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Blockage of melatonin receptors impairs p53-mediated prevention of DNA damage accumulation

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Melatonin has been known to be a chemopreventive agent since its levels inversely correlate with the risk of developing cancer. We have recently shown that melatonin induces p38-dependent phosphorylation of both p53 and histone H2AX. This is associated with a p53-mediated increase in repair of both endogenous and chemotherapy-induced DNA damage. In addition, the inhibition of p38 activities impairs melatonin's capability to induce a p53-dependent DNA damage response and thus its ability to maintain genome integrity. Since melatonin-induced p53 phosphorylation requires an intact p38 phosphorylation cascade and p38 can be activated by G proteins, we supposed that melatonin's activities could be mediated by its G-protein-coupled membrane receptors, MT1 and MT2. Here, we show that the activation of the p53-dependent DNA damage response by melatonin is indeed mediated by MT1 and MT2. As a result, the absence of either receptor impairs melatonin's ability to reduce both cell proliferation and clonogenic potential of cancer cells. In addition, this causes an impairment of the p53-dependent DNA damage response. By providing molecular insight, our findings might have translational impact, suggesting the involvement of melatonin receptors in tumorigenesis.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a mammal pineal hormone mainly secreted by the pineal gland at the hypothalamic level. Melatonin synthesis occurs mainly at night and is tuned by enzymes inhibited by the sunlight (1). Increasing observational evidence associates melatonin concentration to cancer occurrence (2–9). In particular, two epidemiological studies have demonstrated an inverse correlation between overnight urinary levels of melatonin and the incidence of breast cancer (8,9). The first study was conducted within the ORDET cohort and tested the concentration of a melatonin metabolite, 6-sulfatoxymelatonin, in 178 postmenopausal women with incident breast cancer and 710 matched controls (8), whereas the second one refers to a case–control study conducted within the NHIS cohort on 357 postmenopausal women with incident breast cancer and 533 matched controls (9). These and other similar studies suggest that melatonin might be able to induce tumour suppressor pathways *in vivo* (10,11). Molecular studies have shown that melatonin is able to induce both the p53 tumour suppressor and its target gene p21 (10,12–14). In response to a plethora of stressful conditions, especially those causing DNA damage, p53 undergoes post-translational modifications, becomes more stable and its transcriptional activities are enhanced (15–23). Particularly, upon DNA damage, p53 is phosphorylated by a subset of kinases. Ataxia telangiectasia mutant and ataxia telangiectasia related phosphorylate p53 upon DNA double strand breaks, whereas p38 can phosphorylate p53 in response to single strand breaks or in the absence of ataxia telangiectasia mutant (15,22,23). In addition, in the presence of radical oxygen species, both p53 and p38 become phosphorylated (24–29).

We have recently published that melatonin triggers a p38- and PML-dependent phosphorylation cascade, resulting in p53 phosphorylation in Ser-15 (14).

Having a high lipid-water solubility, melatonin is able to enter the cell by diffusing across the cellular membrane and act as a radical scavenger. In the same way, it can cross the nuclear membrane and bind its nuclear receptor RZR/RORα. This is then recruited onto RZR regulatory elements present in the promoters of several genes, such as p21 and 5-lipoxygenase, which are thereby transcriptionally regulated (10). In addition, melatonin regulates signalling pathways by binding to its membrane receptors, MTNR1A and MTNR1B, also known as MT1 and MT2, which exist in the homodimeric and heterodimeric states (30–33). Both MT1 and MT2 are G-protein-coupled membrane receptors which, upon ligand binding, activate G proteins and inhibit cyclic adenosine 3′,5′-monophosphate formation (10,34). It has been shown that both MT1 and MT2 receptors, upon melatonin binding, can also activate MAP kinases, among which c-Jun, in *Chlorocebus aethiops* Cos-7 cells (30). Since we found that melatonin treatment leads to p38 accumulation (14), we investigated whether this was receptor mediated. In the present manuscript, we show that melatonin triggers p53 phosphorylation through the activation of its receptors, MT1 and MT2. In fact, we show that both chemical inhibition and selective gene silencing of the receptors impair melatonin's ability to trigger p38 phosphorylation and accumulation, thereby inhibiting p53 phosphorylation. This pairs with a decrease in melatonin's ability to reduce cell proliferation and prevent DNA damage. Together with recent translational studies showing reduced levels of melatonin receptors in cancer samples as compared with their matched controls (35,36), these findings suggest that the involvement of melatonin receptors in triggering anticancer pathways.

Materials and methods

Cell culture and transfection

HCT116 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, Carlsbad, CA). Cell lines were grown at 37°C, 5% CO_2. Transfections were performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's recommendations, using the following small interfering RNA (siRNA): siGFP 5′-AAGUUCAGCGUGUCCGGGGAG(dTdT)-3′; siMTNR1A 5′-GGAAUACAGGAGAAUUAUA(dTdT)-3′; siMTNR1B 5′-CUAGCUACUUACUGGCUUA(dTdT)-3′ (Eurofins MWG, Ebersberg, Germany). Culture medium was replaced 24 h post-transfection and cells were allowed to grow for additional 72 h before any treatment.

Reagents

Melatonin (M5250) and Luzindole (L2407) were purchased from Sigma–Aldrich (St Louis, MO).

Growth curves and colony-forming assays

For growth curves, 7×10^3 cells/well were seeded in 6-well dishes. Cells were harvested at the indicated times by trypsin detachment and counted automatically using a Guava EasyCyte 8HT flow cytometer (Millipore, Billerica, MA). Cell concentration (cells/ml) is shown on the *y*-axis. With regard to colony-forming assays, 5×10^2 cells were seeded in 35-mm dishes and grown for 15 days. Cells were stained with crystal violet and colonies were counted. To maintain melatonin levels, the medium was changed every 48 h in both experiments.

Western blots

Total protein extracts were prepared by lysing cells in 8 M urea (Bio-Rad, Hercules, CA). Receptors were extracted with 8 M urea/10 mM [(3-cholamidopropyl)-dimethyl-ammonio]1-propanesulfonate (Bio-Rad). All

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protein extracts were quantified by Bradford assay and equal amounts were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (either pre-cast 4–20% Novex Tris-Glycine gels from Life Technologies or home-made gels), transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore) and underwent immunoblot with the indicated antibodies. Antibodies to phospho-p53 Ser-15 (9284, Cell Signaling, Danvers, MA), β-actin (A2288, AC-74, Sigma), p21 (2947, Cell Signaling), nucleolin (ab13541, 4E2, Abcam, Cambridge, UK), MTNR1A (ab87639, Abcam), MTNR1B (ab128469, Abcam), γH2AX (9718, 20E3, Cell Signaling), p38 (9212, Cell Signaling), phospho-p38 Thr180/Tyr182 (9211, Cell Signaling) and p53 (sc126, DO1, Santa Cruz Biotechnology) were diluted in 5% bovine serum albumin in Tris-buffered saline/0.1% Tween-20. Secondary anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad. Images were acquired using a VersaDoc MP instrument (Bio-Rad).

