Research Article

Melatonin inhibits human melanoma cells proliferation and invasion via cell cycle arrest and cytoskeleton remodeling

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ABSTRACT

Among skin cancers, melanoma has the highest mortality rate. The heterogeneous genetic melanoma background leads to a tumor-propagating capacity particularly important in maintaining therapeutic resistance, and tumor recurrence. The identification of efficient molecules able to control melanoma progress represents an important opportunity for new therapeutic strategies, particularly in combination with the current standard-of-care treatments. In this context, several studies have reported the antitumor effects of melatonin against different types of cancer, including melanoma. Here, we describe the underlying mechanisms associated with melatonin's activity in human melanoma cell lines, focusing on cell cycle and cytoskeleton remodeling. Interestingly, while melatonin induced melanocyte DNA replication, melanoma cells exhibited cell cycle arrest in the G1-phase. This phenomenon was associated with cyclin-D1 downregulation or p21 overexpression. The efficacy of melatonin on melanoma cells survival and proliferation was detected using the clonogenic assay, with a decrease in both the number and size of colonies. Additionally, melatonin induced a dramatic cytoskeleton remodeling in all melanoma cell lines, leading to a star-like morphology or cell swelling. The role of melatonin on melanoma cytoskeleton was associated with the actin disruption, with thinning and/or broken actin fibers, and weak and/or loss of paxillin along stress fibers. These data support the observed findings that melatonin impairs melanoma invasion in skin reconstructed models. Together, our results suggest that melatonin could be used to control melanoma growth and support basic and clinical studies on melatonin as a promising immunometabolic adjuvant for melanoma therapy.

Keywords: melanoma, melatonin, cell cycle, cytoskeleton, reconstructed skin.

1. INTRODUCTION

Melanoma is a cutaneous malignancy that originates in the pigment-producing melanocytes in the basal layer of the epidermis (1). Despite the general decline in the incidence of cancer worldwide, melanoma rates continually increase by around 3% annually (2). Depending on melanoma grading, different therapeutic strategies are adopted, from surgical excision to radiation therapy, chemotherapy, targeted-therapy and immunotherapy (3). If not removed, melanoma progresses to metastatic disease with a high mortality rate. Treatment of disseminated melanoma is still a complicated issue, mainly due to its complex and heterogeneous genetic background (4). Although improved knowledge of both cancer immunology and the molecular pathogenesis of melanoma result in major improvements in patient outcomes (5, 6), melanoma is typically resistant to currently available therapy. Therefore, new adjuvant therapy, working together with the current standard-of-care treatments, is essential to improve clinical outcomes. In this regard, melatonin (*N*-acetyl-5-methoxytryptamine) has been evaluated in clinical trials as an immunometabolic adjuvant to aid the treatment of cancer patients.

Melatonin is a natural molecule derived from an essential amino acid tryptophan, produced in the pineal gland of humans and other animals mainly in response to darkness (7). It has become a current supplementary substance among people with occasional insomnia or sleep disorders (8). Although the pineal gland is the major source of melatonin production, this indoleamine is also produced in the gastrointestinal tract (9), skin (10), and immune competent cells (11) independent of the light/dark cycle (12), and probably in the mitochondria of every cell (13). In addition to the role of melatonin in the control of human circadian rhythms and endocrine function, melatonin has a myriad of non-chronobiotic effects (14, 15). Experimental data show that melatonin inhibits tumorigenesis due to its antiproliferative and cytotoxic properties in numerous types of tumor cells (10, 16, 17). Additionally, long-term administration of melatonin in humans (18) and animal models (19) demonstrated no side effects and also showed some protections against the recurrence of the malignant tumors. Melatonin also aided the therapeutic efficacy of cancer chemotherapy due to its chemo-sensitization effects, particularly in patients with poor clinical status (20).

Our group has previously reported that melatonin is synthesized by human melanoma cell lines and skin cells after treatment with 1-methyl-tryptophan (10), a known inhibitor of the enzyme indoleamine 2,3-dioxygenase (IDO) (21). Moreover, melatonin also downregulated the tryptophan metabolite, kynurenine (KYN), in fibroblasts, keratinocytes, melanocytes, and melanoma cells (10). Notably, induction of IDO-mediated tryptophan catabolism, together with its downstream products such as KYN, is an important immunoregulatory mechanism underlying cancer immune evasion (22). Here, we exploit the mechanisms associated with melatonin upon *in vitro* melanoma treatment. We show that melatonin decreases the proliferation of a panel of human melanoma cell lines with distinct genetic backgrounds (4) by disrupting cell cycle and cytoskeleton remodeling. Our data also indicate that melatonin switches the microfilament phenotypes from invasive migratory melanoma to non-migratory cells, highlighting the implications of melatonin with the cytoskeleton as a therapeutic target for melanoma cells.

