM melatonin was significantly inhibited compared with the control ($P < 0.01$ and $P < 0.01$, respectively; Figure 1D); highlighting the growth inhibitory potential of melatonin.

Melatonin inhibits cell viability in 5-FU resistant CRC cells

To evaluate the antitumor effects of melatonin in 5-FU resistant cells, we established chemoresistant cell lines by culturing HCT116 (which is one of the cells lines with microsatellite instability) and SW480 (a known microsatellite stable) cell lines (33), in culture media containing increasing concentrations of 5-FU, and maintaining them with 10 μM 5-FU (Figure 2A). To confirm the acquired 5-FU resistance of these cells, we analyzed their cell viability at various concentrations of 5-FU in comparison with their parental cells using the MTT assay (Figure 2B). Both HCT116-5FU and SW480-5FU cell lines exhibited IC_{50} values of 811.4 and 274.4 μM, respectively, which were 49-fold and 35-fold higher compared with their isogenic parents controls, highlighting that both cell lines were significantly chemoresistant to 5-FU.

We next added melatonin to the resistant cell lines and analyzed cell viability using the MTT assay. The assay revealed that melatonin inhibited cell growth and proliferation in both 5-FU resistant CRC cell lines in a dose-dependent manner (Figure 2C). These findings were consistent with the effect seen of melatonin on the corresponding non-5-FU resistant CRC cell lines (Figure 1A).

Melatonin induces apoptosis, inhibits colony formation and wound healing in 5-FU resistant CRC cells

The apoptosis assay with 5-FU resistant cells treated with melatonin revealed that the numbers of apoptotic cells significantly increased in both HCT116-5FU and SW480-5FU cells treated with 1 mM melatonin in comparison with the controls treated with DMSO ($P < 0.01$ and $P < 0.01$, respectively; Figure 3A). In addition, western blotting of cell lysates showed a significantly decreased expression of poly ADP-ribose polymerase-1 and procaspase-3 at 48 h after treatment with melatonin in

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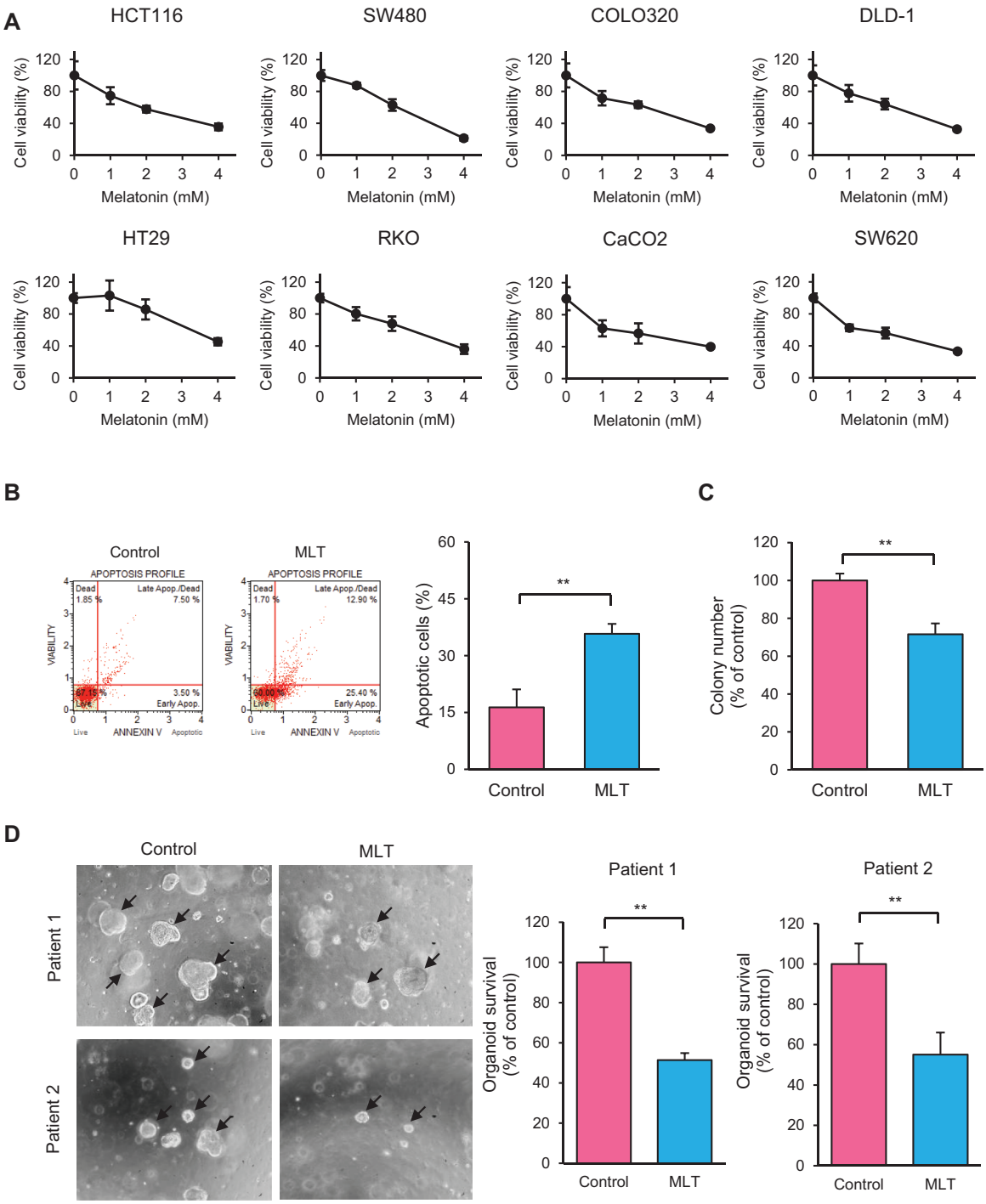