Comet assays

Following transfection with the indicated siRNA, cells were treated with melatonin for 2h and then irradiated at a dose of 0.05 J/cm^2 using a Bio-Sun irradiation apparatus (Vilbert Lourmat, Marne-la-Vallée, France) as described previously (37) and allowed to repair DNA for 4h. After treatment, cells were detached with trypsin and embedded in 1% low melting agarose (Sigma) in phosphate-buffered saline and spread onto microscopy slides coated previously with 1% agarose (Bio-Rad). Cells were lysed in the lysis solution (2.5M NaCl, 100mM ethylenediaminetetraacetic acid, 10mM Tris base, 8g/l NaOH, 1% Triton X-100, 10% dimethyl sulfoxide) for 1h at room temperature and then run in running solution (300mM NaOH, 1mM ethylenediaminetetraacetic acid, pH 13.0) for 30min at 25V and 250 mA. DNA was equilibrated with 0.4M Tris (pH 8.0) and slides were dried with methanol. DNA was stained with propidium iodide (Sigma) and pictures were taken using ×60 magnification on an Axiovert 200M microscope and Axiovision acquisition program (Zeiss). At least 300 cells were scored for each slide.

Mice xenografts

Four-week-old CD1 nude mice (n = 7/each group, Charles River) were subcutaneously injected with either HCT116 p53 wt or HCT116 p53$^{-/-}$ cell lines (2×10^6 cells/mouse) treated with 1 µM melatonin or vehicle (ethanol) for 72h, changing the medium every 24h. Tumour volume was evaluated twice a week. Animals were killed and xenograft excised after 2 weeks from the injection. Tumour volume (mm^3) was calculated as follows: $0.5 \times D_1 \times D_2$, where D_1 and D_2 are the larger and smaller diameters measured by caliper. All tumorigenicity assays were carried out according to the guidelines set by the Internal Ethical Committee.

Results

Melatonin reduces DNA fragmentation through its membrane receptors

We have shown that melatonin reduces DNA fragmentation following treatment with DNA damaging agents, such as chemotherapeutic drugs and ionizing radiation (14). In addition, we found that this event is mediated by p38 mitogen-activated protein kinase activating p53 (14). It is well known that melatonin can activate signalling pathways by binding to its membrane G-protein-coupled receptors MT1 and MT2 with high affinity (10,30,32–34). Since MAP kinases, such as c-Jun, are known to be activated by G proteins (30), we sought to investigate whether the p53- and p38-dependent reduction in DNA fragmentation by melatonin was triggered by receptor signalling. To this purpose, we selectively inhibited the expression of each melatonin receptor by siRNA in both HCT116 and MCF-7 cell lines and treated them with 1 µM melatonin for 2h to induce DNA repair proteins (14). Cells underwent UVB irradiation at a sublethal

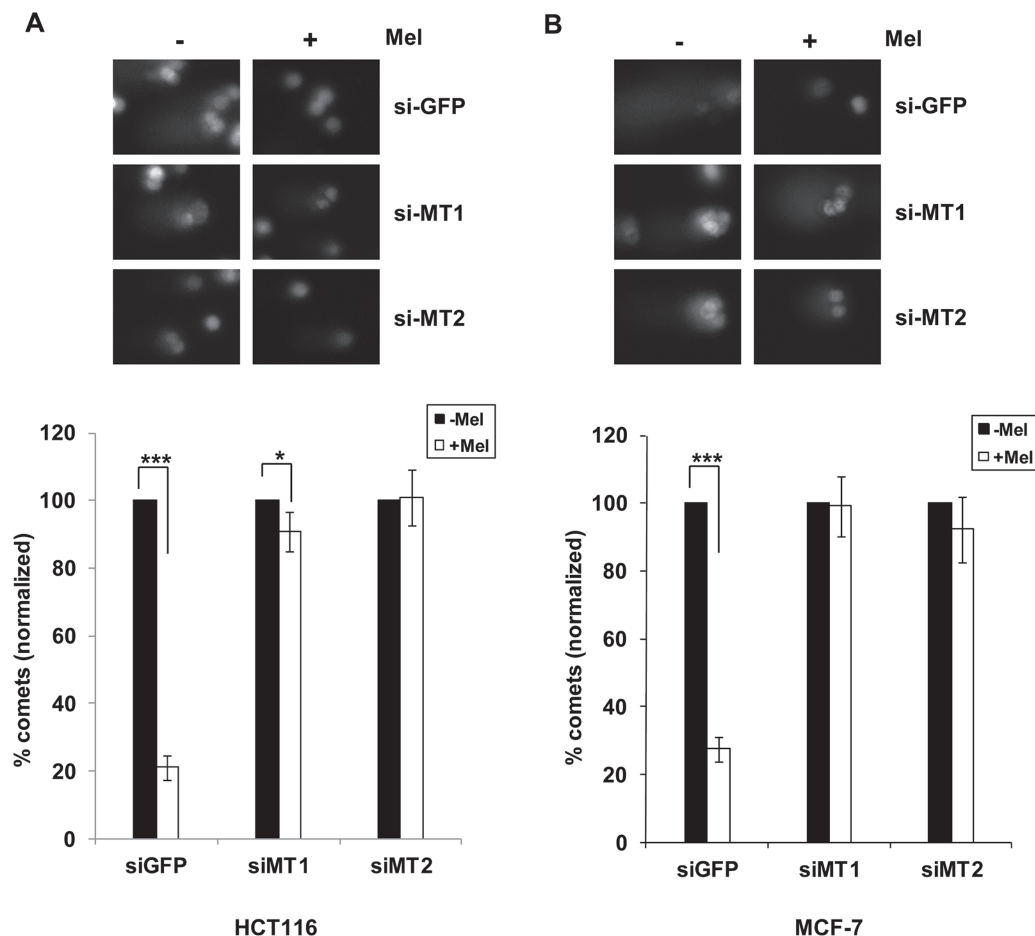


Fig. 1. Induction of DNA repair by melatonin occurs through melatonin membrane receptors. HCT116 (**A**) and MCF-7 (**B**) cells were transfected with the indicated siRNA. Ninety-six hours post-transfection cells were either treated or not treated with 1 µM melatonin for 2h. Cells were subjected to irradiation with 0.05 J/cm^2 UVB and allowed to repair for 4h. Cells were then subjected to comet assay. Histograms show the percentage of comets normalized to control. ***P < 0.001; *P < 0.05.

