The ubiquitin-specific protease 8 antagonizes melatonin-induced endocytic degradation of MT1 receptor to promote lung adenocarcinoma growth

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Highlights

- Melatonin can induce the downregulation of the MT1 receptor.
- MT1 receptor internalization incurred by melatonin follows the canonical endolysosomal pathway.
- The ubiquitin-specific protease 8 antagonizes the endocytic degradation of the MT1 receptor.
- The suppression of ubiquitin-specific protease 8 potentiates the cancer-inhibitory effects of melatonin in vitro.
- Combination of USP8 inhibition and melatonin treatment effectively deters tumor growth in xenograft mouse models.

Graphical Abstract

Introduction: The human genome encodes two melatonin receptors (MT1 and MT2) that relay melatonin signals to cellular interior. Accumulating evidence has linked melatonin to multiple health benefits, among which its anticancer effects have become well-established. However, the implications of its receptors in lung adenocarcinoma have so far remained incompletely understood.

Objectives: This study aims to investigate the response of the MT1 receptor to melatonin treatment and its dynamic regulation by ubiquitin-specific protease 8 (USP8) in lung adenocarcinoma.

Methods: The mRNA levels of MT1 and MT2 receptors were analyzed with sequencing data. The expression and localization of the MT1 receptor with melatonin treatment were investigated by immunoblotting, immunofluorescence and confocal microscopy assays. Endocytic deubiquitylases were screened to identify MT1 association. The effects of USP8 were assessed with shRNA-mediated knockdown and small
Introduction

Melatonin (N-acetyl-5-methoxytryptamine), an indoleamine neurohormone generated primarily by the pineal gland, has been revealed to participate in a diverse range of physiological processes [1]. In addition to its essential roles in regulating circadian rhythms, melatonin demonstrates anti-inflammatory and antioxidant characteristics linking it to additional beneficial roles such as cardiovascular protective and immunomodulatory functions [2–4]. Furthermore, it is worth noting that accumulating evidence from diverse experimental settings infers the oncostatic activities of melatonin towards a variety of malignancies, including lung, prostate, breast, renal and colorectal cancers [5].

Lung cancer has presented a formidable threat to human health, with both highest incidence and mortality among human neoplasms recorded globally [6]. Non-small cell lung cancer (NSCLC) is the principal subtype of human lung cancer, comprising about 80–85% of all cases. Histologically, NSCLC can be divided into 3 categories, i.e. lung adenocarcinoma (LUAD), squamous carcinoma (LUSC), and large cell carcinoma (LCC), with the former two recognized as the most frequently diagnosed subtypes (LUAD and LUSC) of human lung cancer. Importantly, the anticancer effects of melatonin towards NSCLC have been well established, which include the suppression of tumor cell proliferation, invasion and metastasis, as well as the induction of apoptosis, immunomodulation and synergy with chemotherapeutic agents [7].

The human genome encodes two G-protein-coupled receptors that act as melatonin receptors (MTNR1A and MTNR1B for MT1 and MT2, respectively). Although the oncostatic activities of melatonin against a wide array of cancer types have been reported, the involvement of both MT1 and MT2 receptors in the cancer-suppressive functions of melatonin has so far remained less understood [5,8]. Intriguingly, the melatonin MT1 and MT2 receptors were shown to undergo melatonin-triggered internalization that was associated with physiological and pathological implications [9–11]. However, the dynamic regulation of MT1 and MT2 receptor endocytosis and the potential contribution to the anticancer effects of melatonin remain underinvestigated until now.

The current study reports the dynamic regulation of melatonin-induced MT1 receptor endocytosis in lung adenocarcinoma cells. The ubiquitin-specific protease 8 (USP8) counteracts the endocytic degradation of MT1 receptor incurred by melatonin treatment. In lung adenocarcinoma, USP8 depletion renders A549 and H358 cells more susceptible to melatonin-mediated inhibition, while pharmacological USP8 inhibition significantly enhances the tumor-suppressive effects of melatonin. Collectively, our observations uncover novel aspects of the endocytic regulation of the melatonin-MT1 receptor signaling axis and hold potential for future investigation of the oncostatic efficacy of melatonin.