2. MATERIAL AND METHODS

2.1. Cells culture conditions and treatments.

The human melanoma cell lines, identified as SK-Mel-5, SK-Mel-19, SK-Mel-28, SK-Mel-29, SK-Mel-103, SK-Mel-147, G-361, and UACC-62, were cultured in Dulbecco's modified

Eagle's media (DMEM) (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), penicillin (50U/ml) and streptomycin (50µg/ml) (Invitrogen; USA). Primary human fibroblasts and melanocytes were obtained from neonatal foreskins (Hospital Niño Jesús, Madrid, Spain). Melanocytes were cultured in 254CF media (#M-500-254CF, Cascade Biologics, Portland - USA) supplemented with Human Melanocyte Growth Supplement (#S-002-5, Cascade Biologics, USA); fibroblasts were cultured in DMEM supplemented with 10% FBS. All cells were maintained at 37 °C under 5% CO_2. To prepare the samples, cells were seeded in an experimental plate and cultured in the proper media for 24 hours until achieving 50% confluence. Then, melatonin (Sigma-Aldrich, Saint Louis, MO, USA) was added to the cell culture for 24 hours or more (1mM final concentration). The melatonin solution was prepared as followed: melatonin (milligrams) was previously dissolved in dimethyl sulfoxide (DMSO) in a representative volume of 0.5% of the final volume of the solution. After that, the soluble melatonin was resuspended in supplemented DMEM at a final volume to reach the concentration of 1mM of melatonin. The vehicle treatment, used for the control group assays, was composed of the supplemented DMEM with 0.5% DMSO. Under these experimental conditions, neither the melatonin nor the vehicle solution induces cell death (data not shown).

2.2. Cell cycle analysis by detection of BrdU incorporation.

Actively growing cells, treated with or without 1mM of melatonin for 48 hours in 6-well cell culture plate, were pulsed for one hour with 10µM BrdU (Sigma-Aldrich, USA). Then, cells were detached with trypsin, poured into a FACS tube, and centrifuged for 5 minutes, 250g at room temperature. The supernatant was discarded, and cells were treated according to BD bioscience protocol (www.bdbiosciences.com/ds/is/tds/23-1349.pdf) for further Anti-BrdU monoclonal antibody staining, for 30 minutes at 4 °C (cat. Nº 347583, BD Bioscience, USA). Then, cells were washed with PBS and treated with 1µl of RNAse (100mg/ml, Qiagen, Hilden, Germany) and 150µl of propidium iodide solution (50µg/ml in PBS; Sigma-Aldrich, USA) at RT, in the dark, for 30 minutes. After appropriated washing, cells were analyzed by flow cytometry using the equipment of FACS Canto II (Becton Dickinson and Company®, USA). As a result, the G1, S, and G2/M populations can be clearly differentiated and quantitated, respectively. 20,000 single-cell events are normally acquired. Experiments were done in triplicate and repeated twice.

2.3. Protein immunoblotting.

Melanoma cell lines were treated with melatonin (1mM) for 48 and 72h and then, the total protein was extracted from cells using RIPA buffer. Next, proteins were electrophoretically separated by SDS–Page (12% polyacrylamide gel) and subsequently transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). After blocking with 5 % skim milk in TBT-T buffer (500 mM Tris, 60 mM KCl, 2.8 M NaCl, 1% Tween 20, pH 7.4), membranes were incubated with primary antibodies. The secondary antibodies used were anti-rabbit or anti-mouse (GE Healthcare, Buckingham shire, UK). Protein bands were detected using the ECL system (GE Healthcare, UK). Primary antibodies included: anti-α-Tubulin (T9026, Sigma-Aldrich, USA) anti-p21 (sc-397, Santa Cruz Biotechnology, Texas, USA), and anti-cyclin D1 (NeoMarkers, Fremont, USA). The α-Tubulin blots were used as loading samples control. Two independent experiments were performed.

2.4. Clonogenic assay.

Six hundred cells were seeded into 60 mm plates and cultured for 18-24 hours. Then, the medium was removed and replaced with fresh medium containing 1mM of melatonin. The melatonin-containing medium was replaced every 48 hours. After 15 days, cells were washed with PBS and stained as described before (10). Experiments were done in triplicate and repeated twice.

2.5. Immunofluorescence for analysis of cytoskeletal alterations.

Melanoma cells were seeded in glass coverslips placed into a 24 well plate. After 18 hours incubation, cells were treated with 1 mM of melatonin for 48 hours. Next, cells were fixed in 4% paraformaldehyde and were processed for immunofluorescence, according to pre-established manufacturer's protocol. The paxillin antibody (clone 5H11, Millipore, USA) was used to visualize focal adhesions. Alexa Fluor 568 Phalloidin (Invitrogen; USA) was added to visualize F-actin. Preparations were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, USA). Confocal images were collected with a confocal TCS-SP5-WLL (AOBS-UV) spectral microscope (Leica Microsystems, Wetzlar, Germany). Experiments were done in duplicate and repeated twice.