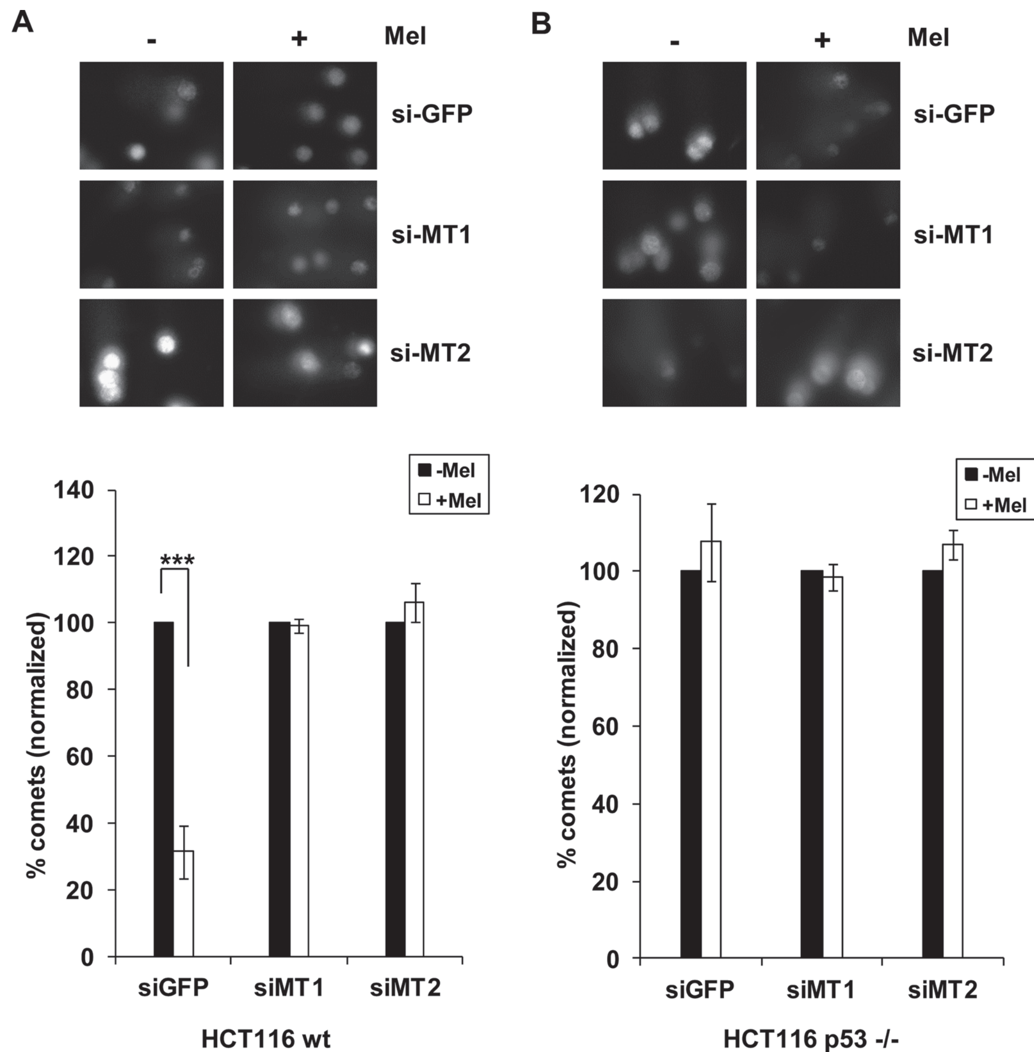


Fig. 2. Melatonin membrane receptors trigger p53-dependent DNA repair. HCT116 wt (**A**) and HCT116 p53$^{-/-}$ (**B**) cells were transfected with the indicated siRNA and either treated or not with 1 µM melatonin for 2 h. Cells were subjected to irradiation with 0.05 J/cm^2 UVB and allowed to repair for 4 h. Cells were then subjected to comet assay. Histograms show the percentage of comets normalized to control. ***$P < 0.001$.

dose and were allowed to repair damaged DNA. As shown previously with chemotherapeutic drugs and gamma-irradiation (14), melatonin was able to induce repair of fragmented DNA following UVB irradiation as well, both in HCT116 (Figure 1A) and in MCF-7 (Figure 1B). However, in the absence of either MT1 or MT2, the number of cells showing DNA fragmentation was not affected by melatonin treatment in either of the cell lines (Figure 1A and B). In addition, comet assays were performed in both HCT116 (Supplementary Figure S1A, available at *Carcinogenesis* Online) and MCF-7 (Supplementary Figure S1B, available at *Carcinogenesis* Online) by either pre-treating or not pre-treating the cells with 1 nM luzindole, a melatonin antagonist binding to and inactivating both MT1 and MT2 receptors, which is known to inhibit both receptors, though it has a 25-fold higher affinity for MT2 (38). Luzindole impaired melatonin's ability to reduce DNA fragmentation following UVB irradiation (HCT116 cells in Supplementary Figure S1A, available at *Carcinogenesis* Online and MCF-7 cells in Supplementary Figure S1B, available at *Carcinogenesis* Online). Both the approaches indicated that receptor function is necessary for melatonin's ability to induce repair of fragmented DNA following UVB irradiation.

Receptor-mediated reduction of DNA damage is p53 dependent

We have shown previously that melatonin's ability to reduce DNA fragmentation was impaired in cells lacking p53 (14). In order to understand whether receptor-mediated DNA repair induced by melatonin was triggered mainly by p53, we performed comet assays (as described for Figure 1) in syngenic HCT116 cells, either wild-type (wt) or *null* for p53. As for other types of DNA damage (14), melatonin was not able to induce repair of fragmented DNA caused by UVB irradiation in the absence of p53 (Figure 2B). Moreover, although removal of either MT1 or MT2 by siRNA impaired melatonin's ability to reduce DNA fragmentation in HCT116 wild-type cells, it did not have any further effect in HCT116 p53 *null* cells (Figure 2A and B) demonstrating that removal of either receptor impairs the activation of p53 by melatonin. Of note, HCT116 p53 *null* cells have been reported to express two p53 isoforms (39–41). Thus, our results are consistent with the need of full-length p53 for DNA repair.

Blockage of melatonin receptors impairs melatonin's ability to reduce cell proliferation

Melatonin has proven to be able to inhibit proliferation of cancer cells (12–14). Here, we investigated whether activation of receptors was involved in this process. In order to do this, we silenced MT1 or MT2 expression in MCF-7 (Figure 3A), HCT116 p53 wt (Figure 3B) and HCT116 p53 *null* (Figure 3C) cells. Colony-forming assays showed that depletion of either receptors by siRNA impaired melatonin's ability to inhibit long-term proliferation (Figure 3A and B). The same effects were observed in short-term proliferation, as assessed by growth curves in both HCT116 (Figure 3D) and MCF-7 (Figure 3E). In the absence of