Materials and methods

Cell culture

All cell lines used were purchased from the American Type Culture Collection (ATCC, Manassas, USA). HEK293T cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) media, and non-small cell lung cancer A549 and H358 cells were maintained in RPMI-1640 media. Culture media were supplemented with 10% of fetal bovine serum (ExCell Bio, Shanghai, China) and 1% penicillin/streptomycin (Thermo-Fisher Scientific, Waltham, USA). The stable knockdown cell lines were cultured in media containing 2 µg/ml of puromycin to maintain positive selection. Cells were kept in a humidified atmosphere at 37 °C with 5% CO₂ enabled within the cell culture incubator (3111, Thermo, Waltham, USA). Cell culture plastic wares were obtained from Guangzhou Jet Bio-Filtration Co., Ltd (Guangzhou, China).

Antibodies and other reagents

Mouse anti-MT1 antibody (sc-390328, 1:1,000) was purchased from Santa Cruz Biotechnology (Dallas, USA). Rabbit anti-MT1 antibody (D160637, 1:1,000) was obtained from Sangon Biotech (Shanghai, China). Mouse anti-USP8 (67321–1-Ig, 1:20,000) and anti-GAPDH (60004-1-Ig, 1:10,000) antibodies were purchased from Proteintech (Wuhan, China). Mouse anti-Tubulin antibody (T5168, 1:100,000) was purchased from Sigma (Darmstadt, Germany). Mouse anti-LAMP2 antibody (ab25631, 1:50) was obtained from Abcam (Cambridge, UK). Mouse anti-Ki-67 (610969, 1:400) and anti-EEA1 (610456, 1:500) antibodies were purchased from BD Medical Technology (Franklin Lakes, USA). Secondary infrared-labeled goat anti-mouse (926–68070, 1:15,000) and anti-rabbit (926–32211, 1:15,000) antibodies for immunoblotting were obtained from LICOR (Lincoln, USA). Alexa Fluor® 488- and 594-labeled secondary antibodies for immunofluorescence (A11001 and A11005, 1:1,000) were purchased from Invitrogen (Carlsbad, USA). Melatonin was obtained from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). The GFP-MT1 construct (T5168, 1:100,000) was purchased from Sigma (Darmstadt, Germany). Mouse anti-LAMP2 antibody (ab25631, 1:50) was obtained from Abcam (Cambridge, UK). Mouse anti-Ki-67 (610969, 1:400) and anti-EEA1 (610456, 1:500) antibodies were purchased from BD Medical Technology (Franklin Lakes, USA). Secondary infrared-labeled goat anti-mouse (926–68070, 1:15,000) and anti-rabbit (926–32211, 1:15,000) antibodies for immunoblotting were obtained from LICOR (Lincoln, USA). Alexa Fluor® 488- and 594-labeled secondary antibodies for immunofluorescence (A11001 and A11005, 1:1,000) were purchased from Invitrogen (Carlsbad, USA). Melatonin was obtained from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). The GFP-MT1 construct has been previously described, which is a generous gift from Prof. Nai-ming Zhou (Zhejiang University, China) [11]. The USP8 inhibitor was purchased from MedChem Express (Shanghai, China). Puromycin was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Unless specified, all chemicals were purchased from Sigma (Darmstadt, Germany).

Immunoblotting

Immunoblotting assays were conducted as previously described [12]. In brief, cell lysates were acquired using “RIPA” buffer (1% Tri-
ton X-100, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS) supplemented with Na3VO4 (2 mM) and PMSF (1 mM). Protein concentrations were determined using BCA protein assays (Takara, Beijing, China). Equal amounts of lysates (30–50 μg) were loaded and resolved by 8% SDS-PAGE gels. Protein samples were then transferred to nitrocellulose blots (Merck Millipore, Darmstadt, Germany). Samples were subsequently blocked in 4% of fat free milk in phosphate-buffered saline (PBS) for an hour before incubated with primary antibodies at 4 °C for 4 h. Next day, membranes were washed with PBS/T (PBS with 0.1% of Tween 20) twice and then incubated with secondary antibodies for an hour, followed by two washes with PBS/T and once with PBS. Finally, blots were scanned on an infrared imaging platform (LI-COR Odyssey, Lincoln, USA) and acquired images were further analyzed using the Image Studio Programme (Version 4.0). Protein band intensities were measured with background subtraction and normalized to corresponding loading control (GAPDH) before plotted. Each experiment was repeated three times with biological replicates.