Figure 1. Melatonin inhibits the growth of CRC cells and patient-derived organoids. **(A)** The cell viability for eight CRC cell lines treated with different concentrations of melatonin (1–4 mM) for 72 h was determined by an MTT assay ($n = 5$). **(B)** Apoptosis in HCT116 cells at 48 h after treatment with DMSO (control) or 1 mM melatonin was measured by apoptosis assay ($n = 3$). **(C)** A colony formation assay was used to assess clonogenicity of HCT116 cells treated with DMSO (control) or 1 mM melatonin ($n = 3$). **(D)** Effect of melatonin on patient-derived tumor organoids. Images show patient-derived organoids treated with DMSO (control) or 2 mM melatonin ($n = 3$). Bar graphs show organoid survival (% of control). MLT, melatonin. **$P < 0.01$.

comparison with the control cells treated with DMSO (Figure 3B), suggesting that melatonin induces apoptosis in 5-FU resistant cells. Furthermore, melatonin significantly inhibited clonogenic survival ($P < 0.01$) and wound healing (200 μM, $P < 0.05$; 1 mM, $P < 0.01$) in HCT116-5FU cells (Figure 3C and D). These results suggest that melatonin may inhibit cancer stem cells and cell migration in 5-FU resistant cells in addition to apoptosis.

Melatonin sensitizes 5-FU resistant CRC cells to 5-FU

To evaluate the effects of melatonin on 5-FU sensitivity in the parental and 5-FU resistant HCT116 and SW480 cells, we examined cell viability of the cells treated with a combination of 5-FU and/or melatonin. We observed that in parental HCT116 and SW480 cells, melatonin did not increase the 5-FU cytotoxicity (not significant and $P < 0.05$, respectively; Figure 4A) and