2.6. Skin reconstructs containing melanoma.

The human skin reconstructs containing melanoma were produced as previously described with some modifications (23–25). The reconstructed skins were performed in two steps. Firstly, a dermal equivalent was prepared using 1.2×10^5 of primary human fibroblasts embedded in a collagen mixture consisting of 50 µL/mL of FBS (Fetal Bovine Serum, Life Technologies, South America), 750 µL/mL of type I collagen (BD Biosciences, San Jose, California, USA), 100 µL/mL of HAM-F12 medium 10X (GIBCO, Life Technologies, Grand Island, NY, USA) and 100 µL/mL of reconstitution buffer 10X (0.05 M NaOH, 2.2% $NaHCO_3$, 200 mM HEPES). Secondly, after the dermal equivalent polymerization, 2×10^5 of primary human keratinocytes, 0.56×10^4 of primary human melanocytes, and 10^5 of SK-Mel-147 were plated on the dermal equivalent layer. Following a contraction of the collagen gel (24 hours later), the entire structure was transferred to a steel grid to allow for an air-liquid interface while maintaining contact with the Raft medium consisting of 67.5% DMEM (GIBCO, Life Technologies, USA), 22.5% HAM-F12 medium (GIBCO, Life Technologies, USA); 10% FBS (Life Technologies, South America), 5µg/mL apo-transferrin (T-1147, Sigma-Aldrich, USA), 5µg/mL insulin (I-1882, Sigma-Aldrich, USA), 0.4mg/mL hydrocortisone 21-hemisuccinate (H-4881, Sigma-Aldrich, USA), 1ng/mL EGF (human epidermal growth factor, 13247-010; (GIBCO, Life Technologies, USA) and 0.1nM cholera toxin (3012, Sigma-Aldrich, USA). Subsequently, the culture was maintained at the air-liquid interface for 10 days to allow the complete keratinocyte stratification and differentiation. Melatonin (1mM) was added to the liquid interface for skin reconstructs treatment for an additional 48 hours. The human skin reconstructs were washed with PBS pH 7.4, fixed in ethanol 70% (1 hour), and embedded in paraffin for histological analysis. Experiments were done in triplicate and repeated twice.

2.7. Statistics.

The statistically significant differences in the mean values of all experimental groups were calculated using a one-way *ANOVA* followed by Newman-Keuls multiple comparison test P value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Melatonin induces melanoma cell cycle arrest via modulation of cyclin-D1 and p21 expression.

Since melatonin has a potential impact on tumor cell proliferation, we investigated the effect of this molecule on three different human melanoma cell lines, SK-Mel-19, SK-Mel-147, and UACC-62, with distinct genetic backgrounds (4). The cell cycle progression was analyzed by the incorporation of BrdU and propidium iodate staining. After 48 hours of 1 mM melatonin treatment, cell analysis revealed that melatonin induced cell cycle arrest in the G0/G1 phase in all melanoma cells, while slightly increasing melanocytes proliferation (Figure 1A-B). Melatonin elevated the G0/G1 phase distribution in approximately 12%, 7%, and 13% for SK-Mel-19, SK-Mel-147, and UACC-62, respectively, this phenomenon was accompanied by S phase reduction of 10%, 6,5%, and 11%, respectively. The G2 phase was unaffected by melatonin for all cells analyzed.

The cell cycle disturbance was confirmed by analyzing the expression of proteins involved in the G0/G1 phase arrest by Western blot. Interestingly, melatonin downregulated the expression levels of cyclin D1 and p21 in SK-Mel-19, while it upregulated the expression levels of those proteins in SK-Mel-147 and UACC-62 (Figure 1C-D). Our data suggest that melatonin abrogates the cell cycle checkpoints at critical phases which limits melanoma cell proliferation and probably switches those cells into a less invasive phenotype, independent of their genetics and phenotypic heterogenicity.

3.2. Melatonin highly impacts on melanoma cell proliferation, clonogenicity, and morphology.

Considering the phenotypic plasticity of melanoma tumors, we tested how melatonin acts on the survival capacity of a higher panel of melanoma cell lines. Thus, we proceeded with the clonogenic assay, which enables an assessment of cells colony-forming ability. Notably, the survival capacity of 6 out of 8 melanoma cell types was impaired following melatonin treatment, whereupon melatonin decreased both the density and the number of the melanoma colonies (Figure 2A). Interestingly, we observed a heterogeneous cell growth inhibition among the different cell lines, suggesting that genetic background might play a role in responsiveness to melatonin treatment. Furthermore, melatonin slightly inhibited cell growth of the melanoma cells line, SK-Mel-19, and SK-Mel-29, compared to the robust effect on other cell lines (Figure 2A).

Throughout the cellular assays, we observed that metastatic melanoma cells underwent a significant morphological change in the presence of melatonin (Figure 2B). SK-Mel-5 showed melanocyte-like morphology, while SK-Mel-19, SK-Mel-28, and SK-Mel-29 exhibited a star-like morphology, with cytoplasm extensions similar to cytoplasmic extensions that radiate from the cell body of dendritic cells. SK-Mel-103 and SK-Mel-147 cells became widespread and larger, while G-361 and UACC-62 cells were slender and fusiform (Figure 2B). The morphological features acquired of the cells after treatment with melatonin certainly have a direct impact on the proliferative capacity of these cells, as observed in the clonogenic assay and/or in the cell cycle assay.