**Immunofluorescence**

Cells were grown on coverslips placed in 3.5-cm culture plates. Previous immunofluorescence procedures were followed [13]. After indicated treatment, cells were washed with PBS prior to fixation with 4% paraformaldehyde. After permeabilization with 0.2% of Triton X-100 in PBS, cells were treated with 2% of bovine serum albumin (BSA) in PBS at room temperature for an hour. Then samples were incubated with specific primary antibody for an hour at room temperature, followed by secondary antibody for 30 min. Finally, samples were washed in PBS and distilled water, and then coverslips were mounted onto glass slide with Mowiol (Sigma). The experiment was conducted with three biological repeats. Immunofluorescence staining was captured using a Leica fluorescence microscope (SP8, Wetzlar, Germany).

**Colony formation assay**

Cultured A549 (at a seeding density of 1, 000 cells per well) and H358 (at a seeding density of 4, 000 cells per well) cells were plated into 6-well plates that were maintained in the cell culture incubator. Cells were treated with 1 mM of melatonin and media were changed every two days. After two weeks, colonies were methanol fixed and stained in 0.1% of crystal violet. Images were acquired on a Bio-Rad ChemiDoc XRS + platform (Hercules, USA) and finally analyzed with the ImageJ programme. The experiments were carried out with three biological replicates.

**MTT assay**

Lung adenocarcinoma A549 and H358 cells were seeded into 96-well plates at a seeding density of 4,000 cells per well and incubated overnight. The following day, cells were treated with 1 mM of melatonin for 24 h, before adding MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into each well and subsequent incubation for 3 h. Then 150 μl of DMSO were added into each well and absorbance (570 and 630 nm) was recorded using a benchtop spectrometer (Perkin Elmer, Enspire2300, Waltham, USA). The experiment was repeated with three biological replicates.

**Stable cell lines**

Cultured HEK293T cells were transfected with shRNA-expressing pLKO.1 plasmids (empty vector control or each of two shRNAs targeting USP2, USP8, AMSH, and AMSH-LP), along with packaging plasmids (three-plasmid system) as per the manufacturer’s instructions (Sigma). Lentiviruses were collected after 48 h and used to infect A549 and H358 cells. Positive expression cells were selected using treatment with 2 μg/ml of puromycin and knockdown efficiency was confirmed with standard semi-quantitative RT-PCR assays. USP8 knockdown efficiency was further evaluated by Western blotting. DUB-specific shRNA sequences were described previously [14].

**Xenograft mouse model**

Animal experiments were conducted in accordance with the 1996 National Institute of Health Guide for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Dalian Medical University. Six-week-old female nude mice (Balb/c background, Charles River, Beijing, China) were randomly distributed into two groups (10 mice/group). A549-pLKO.1 (vector control) and A549-USP8-sh1 (USP8 shRNA1-expressing) stable cells (2 million/mouse) were subcutaneously implanted into mice from two groups separately. Mice were maintained in a pathogen-free condition during the entire experiment and tumor volume was measured with a vernier caliper every 2 days during the whole 37-day period before reaching size limitation. After one week, mice from each group were further divided into two groups (5 mice/group), which were administered with melatonin (25 mg/kg) or DMSO as vehicle control by intraperitoneal injection at 7 pm every day for 30 days [15]. Tumor volume was estimated according to the formula: tumor volume (mm3) = (length) × (width)² × 0.5.

**Immunohistochemistry**

Xenograft tumors were fixed in 4% paraformaldehyde overnight before paraffin-embedding. Tissue sections (4 μm) were prepared and dried at 56 °C for 4 h before deparaffinized and rehydrated with graded alcohol. Following incubation with blocking solution using the IHC assay kit (ZSGB-Bio, Beijing, China), tissue sections were treated with primary antibody for overnight at 4 °C. Next day, tissue sections were stained using the IHC assay kit as per the manufacturer’s instructions. Samples were visualized and images were acquired using a Leica microscope (DMI4000B, Wetzlar, Germany). Randomly chosen fields from each mounted section were analyzed and quantitated with the Image-Pro Plus programme (version 6.0).

**Statistics**

Experiments were repeated for at least three times with biological replicates. Quantification data were analyzed by one-way ANOVA or Student’s t-test using GraphPad Prism (version 7) for multiple group or paired sample comparisons, respectively. Individual data points were presented in quantification plots, with p < 0.05 considered as statistically significant.