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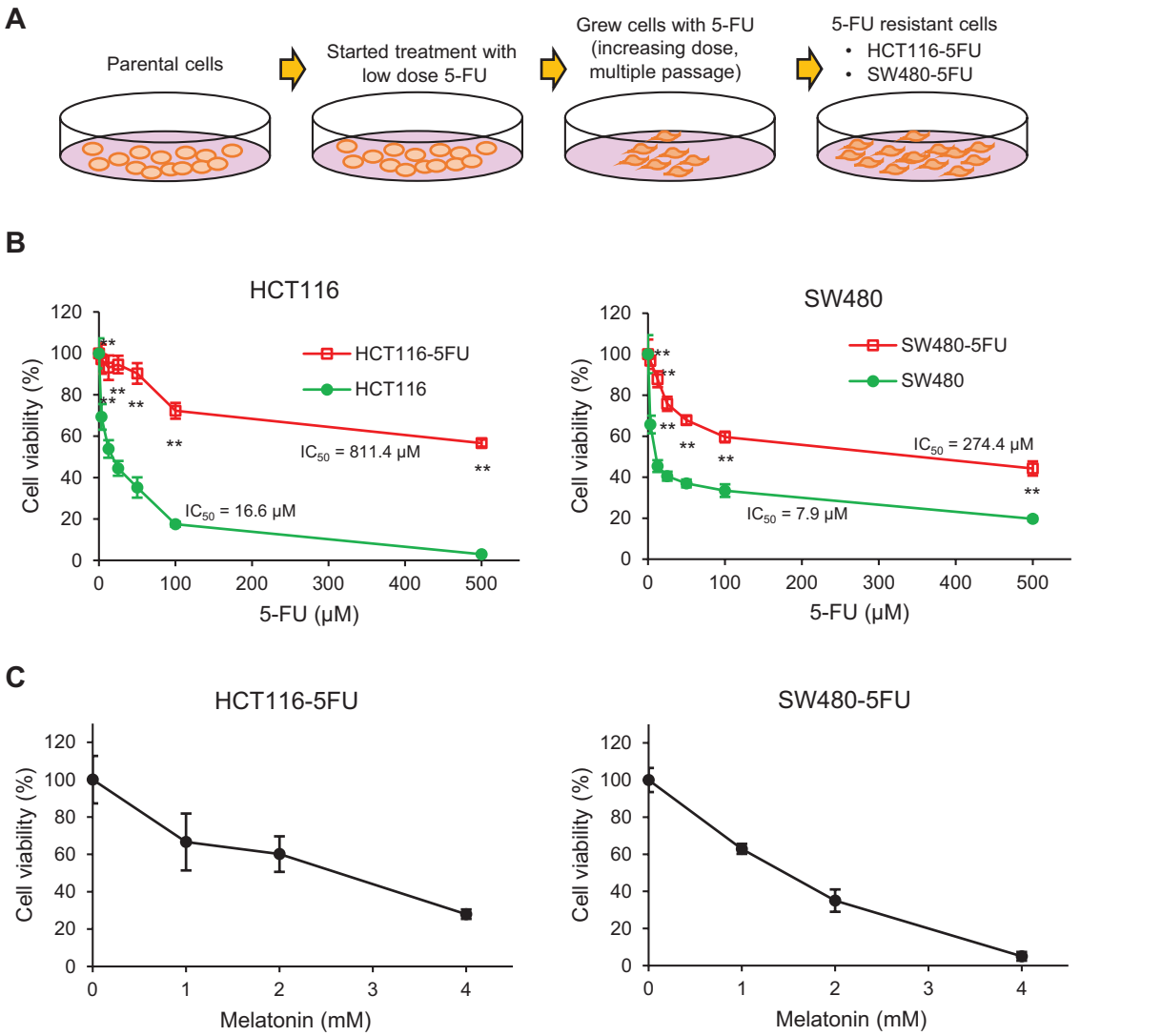

Figure 2. Melatonin inhibits viability of 5-FU resistant CRC cell lines. (**A**) Schematic for the establishment of 5-FU resistant CRC cells. Briefly, parental HCT116 and SW480 cells were cultured in Iscove's Modified Dulbecco's Medium containing increasing concentrations of 5-FU until they acquired resistance to 5-FU. (**B**) MTT assay was performed to compare the cell viability for parental HCT116 and SW480 cells and their 5-FU resistant cells when cultured with various concentrations of 5-FU ($n = 5$). (**C**) Cell viability determined by MTT assay for HCT116-5FU and SW480-5FU cells treated with different concentrations of melatonin (1–4 mM; $n = 5$). HCT116-5FU, 5-FU resistant HCT116; SW480-5FU, 5-FU resistant SW480. *$P < 0.05$, **$P < 0.01$.

isobologram analyses showed no synergism between 5-FU and melatonin in parental cell lines (CI = 1.32 and 1.18, respectively; Figure 4B). On the other hand, in HCT116-5FU and SW480-5FU cells, melatonin enhanced 5-FU cytotoxicity significantly ($P < 0.01$ and $P < 0.01$, respectively; Figure 4C; CI = 0.80; CI = 0.84; Figure 4D). These results suggest that melatonin has the ability to resensitize 5-FU resistant cells to 5-FU.