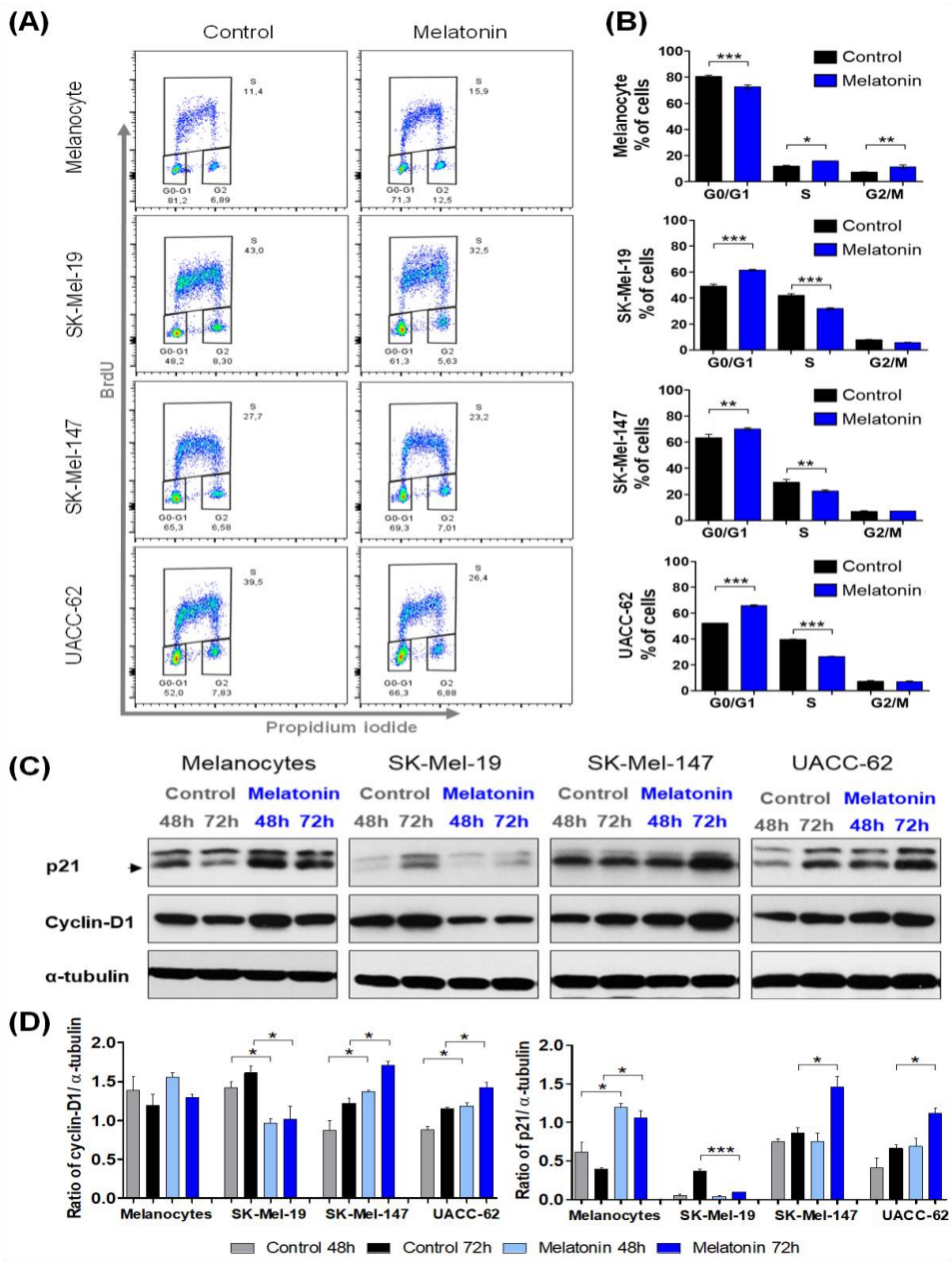

Fig. 1. Effects of melatonin on melanoma cell cycle arrest on G0/G1 phase via regulation of p21 and cyclin-D1 expression.

(*A) Gate strategy for cell cycle analysis from representative samples of human melanocytes and melanoma cell lines subjected with or without melatonin treatment for 48 hours. (B) Melanoma cells treated with 1 mM of melatonin for 48 hours and assayed for detection of BrdU incorporation in actively growing melanoma cells. Cell cycle analysis revealed that melatonin induced cell cycle arrest in the G0/G1 phase in melanoma cells, while a slight proliferation increase was observed for melanocytes. Data are means ± SEM of three independent experiments. (C) Immunoblots of total cell extract isolated from melanocytes, SK-Mel-19, SK-Mel-147 and UACC-62 treated for 48h and 72h with 1 mM of melatonin for the relative levels of p21 and Cyclin-D1 proteins. Melatonin downregulated the expression levels of p21 and cyclin D1 in SK-Mel-19, while upregulated the expression levels of them in SK-Mel-147 and UACC-62. (D) Quantitative analysis of p21 and cyclin D1 expression relative to α-tubulin in melatonin-treated melanocytes and melanoma cell lines. Data correspond to means ± SEM of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