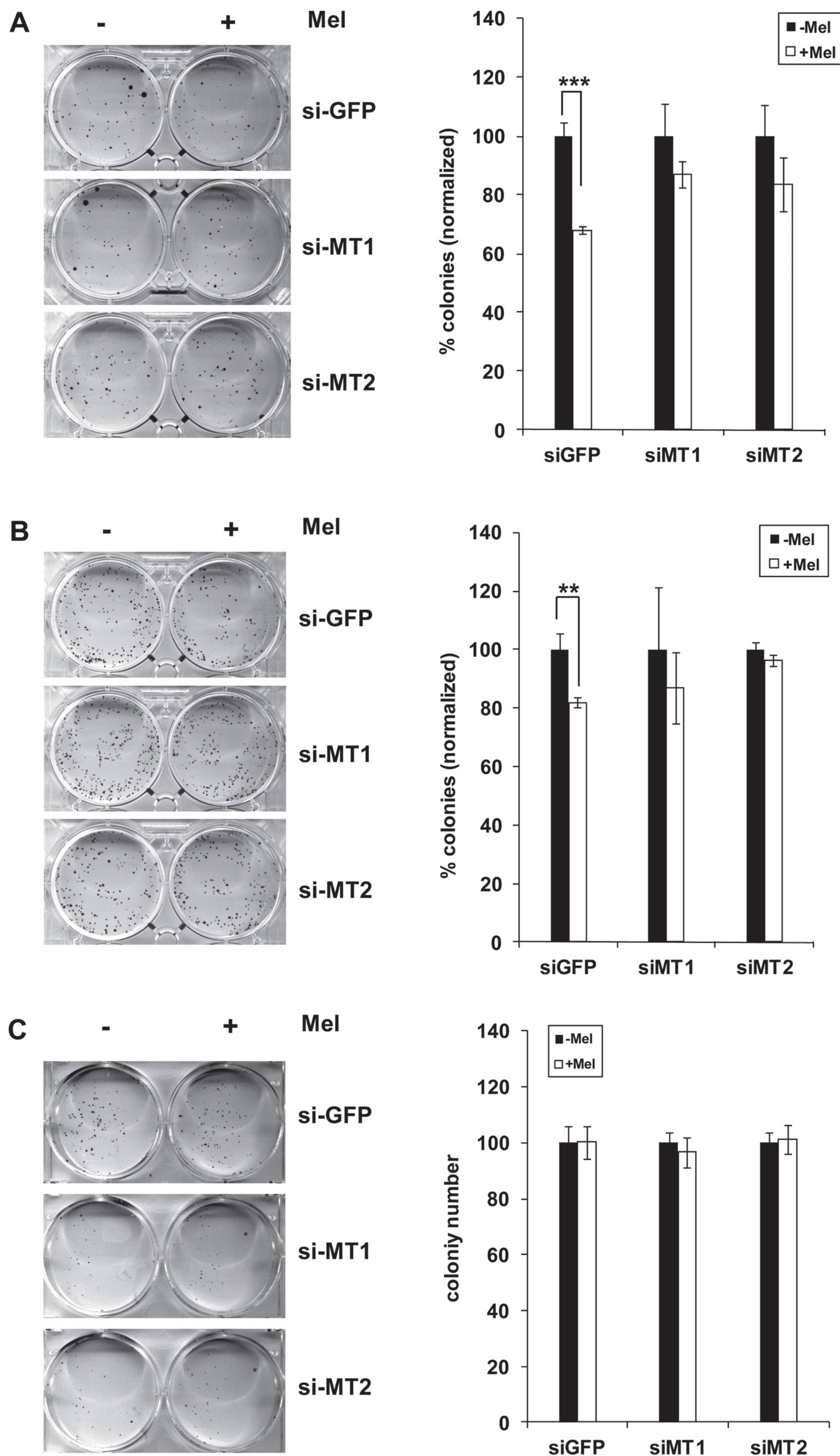
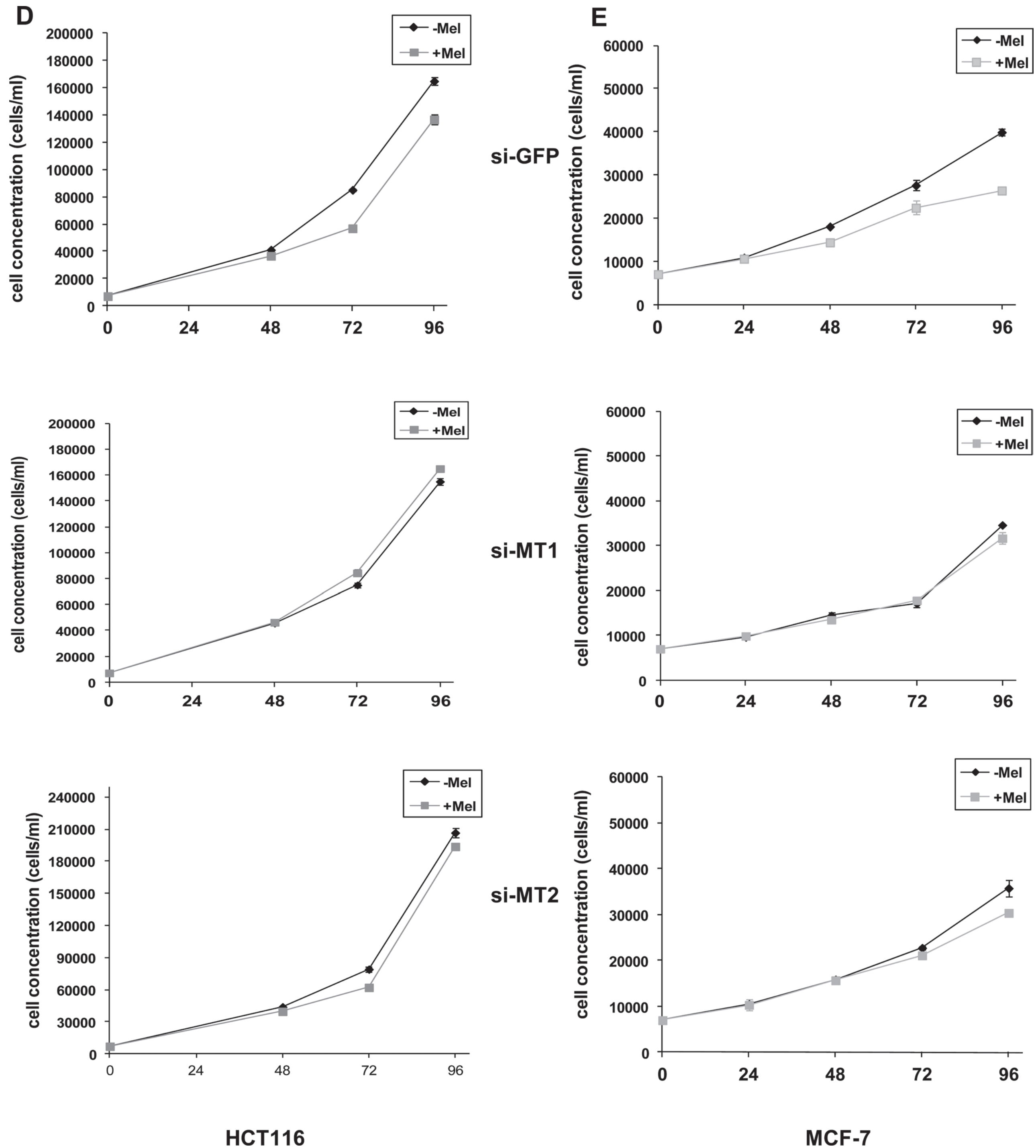


Fig. 3. Melatonin inhibition of cell proliferation is receptor mediated. MCF7 (**A**), HCT116 wt (**B**) and HCT116 p53$^{-/-}$ (**C**) cells were transfected as in Figure 1, seeded and allowed to form colonies for 15 days. Culture medium (without or with 1 µM melatonin) was replaced every second day to keep melatonin levels constant. Colonies were stained and counted. Histograms show the percentage of cells relative to untreated cells. ***$P < 0.001$; **$P < 0.01$. HCT116 (**D**) and MCF-7 (**E**) cells were seeded and allowed to grow for the indicated time. Culture medium (without or with 1 µM melatonin) was replaced every second day to keep melatonin levels constant. Cell number was counted with the Guava EasyCyte 8HT flow cytometer. Cell concentration is shown in the graphs.



p53, removal of melatonin receptors did not have any effect on cell proliferation (Figure 3C). Besides the genetical approach, we chemically inhibited both MT1 and MT2 functions by using luzindole. Melatonin inhibited long-term cell growth only in the presence of p53 and in the absence of luzindole both in HCT116 (Supplementary Figure S2A and B, available at *Carcinogenesis* Online) and in MCF-7 (Supplementary Figure S2C and D, available at *Carcinogenesis* Online) cells. These experiments showed that intact receptor function mediated the reduction in cell proliferation by melatonin.

To explore the antitumorigenic potential of melatonin, we subcutaneously inoculated immunodeficient CD1 mice with human HCT116 p53 wt and HCT116 p53 *null*, which have been either treated or not treated with melatonin for 72 h. All mice were killed 21 days after the injection. As shown in Figure 4A, melatonin treatment induced a specific inhibitory response and robustly interfered with tumour growth in HCT116 p53 wt, as shown by the distribution of tumour volumes among the four groups of HCT116 p53 wt and HCT116 $p53^{-/-}$ injected mice at the time of the last measurement (Figure 4A). This difference was due to a melatonin-induced reduction of proliferation in p53-expressing cells, as demonstrated by the decrease of Ki-67 protein expression in the HCT116 p53 wt xenografts, which was not observed in HCT116 $p53^{-/-}$ xenografts (Figure 4B).