Melatonin increases miR-215-5p, which subsequently leads to decreased TYMS in 5-FU resistant cells

Previous studies have shown that upregulation of TYMS is one of the key mechanisms for 5-FU resistance in cancer cells and that its inhibition can enhance therapeutic responsiveness to 5-FU (7–9). Thus, we first evaluated the expression of *TYMS* mRNA in both parental cells and 5-FU resistant cells to confirm whether *TYMS* expression indeed is increased in 5-FU-resistant cells. qRT–PCR showed that the expression levels of *TYMS* mRNA in 5-FU resistant cells were significantly higher than in their parental cells (HCT116, $P < 0.01$; SW480, $P < 0.01$; Figure 5A), in line with previously published studies (34–36). Next, we analyzed the expression of *TYMS* in 5-FU resistant cells cultured with or without melatonin (Figure 5B). In HCT116-5FU and SW480-5FU cells treated with 1 mM melatonin, the expression levels of *TYMS* mRNA were significantly reduced to 55% and 43%, respectively, compared with the DMSO control ($P < 0.01$ and $P < 0.05$, respectively). In addition, we analyzed TYMS protein expression in 5-FU resistant cells treated with various concentrations of melatonin. TYMS protein was significantly downregulated by melatonin in a dose-dependent manner (HCT116-5FU treated with 1 mM and 2 mM melatonin, $P < 0.01$ and $P < 0.01$, respectively; SW480-5FU treated with 1 mM and 2 mM melatonin, $P < 0.01$ and $P < 0.01$, respectively; Figure 5C). These results illustrate that melatonin sensitizes 5-FU resistant CRC cells to 5-FU by downregulating TYMS levels.

There is some suggestion that miRNAs may mediate the function of melatonin in human cells (37–39). In this regard,

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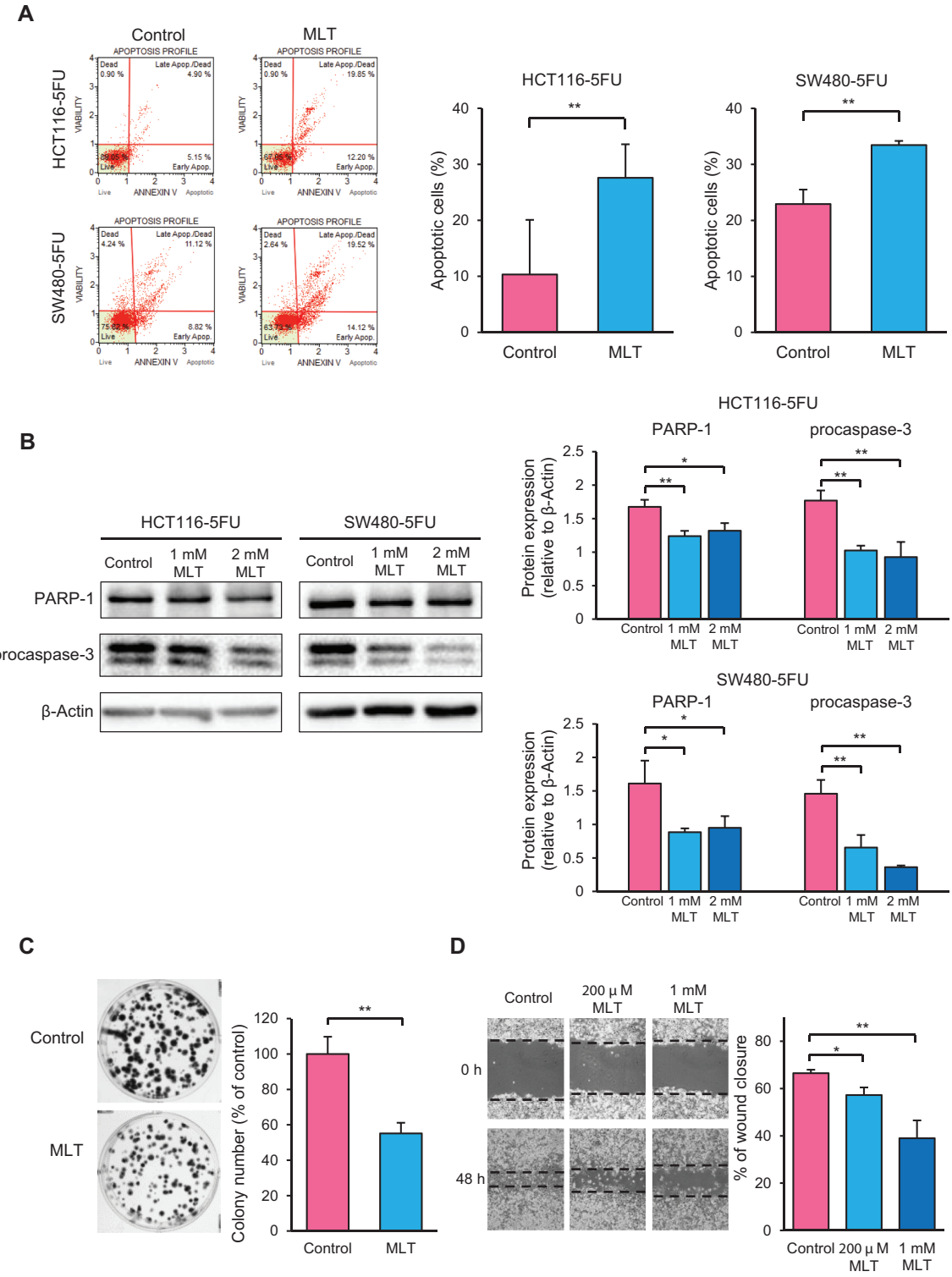