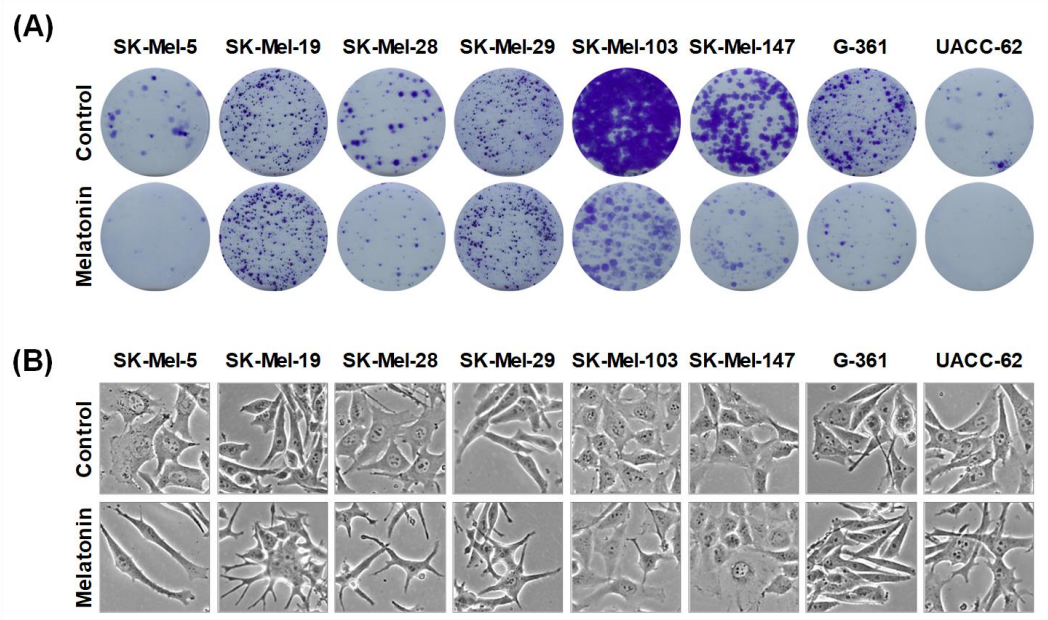

Fig. 2. Effects of melatonin on melanoma cell proliferation and cell cytoskeleton.

(A) Clonogenic assay performed to study the effectiveness of melatonin on the survival and proliferation of melanoma cells. These are representative images of metastatic melanoma panel after treatment with 1mM of melatonin for 15 days. Six of eight melanoma cell types were compromised under melatonin treatment, with a decrease in both cell density and the number of colonies. (B) Bright-field microscopy images showing the morphological changes of metastatic melanoma cells after treatment with 1mM of melatonin for 48 hours. Results are summary of three independent experiments.

3.3. Melatonin provokes actin disruption and focal adhesion downregulation, inducing metastatic melanoma cytoskeleton remodeling.

We then turned to understand the mechanism by which melatonin influences cell morphology, considering that cell movement is driven by dynamic cytoskeletal-dependent features, such as a highly cell polarized structure and focal adhesion complexes. For that, a panel of eight melanoma cell lines was treated with melatonin for 48 hours, and then stained for actin (phalloidin) and focal adhesions (paxillin). The polarized cortical actin organization and the focal adhesions were visualized in each cell control group (vehicle). Melanoma cells showed an actin-rich stress fiber distinctly arranged long microtubules spreading out and intermingling in the center of the cells, and lamellipodia and/or filopodia at their periphery (Figure 3). In addition, the distribution and expression levels of paxillin were much higher in melanoma cell lines (Figure 3) than in melanocytes (Figure S1), suggesting that paxillin plays an important role in the maintenance of a malignant melanoma phenotype. In contrast, the diversity of microfilament phenotypes elicited by melatonin included actin depolymerization, characterized by few microfilament stress fibers, punctate actin pattern, and enhanced actin staining at the cell membrane edges. Furthermore, the morphological signature of paxillin was completely lost in all treated melanoma cells (Figure 3A-H). In turn, the actin organization and the focal adhesions from melanocytes were unaffected by melatonin (Figure S1). Altogether, our data suggest that reduced paxillin accumulation in cytosolic puncta and actin-cytoskeleton reorganization elicited by melatonin might be associated with the impaired melanoma cells adhesion and motility and could partially contribute to a decrease of melanoma tumorigenic features.