Melatonin membrane receptors trigger p53 activation

Melatonin has been shown to induce a p38-dependent p53 phosphorylation in Ser-15 (14). However, little is known about the mechanisms

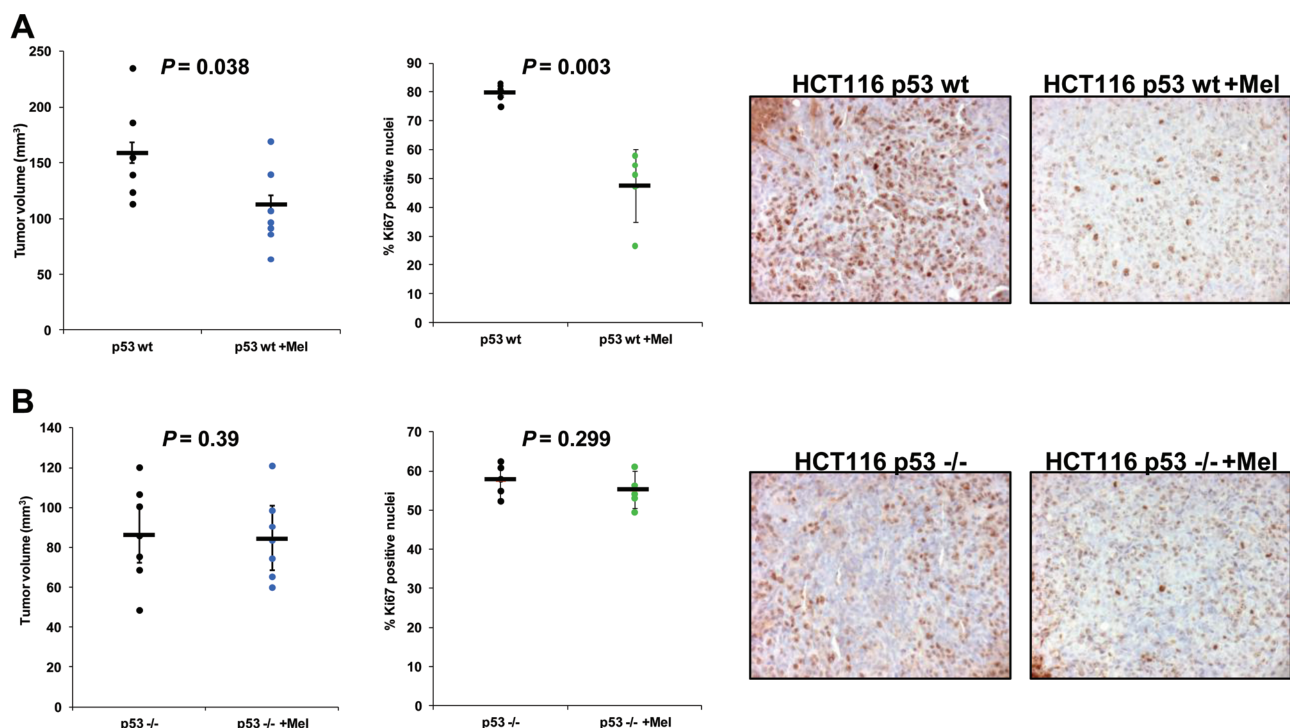


Fig. 4. Melatonin reduces the growth of cancer cells in xenograft models. HCT116 wt (**A**) and HCT116 $p53^{-/-}$ (**B**) cells were treated with 1 µM melatonin or vehicle for 72 h and subcutaneously injected into CD1 mice. Dot plots show tumour volume (left panel) and proliferation index (right panel) as assessed by Ki-67 immunostaining, for which sample images are shown. *P*-values are indicated on the graphs.

underlying the activation of p38 and p53 by melatonin. We deduced that, since melatonin's chemopreventive activities such as reduction of cell proliferation and DNA fragmentation were receptor mediated, p53 activation by melatonin could occur through receptor activation as well. In order to ascertain whether melatonin receptors were involved in p38 activation and p53 phosphorylation, we performed immunoblots in both HCT116 and MCF-7 cells depleted for either MT1 or MT2 by siRNA. As shown in Figure 5A and B and Supplementary Figure S3A and B, available at *Carcinogenesis* Online, melatonin induced an increase in p38 protein levels as well as phosphorylation of both p38 in Thr180/Tyr1182 and p53 in Ser-15. Depletion of either MT1 or MT2 impaired melatonin's ability to induce p38 and p53 phosphorylation. This indicated that these events are caused by a signal transduction mediated by the binding of melatonin to its receptors. We performed the same kind of experiment following chemical inhibition of melatonin receptors MT1 and MT2 with luzindole. Inhibition of melatonin receptors by this approach impaired the signal transduction pathway triggering p53 phosphorylation (Figure 5C and D and Supplementary Figure S3C and D, available at *Carcinogenesis* Online). In addition, we knocked down simultaneously MT1 and MT2 receptors in both the cell lines. Similar to luzindole treatment, lack of the receptors impaired melatonin-induced signal transduction (Figure 5E and F and Supplementary Figure S3E and F, available at *Carcinogenesis* Online).

p38 and p53 are phosphorylated following DNA damage and radical oxygen species generation (24–29). In order to investigate whether melatonin receptors could trigger p38 activation in response to DNA damage, we tested the impact of simultaneous knock-down of MT1 and MT2 on p38 phosphorylation following UVB irradiation. UVB irradiation induced a massive phosphorylation of p38, which was completely impaired in the absence of melatonin receptors (Figure 5G and Supplementary Figure S3G, available at *Carcinogenesis* Online). This further strengthened our observation that melatonin receptor signalling triggers a p38- and p53-dependent DNA damage response.

It is worth noting that silencing of either receptor induced an increase in p38 and p53 levels (Figure 5A and B, E and F), possibly due to generation of DNA fragmentation (data not shown). Treatment of cells with melatonin following silencing of MT1 induced a decrease in both p38 and p53 phosphorylation, whereas this did not happen when MT2 was knocked down (Figure 5A and B and Supplementary Figure S3A and B, available at *Carcinogenesis* Online). It has been reported that melatonin receptors have a different ability to trigger phosphorylation upon melatonin binding (31), with MT1 being more effective than MT2. In addition to inducing a receptor-mediated phosphorylation cascade, melatonin acts as a radical scavenger within the cells, thereby reducing the phosphorylation of p38 and p53. We believe that, in the absence of MT1, MT2 activation by melatonin cannot cope with melatonin potential as a radical scavenger and this results in reduction of radical oxygen species-induced phosphorylation of p53 and p38.