Figure 3. Melatonin induces cell apoptosis and inhibits colony formation and wound healing in 5-FU-resistant CRC cell lines. (**A**) An apoptosis assay was used to determine cell death at 48 h after treatment with DMSO (control) or 1 mM melatonin in HCT116-5FU and SW480-5FU cells ($n = 3$). (**B**) Western blotting shows the expression of poly ADP-ribose polymerase and caspase-3 in 5-FU resistant cells after treatment with 1 mM and 2 mM melatonin ($n = 3$). (**C**) A colony formation assay was used to assess clonogenicity of HCT116-5FU cells treated with DMSO (control) or 1 mM melatonin ($n = 3$). (**D**) Wound healing assay to assess the migration of HCT116-5FU cells treated with melatonin. Images (×100 magnification) show HCT116-5FU cells treated with melatonin (0, 200 μM and 1 mM; $n = 3$). Bar graphs show % of wound closure after 48 h of treatment. MLT, melatonin; HCT116-5FU, 5-FU-resistant HCT116; SW480-5FU, 5-FU-resistant SW480. *$P < 0.05$, **$P < 0.01$.

evidence exists that *TYMS* mRNA can be targeted by several miRNAs, including miR-203 and miR-433, which enhance chemosensitivity to 5-FU (40,41). Hence, to identify novel miRNAs induced by melatonin that could potentially regulate TYMS expression in CRC, we carried out *in silico* analysis using three different prediction algorithms (TargetScan,