**Results**

**Melatonin-triggered MT1 endocytosis is associated with receptor degradation**

Accumulating RNA sequencing data from cancer consortium projects have tremendously enhanced our understanding about the transcriptomic characteristics of various types of cancer [16]. Using the web-based GEPIA (Gene Expression Profiling Interactive Analysis) platform, we analyzed the RNA expression levels of both melatonin receptors in non-small cell lung cancer (LUCAD and LUSC)
and non-cancerous samples [17]. As demonstrated in Fig. 1A and B, in general, the expression of MT₁ receptor appears higher than MT₂ in both LUAD and LUSC specimens, with higher average levels of MT₁ observed in LUAD than in LUSC. Therefore, we decided to focus on the MT₁ receptor in LUAD for subsequent investigation.

We treated lung adenocarcinoma A549 and H358 cells with pharmacological dose of melatonin before performing immunoblotting analyses [18]. Results from immunoblotting assays show that melatonin exposure led to a gradual reduction in the protein levels of MT₁ receptor over a 12 h period in both cell lines (Fig. 1C and D). Such quick responses of MT₁ protein abundance to melatonin treatment within hours indicated that changes likely occurred at the protein level rather than at the mRNA level of MT₁ expression [19]. To acquire an optimal visualization of MT₁ receptor localization in the cell, we exogenously expressed GFP-tagged MT₁ receptor in HeLa cells and performed fluorescence investigation following melatonin treatment. In untreated cells, MT₁ receptor was observed on both the plasma membrane and within the cell in the cytoplasm, while melatonin addition markedly enhanced MT₁ internalization leading to its significantly reduced distribution to the surface (Fig. 1E). In light of the evidence that cell surface receptors are frequently turned over by endocytic degradation through lysosomes, we posited that internalized MT₁ receptor was possibly routed through endosome system for lysosomal degradation [20]. Indeed, when the cells were incubated in chloroquine to impede lysosomal function, we observed enhanced GFP fluorescence signal within the cell following melatonin incubation, intimating accumulated MT₁ receptors that were unable to go through lysosomal destruction (Fig. 1F) [21].

**Internalized MT₁ receptor is sorted through the canonical endo-lysosomal route**

To gain more mechanistic insights into the intracellular trafficking of internalized MT₁ receptor, we conducted immunofluorescence and confocal microscopy experiments to investigate the colocalization of endocytosed MT₁ receptor with established markers of early endosomes (Early endosome antigen 1, EEA1) and late endosomes/lysosomes (Lysosome-associated membrane glycoprotein 2, LAMP2), respectively [21]. As described in Fig. 2A and B, in untreated cells, GFP-tagged MT₁ receptor appeared to be largely separate from both EEA1 and LAMP2 staining, illustrating only sparse colocalization of the MT₁ receptor with endosomal structures under steady state conditions that likely represented constitutive receptor turnover by endocytosis. However, following 6 h of melatonin treatment, internalized MT₁ receptor showed widespread colocalization with the early endosomal marker EEA1, as well as evident colocalization with the late endosomal marker LAMP2 (Fig. 2A and B). After 24 h of melatonin exposure, the colocalization of the MT₁ receptor with EEA1 was dramatically attenuated (Fig. 2A), whilst its association with the late endosomes and lysosomes persisted as revealed by LAMP2 staining (Fig. 2B), suggesting that the majority of MT₁ receptor was already trafficked through early endosomes to reach late endosomes or lysosomes. Furthermore, we treated cells with chloroquine to block lysosomal function aiming to investigate the potential degradation of internalized MT₁ receptor following melatonin incubation. As illustrated in Fig. 2C, over a 12 h incubation period, inhibition of lysosomal degradation imposed by chloroquine treatment led to a significant accumulation of the MT₁ receptor on structures marked with LAMP2 staining, indicating a buildup of unprocessed MT₁ receptor that was sorted to the lysosome incurred by melatonin treatment. Hence, these observations collectively show the dynamic transportation of the MT₁ receptor along the canonical endosomal track for destruction in the lysosome following melatonin treatment, which also complies with the well established models of endocytic degradation of cell surface receptors [22–24].