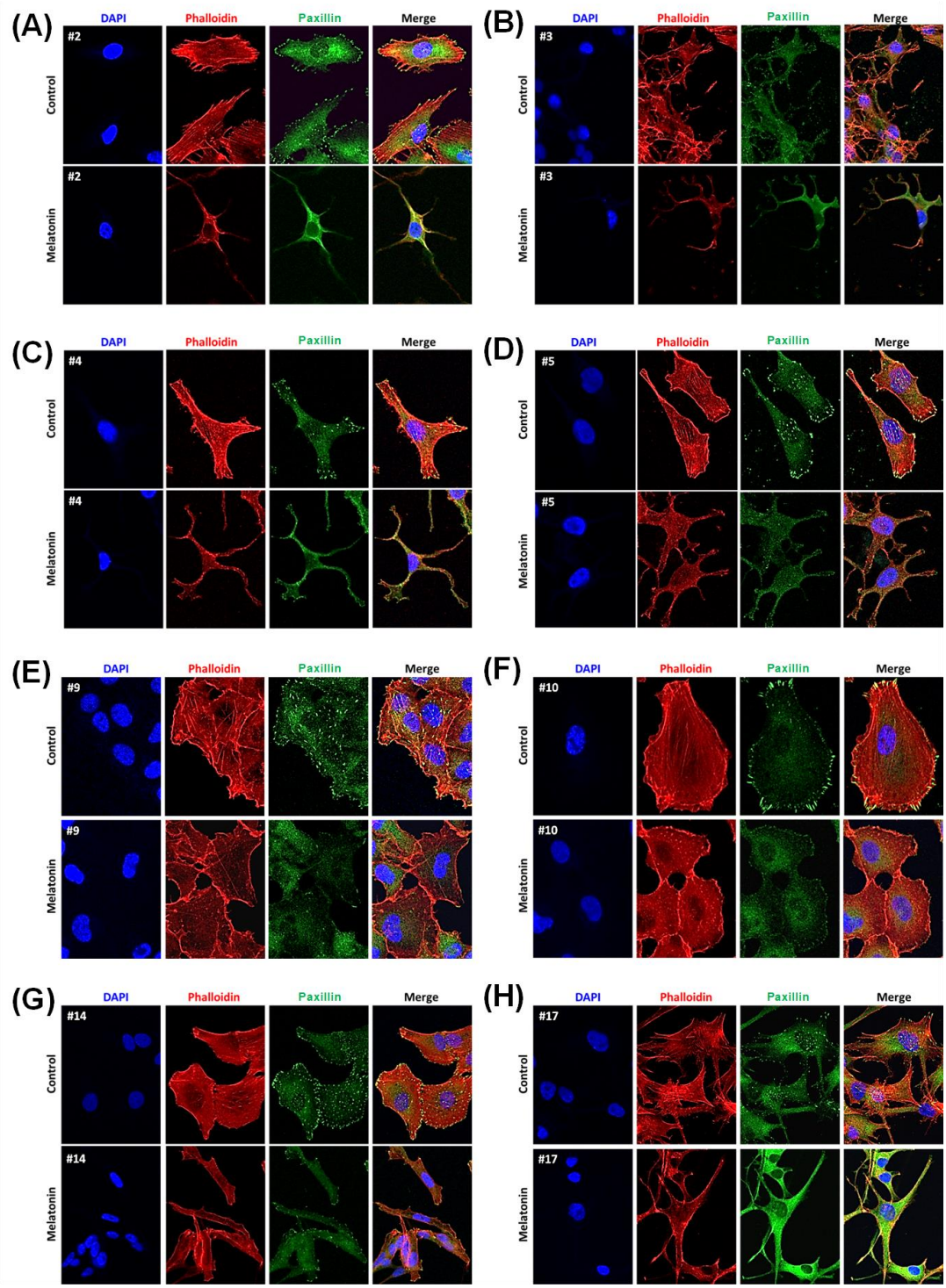

Fig. 3. Effects of melatonin on cytoskeleton remodeling of metastatic melanoma cells.

(A) Confocal imaging of a panel of melanoma cells treated with or without 1 mM of melatonin for 48h. F-actin was visualized by phalloidin (red), focal adhesions by paxillin (green) and nuclei by DAPI (blue) antibody staining. All melanoma cells showed actin disruption, with thinning and/or broken actin fibers, and weak and/or loss paxillin staining along stress fibers. #2 – SK-Mel-5; #3 – SK-Mel-19; #4 – SK-Mel-28; #5 – SK-Mel-29; #9 – SK-Mel-103; #10 – SK-Mel-147; #14 – G-361; #17 – UACC-62. Experiments were done in duplicate and were repeated twice.

3.4. Melatonin impairs invasive processes of the highly metastatic melanoma cell line in skin reconstructed models.

To evaluate the effects of melatonin on melanoma invasion in a robust and complex experimental model, we used a human reconstructed skin model with the highly metastatic melanoma cell line, SK-Mel-147, to evaluate the interaction of melanoma-stroma cells after melatonin treatment. In this context, the cell line SK-Mel-147 was chosen due to its pronounced proliferative and migratory ability compared to the other melanoma cell lines (4). In Figure 4, we observed fewer invasion sites of melanoma cells when the reconstructed skin is subjected to melatonin treatment, with none or few melanoma cells invasion into the stroma (dermis). Additionally, regarding the epidermis, by histological analysis, we showed a differentiated keratinized stratified squamous epithelium organization of reconstructed skin treated with melatonin and without the addition of melanoma (Figure S2). Therefore, melatonin significantly impacts the invasive behavior of SK-Mel-147 from the epidermis to the dermis, leading to a reduction of the invasive potential of those cells on reconstructed skin.