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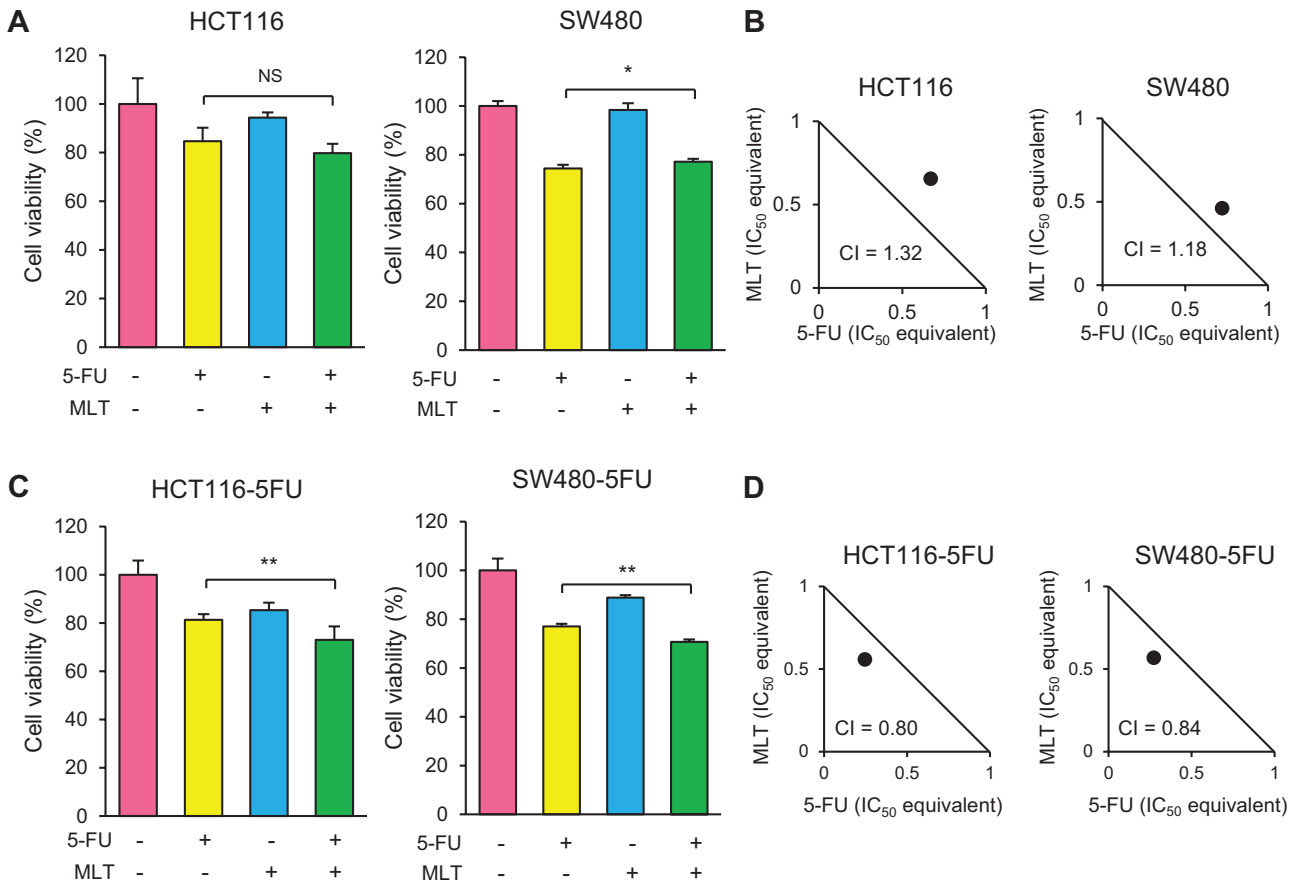

Figure 4. Melatonin resensitizes 5-FU resistant cells to 5-FU. (**A**) The cell viability of HCT116 and SW480 cells treated for 72 h with 5 μM 5-FU, 750 μM melatonin, alone or in combination was determined by an MTT assay ($n = 5$). (**B**) IC_{50} isobolograms for 5-FU- and melatonin-treated HCT116 and SW480 cells. (**C**) The cell viability of HCT116-5FU and SW480-5FU cells treated for 72 h with 100 μM 5-FU, 200 μM melatonin, alone or in combination was determined by MTT assay ($n = 5$). (**D**) IC_{50} isobolograms for 5-FU- and melatonin-treated HCT116-5FU and SW480-5FU cells. MLT, melatonin; HCT116-5FU, 5-FU resistant HCT116; SW480-5FU, 5-FU resistant SW480; NS, not significant. $^{*}P < 0.05$, $^{**}P < 0.01$.

www.targetscan.org; miRTarBase, http://mirtarbase.mbc.nctu.edu.tw and microRNA.org, http://www.microrna.org/). We found 138 miRNAs from TargetScan, 14 miRNAs from miRTarBase and 6 miRNAs from microRNA.org that were identified as potential candidates. From the intersection of these three clusters, miR-215-5p was identified as the most promising miRNA candidate responsible for regulating *TYMS* expression (Figure 5D). The 5′-end of miR-215-5p contains a seven nucleotide stretch complementary to the 3′-end of the *TYMS* mRNA (Figure 5E). Furthermore, miR-215-5p is known as a tumor suppressor (22,42,43) and a previous study using a luciferase reporter assay demonstrated that miR-215-5p regulates *TYMS* expression (24). We performed qRT–PCR for miR-215-5p and found that the expression level of miR-215-5p significantly increased after melatonin treatment of HCT116-5FU cells (1 mM, $P < 0.05$; 2 mM; $P < 0.01$; Figure 5F). Considering that there is experimental evidence that miR-215-5p reduces the expression of *TYMS* in CRC cells (24,44), our results help provide a novel connection between melatonin treatment and downregulation of *TYMS* expression through melatonin-induced overexpression of miR-215-5p (Figure 5G).