Discussion

Epidemiological studies have clearly demonstrated that high levels of melatonin decrease the risk of developing various types of cancer, among which breast and colon cancer (2–9). Only recently some light has been shed on the mechanisms underlying melatonin's chemopreventive actions (10,12–14). In particular, melatonin has been shown to regulate the expression of both p53 and p21 (13) and to increase p38-mediated p53 phosphorylation in cancer cells (12,14). In experimental studies, the induction of the p38 and p53 pathways has different outcomes according to the dose of melatonin used. When cells are treated with doses of melatonin close to either physiological levels or levels reached during the treatment of sleep disorders (1 nM to 0.5 µM), p53 is activated through phosphorylation in Ser-15 and cells undergo a transient cell cycle arrest, during which they can repair damaged DNA (14). Conversely, when cells are treated with higher doses of melatonin (0.5–2 mM), p53 phosphorylation as well as total p53 levels are markedly increased and apoptosis is induced (12). This is not surprising since, in response to stressful conditions, the transcription factor p53 undergoes post-translational modifications, such as acetylation, phosphorylation and sumoylation (15–23,42,43). Once modified, p53 binds the DNA and activates the transcription

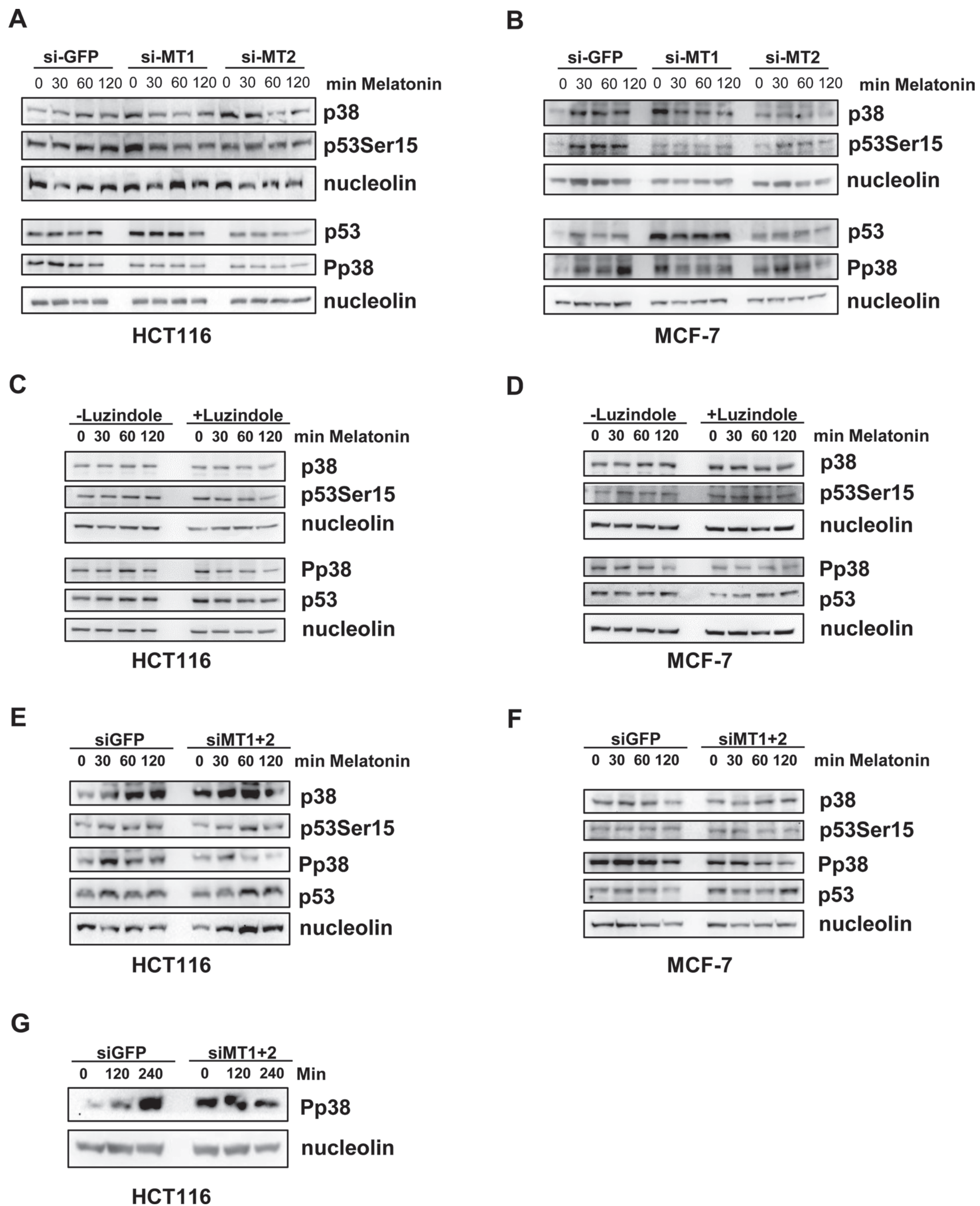


Fig. 5. Blockage of melatonin membrane receptors impairs melatonin-induced activation of p53. HCT116 (**A**) and MCF-7 (**B**) cells were transfected with the indicated siRNA. Ninety-six hours post-transfection cells were treated with 1 µM melatonin for the indicated time. Cell extracts were subjected to immunoblot with the indicated antibodies. HCT116 (**C**) and MCF-7 (**D**) cells were treated with 1 nM luzindole for 15 min, then with melatonin for the indicated time. Cell extracts were subjected to immunoblot with the indicated antibodies. HCT116 (**E**) and MCF-7 (**F**) cells were transfected with the indicated siRNA. Ninety-six hours post-transfection cells were treated with 1 µM melatonin for the indicated time. Cell extracts were subjected to immunoblot with the indicated antibodies. (**G**) HCT116 cells were transfected with the indicated siRNA. Ninety-six hours post-transfection cells were irradiated with 0.05 J/cm^2 UVB. Cell extracts were prepared at the indicated time post-irradiation and were subjected to immunoblot with the indicated antibodies. Western blot signal quantification for each panel is shown in Supplementary Figure S3, available at *Carcinogenesis* Online.

of a plethora of target genes, which can cause a variety of cellular responses, ranging from cell cycle arrest to apoptosis (17,19,43,44). In particular, when cells undergo sublethal DNA damage, p53 is phosphorylated by DNA damage activated kinases, such as ataxia telangiectasia mutant, ataxia telangiectasia related and p38 mitogen-activated protein kinase, and activates genes inducing cell cycle arrest, such as p21 (15,17,21–23). When this occurs, a DNA repair response is induced, in which phosphorylated p53 plays a central role (19,21,44). We have demonstrated previously that melatonin is able to induce a p38-dependent phosphorylation cascade, which determines the phosphorylation of both p53 in Ser-15 and histone H2AX in Ser-139 (14). Here, we show that melatonin triggers a p38- and p53-mediated DNA damage response through activating receptors (Figures 1 and 2). In fact, by both selective silencing of melatonin receptors MT1 and MT2 and their chemical inhibition with melatonin's antagonist luzindole, melatonin's ability to reduce DNA fragmentation in response to ionizing radiation was impaired. Along the same line, both removal and inactivation of melatonin receptors weakened