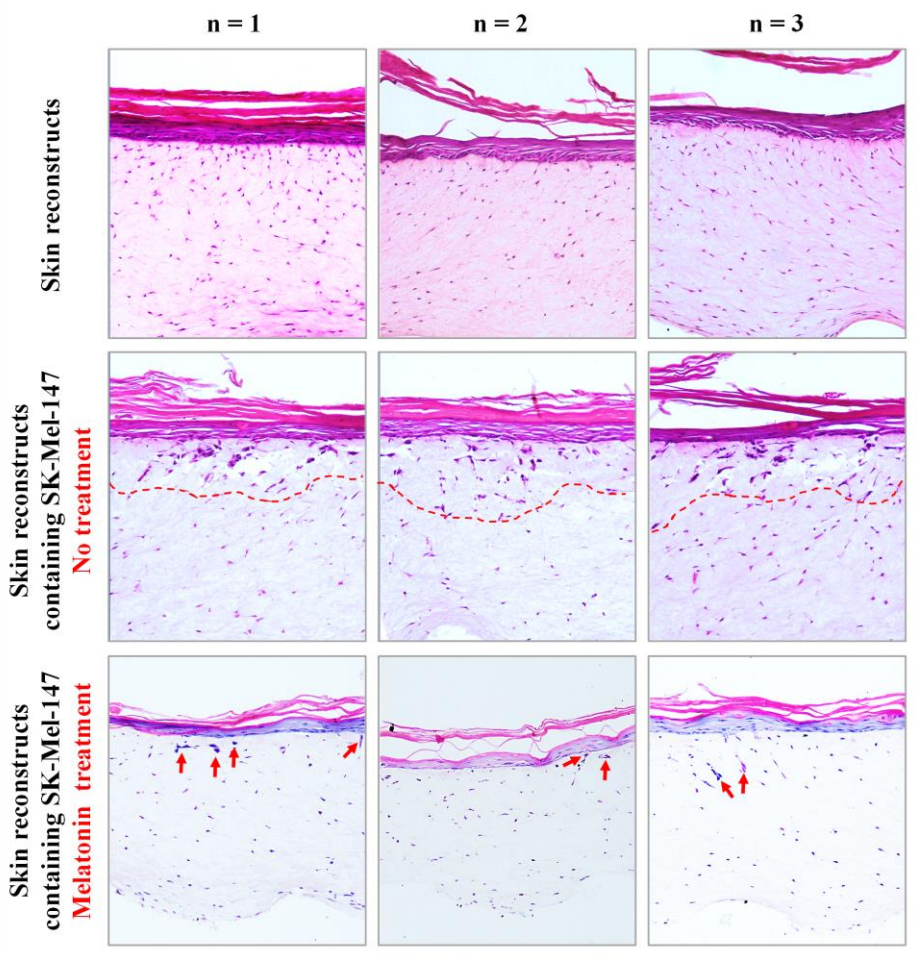

Fig. 4. Effects of melatonin in melanoma invasion.

Bright light microscopy image of skin reconstructs tissue stained by hematoxylin-eosin histological staining after 48h of melatonin treatment. We evaluated the effect of melatonin on SK-Mel-147 invasion, and it was observed that melatonin impairs invasion process of melanoma in skin reconstructed models. Red dot line delimitates the area-containing melanoma, which is represented by the larger and irregular cell intensely stained in purple. The smaller and fusiform cells are fibroblasts. Red arrows indicate melanoma cells in tissue treated with melatonin. Experiments were done in triplicate and were repeated twice.

5. DISCUSSION

Melanoma treatment has become a challenge in terms of clinical outcomes (26). Therefore, a molecule with a wide diversity of actions should be considered when developing pharmacological approaches to target melanoma survival and proliferation. For this proposal, we studied the *in vitro* effects of melatonin on nine different human melanoma cell lines. The results presented in this study demonstrated that besides the effect of melatonin on the cell cycle, this indole markedly impacts the melanoma cytoskeleton, leading to inhibition of cells proliferation and invasion. Our results provide evidence that melatonin enhances p21 and decreases cyclin D1, and disorganizes the paxillin puncta formation and stress fiber polarization, disclosing new insights for understanding its anticancer effect in melanoma cells.

A range of melatonin concentration showed inhibitory properties on the cell cycle in a variety of tumor cell lines (17, 19, 27–29) as well as in an experimental animal model (16, 19). For glioblastoma (28) and prostate cancer cell lines (27), pharmacological concentrations of melatonin induced cell cycle arrest in the G0/G1 phase and reduced the S-phase percentage in a dose-dependent manner (27). In turn, the antiproliferative effect of melatonin on neuroblastoma was related to the increased percentage of cells in the G2/M phase, with the induction of apoptosis by caspase-3 activation (29). Regarding the melanoma cell lines studied here, we did not observe caspases-3, -7, -8, and -9 activation after *in vitro* cell treatment (data not shown). Surely, the lowest cell growth observed under melatonin treatment was related to the reduction of SK-Mel-19, SK-Mel-147, and UACC62 self-renewal, reflected by the truncated cell cycle in the G1-phase. Comparable results were observed for SK-Mel-1 (30). Furthermore, melatonin also suppressed the clonogenic capacity in several of human melanoma cell lines (SBCE2, WM-98, WM-164, and SK-Mel-188) other than those studied here (31).

Examining the downstream protein determinants, a distinct molecular basis for melatonin-induced cell cycle disruption was observed. For the SK-Mel-19 cell line, we observed a decrease in cyclin D1 expression, for the SK-Mel-147 and UACC-62 cell lines we observed an increasing level of p21 under melatonin treatment. Cyclin D1 is a key protein of the G1 to S transition (32) while p21 is known to inhibit cyclin-dependent kinases leading to retinoblastoma protein inactivation and the subsequent cell cycle arrest (33). Interestingly, a positive correlation between p21 expression and the induction of apoptosis in LNCaP cells (34) and MCF7 breast cancer cells (17) had been previously reported whereas we did not observe melanoma cell death using the pharmacological concentration of melatonin utilized in this study. Additionally, one of the antiproliferative activities of melatonin has already been attributed to the transcriptional inhibition of cyclin D1 expression (35).