Discussion

In modern treatment therapies for several cancers, 5-FU remains the standard of care, first line chemotherapeutic drug of choice in patients with CRC (3,4,45). In this study, we established 5-FU resistant CRC cell lines and investigated the cytotoxic action of melatonin on both parental cells and the 5-FU resistant cells. Melatonin strongly inhibited cell viability through the induction of apoptosis not only in parental CRC cells but also in 5-FU resistant cells. In addition, we confirmed that melatonin sensitizes 5-FU resistant cells to 5-FU, regardless of their microsatellite instability or microsatellite stable status, which indicates that a combination of 5-FU with melatonin might further improve therapeutic response rates in patients with advanced CRC.

TYMS is known as the key enzyme influencing 5-FU efficacy in cancer treatment (5). High levels of TYMS expression in tumor tissues are predictive of poor therapeutic response to 5-FU-based chemotherapy, associated with worse overall prognosis. In addition, overexpression of TYMS in tumors is also considered one of the mechanistic drivers of 5-FU resistance in cancer cells (10–12). In this study, we confirmed that the expression levels of *TYMS* mRNA in our 5-FU resistant cells were significantly higher vis-à-vis parental counterpart cells, which is consistent with previous reports (34–36). In this study, we showed that melatonin downregulates both the transcript and protein expression of TYMS in our 5-FU resistant cell lines. Given that knockdown of TYMS is lethal for the cells, we were unable to perform studies to study the effect of 5-FU in chemoresistant cell lines. In addition, in the resistant cell lines, but not the

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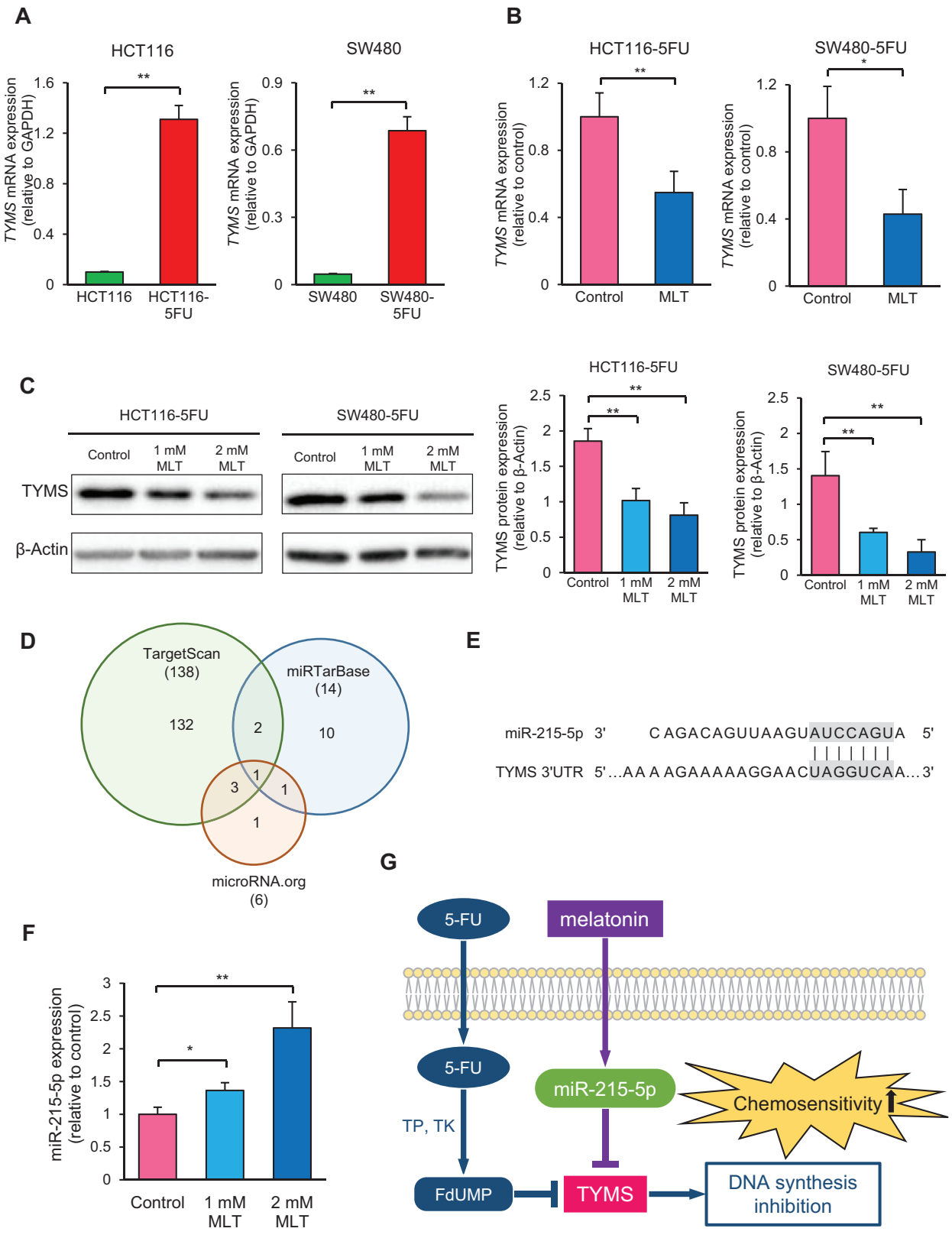