**USP8 obstructs melatonin-induced endocytic degradation of the MT₁ receptor**

The endocytic degradation of a slew of cell surface receptors has been demonstrated as a dynamically regulated process, in which several deubiquitylases are frequently observed to regulate the dynamics of receptor degradation predominantly via catalyzing the removal of ubiquitin moieties off receptors or receptor-associated proteins [25]. Among the deubiquitylase superfamily that contains close to 100 proteins, 4 members (USP2, USP8, AMSH, and AMSHLP) have been shown to localize to the endosome/lysosome system and experimentally revealed to regulate diverse receptor endocytosis procedures [14,26,27]. Therefore, we speculated that certain endosome-associated deubiquitylase might exert function in the regulation of melatonin-incurred endocytic degradation of the MT₁ receptor. To test this hypothesis, we knocked down the expression of each of the four endosome-associated deubiquitylases using an shRNA-based approach (two separate shRNAs per target). While comparing the levels of the MT₁ receptor in these 8 shRNA-expressing stable cell lines to that in vector control A549 cells under steady state conditions, we found that the MT₁ receptor expression was significantly compromised in both USP8 shRNA-expressing A549 cells (Fig. 3A).

We next sought to tease out how USP8 silencing led to reduced MT₁ receptor expression under steady state conditions and thus measured the mRNA levels of the MT₁ receptor in control and USP8-depleted cells with quantitative polymerase chain reaction assays. Since the MT₁ receptor mRNA expression was not significantly affected by USP8 knockdown (Fig. 3B), we reasoned that the reduction in MT₁ receptor occurred post-transcriptionally. Therefore, we carried out cycloheximide chase experiments that measure protein stability through blocking polypeptide synthesis, in order to assess the turnover rates of the MT₁ receptor in control and USP8-depleted cells [28]. As shown in Fig. 3C, in USP8 shRNA-expressing A549 cells, the degradation of MT₁ receptor turned out to be accelerated compared to it was in control A549 cells, suggesting that the enhanced turnover imposed by USP8 depletion was likely the main reason for decreased levels of MT₁ receptor. To examine the influence of USP8 knockdown on melatonin-induced downregulation of the MT₁ receptor, we performed further immunoblotting experiments using these established stable cell lines. As demonstrated in Fig. 3D and E, USP8 silencing led to further MT₁ receptor downregulation incurred by melatonin treatment. Therefore, these findings collectively indicate that USP8 safeguards the MT₁ receptor expression levels under both steady state and melatonin treatment conditions, and accordingly shRNA-mediated USP8 silencing results in attenuated MT₁ receptor expression through enhancing its degradation.

**USP8 depletion sensitizes lung adenocarcinoma cells to melatonin**

Although the anticancer effects of melatonin against lung cancer have become well-documented, the exact roles of the MT₁ receptor in the oncostatic activities of melatonin appear understudied. Given that the melatonin-MT₁ receptor axis has been linked to multiple downstream signaling molecules including G proteins and MAP kinases that are recognized as key factors during tumorigenesis, it is of great importance to understand the roles that the MT₁ receptor and its endocytosis play in cancer cells treated with melatonin. In doing this, we performed cell proliferation and colony formation assays using lung adenocarcinoma cells with or without USP8 knockdown. Considering the evidence that USP8...
also regulates the endocytic degradation of the epidermal growth factor receptor (EGFR) that acts as a pivotal oncogenic driver in lung adenomas, we selected A549 and H358 cell lines that contain activating mutations in KRAS downstream of EGFR and thus can bypass the inhibition on EGFR imposed by USP8 silencing in these assays [29].

The results from MTT assays measuring cell growth over a 24 h period showed that shRNA-mediated USP8 depletion rendered both A549 and H358 cells reduced cell proliferation as compared to control vector-containing cells (Fig. 4A). Consistent with previous findings, melatonin treatment effectively decreased cell propagation in both A549 and H358 lung adenocarcinoma cells, while the extent of growth inhibition imposed by melatonin over a 24 h duration was comparable in A549 and H358 cells with USP8 silencing as revealed by MTT assays (Fig. 4B). However, data from long-term colony formation assays revealed even stronger...
influence of USP8 knockdown on the growth of A549 and H358 lung adenocarcinoma cells, suggesting an accumulative effect of USP8 silencing in these cells (Fig. 4C). Importantly, the results from colony formation assays demonstrated even stronger inhibitory effects of melatonin treatment on both A549 and H358 cells with stable USP8 knockdown, suggesting that USP8 depletion sensitized
lung adenocarcinoma cells to melatonin exposure (Fig. 4D and E). Taken together, these observations hence indicate the potential tumor-supportive role of USP8 in lung adenocarcinoma is likely linked to its impact on the endocytic degradation of the melatonin receptor MT1.