In vitro, often pharmacological concentrations of melatonin seem to be required to inhibit tumor cell growth (17, 27–29, 35). Regarding human melanoma cells, although the nanomolar and micromolar range of melatonin could affect important cellular features as cellular receptors expression, cytosolic binding protein expression, cellular proliferation, and tyrosinase activity (30, 31, 36–39), most of these effects are more evident in an mM range, particularly the anti-proliferative effect (30, 31, 40). Interestingly, SK-Mel-188 and DX3 are less sensitive to melatonin concentration than other human melanoma cell lines, as SBCE2, WM-98, WM-164, and WM-115 (31, 40). These observations call attention to the pharmacological concentrations of melatonin required to obtain robust antitumor effects in melanomas with high metastatic potential, as the SK-Mel- cells (4, 41). Although the physiological serum concentration of melatonin is in a picomolar to a nanomolar range (42), it is important to consider that exogenous melatonin reaches higher levels in xenografted tumor tissue than in animals' plasma, highlighting that melatonin concentrates in the tumor (16). Thus melatonin serum levels may have important biological activities, such as antiangiogenic properties (16), and the pharmacological levels of melatonin in tumor mass could directly impact cell proliferation.

Moreover, it is important to note that the differential melatonin uptake by normal cells or tumor cells may explain the variability of the antiproliferative properties of melatonin in different cell types (43), as well as the higher concentration that melatonin reaches in tumor mass (16). Additionally, melatonin can also be produced by many cells (12, 13), and its effects may vary according to the local concentrations achieved, reaching millimolar concentrations when locally produced by activated immune-competent cells (44–47).

One of the most interesting and unprecedented findings in the work performed here was the potent effect of melatonin on the cytoskeletal remodeling in human melanoma cell lines. The melatonin-induced microfilament phenotypes possibly interfered with the human melanoma invasion in the skin reconstruct model. The role of melatonin as a cytoskeletal modulator has been known for almost three decades (48). Dynamic organization of the cytoskeleton is mandatory for metastatic tumor cells, leading them to become highly migratory and invasive (49). The invading cells attach themselves to the substrate through the focal adhesion contact formed by actin stress fibers and are joined together by adhesion proteins (49). The cytoskeletal modulatory properties of melatonin with subsequent morphological cell change have been demonstrated in prostate cancer cells (27), human mammary epithelial cancer (50), Chinese hamster ovary cells (51), neuroblastoma cells (52), and MDCK cell line (52). At the molecular level, melatonin inhibits the migratory process and cell invasion of breast cancer cells via ROCK-regulated microfilament and cytoskeletal organization that converges in migration to switch anchorage (50). Regarding human melanoma cell lines, melatonin-treated cells showed a less organized cytoskeleton than untreated ones. The downregulation of paxillin and the complete loss of F-actin organization causes melanoma cells to alter in a star-like morphology or cell swelling.

The reduction of paxillin levels found in our work is consistent with previous studies that have shown the importance of this protein in the process of invasion and tumor growth in several organs, such as stomach (53), lung (54), liver (55), and prostate (56). This protein is expressed in focal adhesions, which may interfere with cell migration, increasing the attachments between the neoplastic cells and the extracellular matrix in the tumor microenvironment. High levels of paxillin are associated with poorer prognosis of patients by increasing the invasiveness and tumor angiogenesis (57). Furthermore, the reduced levels of paxillin have an antimetastatic effect on B16-F10 melanoma cells (58). Consequently, the reduction of paxillin levels by melatonin would emphasize a potentially important therapeutic approach to melanoma therapy.

In recent years, numerous cellular and molecular mechanisms have been proposed to explain the oncostatic effects of melatonin. Since human melanoma cells express the membrane melatonin receptors MT-1 or MT-2 (40, 59, 60), there is a range of biological possibilities for the action of melatonin in this type of tumor, either by the binding of melatonin to its membrane receptors or to the intracellular actions targeting distinct molecules, or binding intranuclear receptors (31, 61). Undoubtedly, melatonin is a suitable candidate for the development of new therapeutic approaches against cancer and may improve outcomes in melanoma patients by playing a key role in several aspects of malignancy (62).

In conclusion, our findings establish for the first time a molecular link between melatonin and its effects on human melanoma cell cytoskeleton disruption. This supports the hypothesis that the cytoskeleton may be a therapeutic target for melatonin to block the invasiveness and metastasis of melanoma. Although there is a need for further studies to understand the relationship between melatonin and skin cancer, the results of *in vitro* and *in vivo* studies are highly encouraging. Collectively, we believe that our data may be helpful for the experimental design of future clinical research studies and advancing melatonin as a therapeutic agent for metastatic melanoma.

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AUTHORSHIP

ACRM concepts, designs, develops methodology, acquisitions data; analyzes, interprets data, writes, reviews and/or revises the manuscript as well as conducts administrative, technical, or material support and study supervision. RFS conducts acquisition, analysis and interpretation of data, writing, reviewing and/or revision of the manuscript. MT and RRM conduct acquisition of data. RLP, RP and PCS conduct acquisition of data and writing the manuscript. RJR conducts review and/or revision of the manuscript. AC and MSS conducts administrative, technical, or material support; Review and/or revision of the manuscript as well as study supervision. SSME conducts interpretation of data, administrative, technical, or material support, review and/or revision of the manuscript as well as study supervision.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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