Figure 5. Melatonin suppresses TYMS and increases miR-215-5p in 5-FU resistant cells. (**A**) The expression of *TYMS* mRNA in parental cells and 5-FU resistant cells was determined by qRT–PCR ($n = 3$). (**B**) The expression of *TYMS* mRNA at 48 h after treatment with 1 mM melatonin was measured by qRT–PCR in 5-FU-resistant cells ($n = 3$). (**C**) Western blotting showing the expression of TYMS protein in lysates from 5-FU resistant cells after 48 h of treatment with 1 mM and 2 mM melatonin ($n = 3$). (**D**) Venn diagram showing the number of microRNAs that target TYMS as predicted by TargetScan, miRTarBase and microRNA.org. (**E**) The 3'Untranslated region of *TYMS* mRNA contains a putative binding site for miR-215-5p. (**F**) The expression of miR-215-5p in HCT116-5FU cells 48 h after treatment with 1 mM and 2 mM melatonin as measured by qRT–PCR ($n = 3$). (**G**) Schematic diagram depicting the role of melatonin in 5-FU-resistant CRC cells. MLT, melatonin; TYMS, thymidylate synthase; HCT116-5FU, 5-FU-resistant HCT116; SW480-5FU, 5-FU-resistant SW480; TP, thymidine phosphorylase; TK, thymidine kinase; FdUMP, fluorodeoxyuridine monophosphate. $^{*}P < 0.05$, $^{**}P < 0.01$.

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parental cell lines, we observed a synergism between melatonin and 5-FU treatments, which might be a consequence of TYMS inhibition by melatonin treatment. These results suggest that melatonin may be used in alone or in conjunction with 5-FU to treat chemoresistant CRC.

TYMS mRNA has been reported to bind to several miRNAs, which in turn enhance the cellular chemosensitivity to 5-FU (40,41). miRNAs are short single-stranded non-coding RNAs that posttranscriptionally regulate gene expression by binding to the 3′ untranslated region of target mRNAs (46). Many studies have shown that miRNAs can have both oncogenic and tumor-suppressive effects in cancer (47,48). A previous study showed that melatonin regulates several miRNAs (37–39,49); however, to the best of our knowledge, no studies have interrogated whether melatonin regulates expression of specific miRNAs in 5-FU resistant cancer cells—a paradigm that is fairly common in patients with advance and unresponsive CRCs. In this study, using multiple publicly available prediction algorithms, we found that melatonin specifically upregulates the expression of miR-215-5p, which acts as a tumor suppressor (22,42,43), and has been shown to inhibit the expression of *TYMS* (24,44). Taken together, our results provide the first evidence that overexpression of miR-215-5p is one of the key mechanisms by which melatonin downregulates *TYMS* expression.

In conclusion, we demonstrate that melatonin inhibits the growth of 5-FU resistant CRC cells through upregulation of miR-215-5p and a concomitant downregulation of TYMS. Although chemotherapy is a highly effective treatment for various kinds of cancer, over time, development of resistance is not uncommon. Melatonin is a hormone that has been used as a supplement and can be used safely for treatment of insomnia and regulation of the sleep–wake cycle. Our study suggests that melatonin helps patients with CRC not only as a drug for insomnia, one of several side effects of chemotherapy (50), but also as an inhibitor of impending 5-FU resistance. Although further analyses are required in the future, we are enthused that combined treatment with melatonin and 5-FU might be a potential strategy for patients with advanced CRC in the future.

Supplementary material

Supplementary material is available at *Carcinogenesis* online.

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