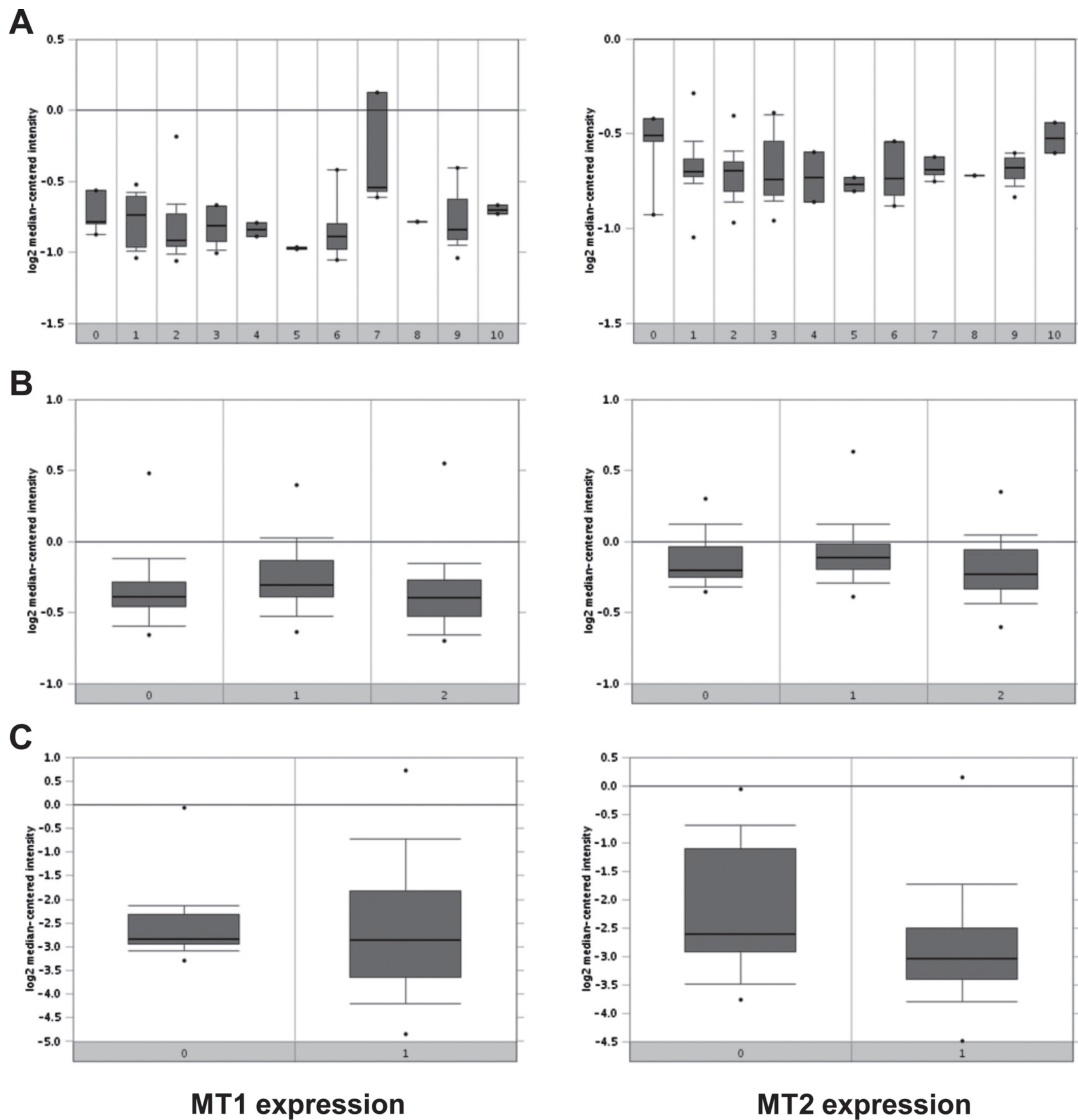
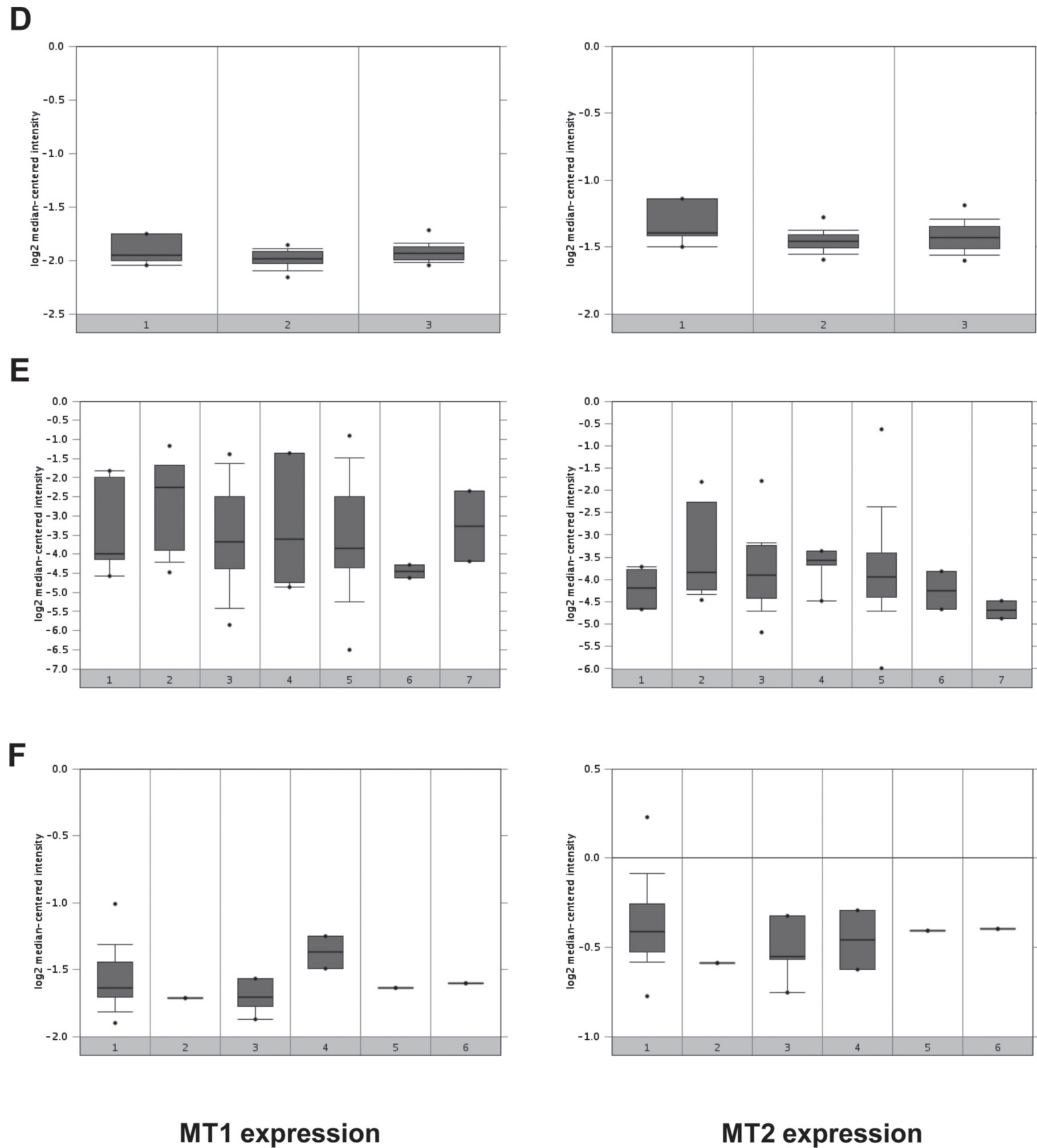