USP8 small molecule inhibitor potentiates the tumor-suppressive effects of melatonin

Since ubiquitin-specific proteases are deemed as actionable targets for pharmacological interference, we next sought to test the
influence of USP8 chemical inhibitor on the \textit{in vitro} growth of lung adenocarcinoma cells in the presence of melatonin treatment \cite{30,31}. Using synthesized 9-[(phenylmethoxy)imino]-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile that is an analogue of 9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile and reported by Colombo \textit{et al.} to have an IC50 of 0.71 \mu M against USP8, we carried out MTT and colony formation assays with or without melatonin treatment at 1 mM. Column charts on the right show the relative sizes of colonies formed in each group, with mean values labeled on top. Individual data points were indicated for quantification plots, with * and ** showing \(p < 0.05\) and \(p < 0.01\), respectively.
USP8 inhibition. The results from MTT assays showed that this USP8 inhibitor exerted a dose-dependent inhibitory effect on the proliferation of A549 cells, which were consistent with data obtained from shRNA-mediated USP8 depletion experiments (Fig. 5A).

Furthermore, data from subsequent MTT assays to measure cell proliferation over a 24 h incubation period under the treatment with melatonin (1 mM), the USP8 inhibitor (0.6 μM), or the combination revealed analogous extent of growth inhibition (Fig. 5B). However, with colony formation assays that recorded long-term cell propagation, treatment with melatonin and the USP8 inhibitor resulted in further decreased cell growth compared to the effects observed with MTT assays, while the combined treatment with melatonin and USP8 inhibitor led to a significantly enhanced defect in the colony formation of A549 cells than single treatment groups (Fig. 5C and D). Therefore, these observations indicate that the pharmacological inhibition of USP8 deubiquitylase could not only elicit a tumor-suppressive effect towards lung adenocarcinoma, but also potentiate the anticancer effects of melatonin.

USP8 silencing enhances the anticancer effect of melatonin in xenograft animal model

Having demonstrated the suppressive roles of USP8 inhibition in lung adenocarcinoma cell growth in vitro, we next turned to investigate the impact of USP8 suppression on the in vivo tumor xenograft formation of lung A549 cells in nude mice. In doing this, A549 cells stably transfected with control and USP8 shRNA-expressing vectors were subcutaneously implanted into athymic nude mice with Balb/c background. Xenograft tumors were recorded and mice were randomized into four groups to receive melatonin or control vehicle: A549 pLKO.1 administered with DMSO or melatonin and A549 pLKO.1-USP8-sh1 administered with DMSO or melatonin. As shown in Fig. 6A and B, USP8 depletion rendered A549 cells attenuated xenograft tumor formation in nude mice, while melatonin treatment displayed inhibitory effects on the in vivo growth of A549 cells with or without USP8 knockdown. As a result, xenograft tumors from the A549 pLKO.1-USP8-sh1 group received melatonin treatment were recorded with the smallest size on average and hence were most significantly suppressed as compared to the control group (Fig. 6C). Interestingly, Western blotting analyses of xenograft samples revealed an upregulation of USP8 expression in melatonin-treated A549 group, suggesting a potential positive regulation of USP8 abundance by melatonin treatment (Fig. 6D and E). Consistent with our in vitro observations, the expression levels of the melatonin MT1 receptor in A549 xenograft tumors were down regulated by melatonin treatment and USP8 knockdown, with the lowest levels on average observed in the xenograft samples from the A549 pLKO.1-USP8-sh1 group treated with melatonin (Fig. 6D and E). Congruently, immunohistochemistry analyses of xenograft tumor samples further confirmed the inhibitory effects of USP8 depletion and melatonin treatment on MT1 expression and tumorigenesis, which were revealed by the staining of MT1, and the proliferation marker Ki-67, respectively (Fig. 6F and G). Taken together, our data from in vivo xenograft animal model study corroborate the tumor-protective implications of USP8 and intimate it as a potential therapeutic target in the treatment of lung adenocarcinoma either as monotherapy or in combination modalities such as with melatonin.

**Fig. 5.** Pharmacological USP8 inhibition sensitizes lung adenocarcinoma cells to melatonin. A A549 cells were treated with the USP8 inhibitor at indicated concentrations for 24 h. Cell viability was examined with MTT assays. Graph shows data from 3 independent experiments. B A549 cells were treated with the USP8 inhibitor (0.6 μM), melatonin (1 mM), or the combination as indicated for 24 h. Cell viability was evaluated with MTT assays. Graph shows data from 3 independent experiments. C Colony formation assays of A549 cells with the treatment of the USP8 inhibitor (0.6 μM), melatonin (1 mM), or the combination as indicated. D Graph shows the quantification of C (n = 3). Individual data points were shown for quantification plots, with * and ** showing p < 0.05 and p < 0.01 relative to untreated controls as indicated, respectively.
Discussion

Lung cancer has remained a leading threat to human health and is recorded with steadily high morbidity and mortality globally [33]. Therefore, this challenge calls for continuous investigation into this malignancy and the development of additional efficient therapeutic strategies [34]. Recent advances in targeted therapy and immune therapy have tremendously improved patient outcomes. However, clinically approved therapeutic targets exemplified by EGFR, Anaplastic Lymphoma kinase...
(ALK), and the PD-1/PD-L1 immune checkpoint axis are only applicable to a small proportion of lung cancer patients, and accordingly additional actionable therapeutic targets are in great request to design potential treatment modalities. The human deubiquitylases (DUBs) are a group of cysteine proteases and metalloenzymes that have been associated with a wide range of cellular activities including the dynamic regulation of cell surface receptors [35,36]. Endocytosis-associated deubiquitylases exemplified by AMSH, USP8, and USP2 have been shown to regulate the dynamic turnover of top therapeutic targets in cancer treatment such as EGFR and ErbB2/HER2 [14,29,37].

The deubiquitylase USP8, also known as ubiquitin isopeptidase Y (UBPY), belongs to the USP subfamily of human DUBs [38]. USP8 was initially identified as a growth-regulated isopeptidase and subsequently revealed to exhibit tumorigenic roles in many cancer types, such as lung cancer, cervical cancer, cholangiocarcinoma, breast cancer, and pituitary tumor [39-41]. Although demonstrating pleiotropic functions to promote tumorigenesis, the endocytic regulation of cell surface receptor dynamics has been recognized as a canonical role of USP8 [42-44]. In this study, we provide evidence that USP8 deters the melanotonin-induced endocytic degradation of the MT1 receptor, thus displaying a tumor-supportive role in lung adenocarcinoma. Although the oncostatic activities of melatonin have been well documented, the involvement and dynamic regulation of its physiological receptors MT1 and MT2 in lung adenocarcinoma appear to be incompletely investigated until now. Since the melatonin receptors are linked to multiple downstream signaling modules that play pivotal roles during tumorigenesis, it is of great significance to fully understand the effects of melatonin treatment. Our observations uncover the obscure aspect of the MT1 receptor dynamics and reveal a positive regulatory role of USP8. We provide evidence that melatonin treatment triggers the internalization and endocytic sorting of the MT1 receptor towards lysosomal degradation acting as a negative feedback mechanism to down tune the melatonin-MT1 receptor signaling output, while the endosomal DUB USP8 antagonizes such negative modulation to spurn cancer cell growth.

Conclusion

Our findings uncover the dynamic regulation of the MT1 receptor endocytosis and hence gain novel insights into the complexity of melatonin treatment on malignant cells. Furthermore, the observations can be exploited to design targeted therapeutic strategies to enhance the efficacy of melatonin, which has been experimentally validated. With the increasing appreciation of the tumor-promoting effects of human DUBs and the susceptibility of these cysteine proteases to pharmacological interference, USP8 holds potential to serve as a leading paradigm from the DUB family proteases as a therapeutic target in cancer treatment [31,45,46].

Compliance with Ethics Requirements

Ethics statement: All procedures followed were in accordance with the 1996 National Institute of Health Guide for the Care and use of Laboratory Animals and the ethical standards approved by the Institutional Animal Care and Use Committee of Dalian Medical University.

CRediT authorship contribution statement

Qianhui Sun: Investigation, Data curation, Formal analysis. Jinrui Zhang: Investigation, Formal analysis. Xiaoxi Li: Investigation.

Guoheng Yang: Investigation. Shaoxuan Cheng: Investigation. Dong Guo: Investigation. Qingqing Zhang: Investigation. Feng Sun: Investigation. Feng Zhao: Investigation. Dian Yang: Formal analysis. Shanshan Wang: Formal analysis. Taishu Wang: Formal analysis. Shuyan Liu: Formal analysis. Lijuan Zou: Conceptualization. Yingli Zhang: Conceptualization, Supervision. Han Liu: Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.01.015.

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