Fig. 6. Expression of melatonin receptors is reduced in colon cancer. Box plots showing reduced MT1 (left) and MT2 (right) expression in tumour versus normal samples in six publicly available casuistries from Oncomine. (**A**) Kaiser casuistry [0. No value (5); 1. Cecum adenocarcinoma (17); 2. Colon adenocarcinoma (41); 3. Colon mucinous adenocarcinoma (13); 4. Colon signet ring cell adenocarcinoma (2); 5. Colon small cell carcinoma (2); 6. Rectal adenocarcinoma (8); 7. Rectal mucinous adenocarcinoma (4); 8. Rectal signet ring cell adenocarcinoma (1); 9. Rectosigmoid adenocarcinoma (10); 10. Rectosigmoid mucinous adenocarcinoma (2); Kaiser colon. Genome Biol 2007/07/05, 105 samples, MTNR1A Information: mRNA, 19 574 measured genes. Reporter information: Human Genome U133 Plus 2.0 Array]; (**B**) Skrypczak casuistry [0. No value (24); 1. Colorectal adenocarcinoma (45); 2. Colorectal carcinoma (36); Skrzypczak colorectal. PLoS One 2010/10/01, 105 samples, MTNR1A Information: mRNA, 19 574 measured genes. Reporter Information: Human Genome U133 Plus 2.0 Array]; (**C**) Hong casuistry [0. No value (12); 1. Colorectal carcinoma (70); Hong colorectal. Clin Exp Metastasis 2010/02/01, 82 samples, MTNR1A Information: mRNA, 19 574 measured genes. Reporter Information: Human Genome U133 Plus 2.0 Array]. Numbers between brackets indicate the number of samples; (**D**) Farmer casuistry [1. Apocrine breast carcinoma (6); 2. Basal-like subtype of invasive breast carcinoma (16); 3. Luminal-like subtype of invasive breast carcinoma (27); Farmer breast. Oncogene 2005/07/07, 49 samples, MTNR1A Information: mRNA, 12 624 measured genes. Reporter Information: Human Genome U133A Array]; (**E**) Desmedt casuistry [1. Breast carcinoma (8); 2. Invasive ductal and invasive lobular breast carcinoma (11); 3. Invasive lobular breast carcinoma (13); 4. Invasive tubular and lobular carcinoma (4); 5. Invasive ductal breast carcinoma (158); 6. Medullary breast carcinoma (2); 7. Mucinous breast carcinoma (2); Desmedt breast. Clin Cancer Res 2007/06/01, 198 samples, MTNR1A Information: mRNA, 12 624 measured genes. Reporter Information: Human Genome U133A Array]; (**F**) Ginestier casuistry [1. Invasive ductal breast carcinoma (45); 2. Invasive lobular breast carcinoma (1); 3. Medullary breast carcinoma (5); 4. Invasive mixed breast carcinoma (2); 5. Tubular breast carcinoma (1); 6. Invasive breast carcinoma (1); Ginestier breast. Clin Cancer Res 2006/08/01, 55 samples, MTNR1B Information: mRNA, 19 574 measured genes. Reporter Information: Human Genome U133 Plus 2.0 Array].



melatonin's activity towards reduction of cell proliferation (Figure 3). Melatonin's anticancer activities can be attributed to p53 activation by p38 (14). Furthermore, intact receptor signalling was necessary for melatonin-induced DNA damage response. Hence, we proposed that blockage of melatonin receptor signalling by either gene silencing or chemical inhibition would result in lack of phosphorylation of p53 following melatonin treatment. Indeed, our experiments showed that melatonin could not induce p53 phosphorylation in Ser-15 in the absence of intact receptor signalling, which paired to a lack in the increase of p38 protein levels (Figure 5). Moreover, we showed that removal of either receptor impaired melatonin's anticancer activities. Based on these results, we could speculate that melatonin triggers its chemopreventive activities by binding to and activating MT1/MT2 heterodimers. This is confirmed by previous studies showing that melatonin-activated signal transduction is stronger when melatonin binds to MT1/MT2 heterodimers as compared with MT1/MT1 and MT2/MT2 homodimers (31). In light of these results, we suggested that melatonin receptors could play an important role in tumorigenesis. In order to ascertain whether they are actually involved in cancer pathogenesis, we interrogated tumour data sets with Oncomine (www.oncomine.org). We used three independent casuistries of colon cancers and three of breast cancers and analysed the transcript levels for MT1 and MT2 in cancer versus normal samples (45–50). The data sets were produced with Affymetrix Human Genome U133 Plus 2.0 Array and the analyses were performed by using Oncomine (www.oncomine.org). For colon cancers, the first (Kaiser, Figure 6A) and the second (Skrzypczak, Figure 6B) casuistries are comprised of 105 samples each; the third one (Hong, Figure 6C) is composed of 82 samples. For breast cancers, the first casuistry (Farmer, Figure 6D) is comprised of 49 samples, the second (Desmedt, Figure 6E) of 198 samples and the third one (Ginestier, Figure 6E) of 55 samples. In all the casuistries, both MT1 and MT2 mRNA expression levels were downregulated in cancer versus normal samples, as shown by box plots, regardless of tumour type.

Moreover, we show that melatonin treatment significantly reduced the tumour volume as well as the Ki-67 proliferation index of p53-expressing cells *in vivo* (Figure 4), confirming the key role of melatonin in the control of cancer cells proliferation.

It has to be noted that published data evidenced an inverse correlation between MT1 expression and tumour development (51–53), whereas others found a positive correlation (54–56). Importantly, some authors showed an inverse correlation between melatonin serum levels and MT1 expression (57), shading the possibility that enhanced expression of melatonin receptors in cancer could be due to lower levels of circulating melatonin.

Several trials have been conducted with melatonin as an adjuvant for chemotherapy in cancer patients (58–60) and systematic reviews on these data have been published (60,61). The main aim of these studies was to understand whether melatonin could have an active role in suppressing tumour growth, whereas little attention was paid to the side effects of chemotherapy. A systematic review considered and analysed also the information related to the physiological response to chemotherapeutic treatments (61). Meta-analysis showed that the use of melatonin as an adjuvant for chemotherapy significantly improved relapse and survival rate as compared with chemotherapy alone. Moreover, melatonin treatment ameliorated chemotherapy side effects, such as immune response and thrombocytopenia.

Our data provide *in vitro* and *in vivo* evidences that melatonin anti-cancer activities, including DNA damage response and inhibition of cell proliferation, are mediated by the activation of the MT1 and MT2 receptors and analyses on sample data sets for breast and colon cancer show that both MT1 and MT2 mRNA levels are reduced in cancer as compared with normal colon samples. This represents important information for both the design and the analysis of trials involving melatonin either as an adjuvant for chemotherapy or as a chemopreventive agent. In fact, when analysing the effects of melatonin on cell growth and relapse-free survival, MT1 and MT2 expression in tumours under study might acquire great importance. It is expected that patients with tumours expressing low levels of melatonin receptors would not respond to melatonin treatment in terms of decreasing tumour progression and increasing relapse-free survival. However, the same patients could benefit from melatonin's ability to reduce bystander effects, increase immune response and ameliorate side effects due to chemotherapy administration (thrombocytopenia, anaemia and asthenia). This is confirmed by the fact that meta-analysis showed that 1 year survival rate was significantly increased by melatonin co-administration with chemotherapy (61).

As for the prospective chemopreventive studies, it could be of interest the analysis of possibly arising tumours in subjects treated with melatonin. In fact, melatonin could demonstrate its effectiveness in preventing the formation of a wide range of tumours, while tumours arising despite the treatment could be due to mutations, hypermethylation or deletion of either MT1 or MT2. In light of these implications, our work might acquire strong relevance for clinical trials involving melatonin both as a chemopreventive agent and as an adjuvant for chemotherapy.

Supplementary material

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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