Melatonin exerts an *in vitro* anti-glioblastoma effect through SIRT1-mediated HMGB1 deacetylation and inhibition of the TLR4/NF-κB pathway

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Abstract. Glioblastoma (GBM) is a highly malignant brain tumor with an unfavorable prognosis, necessitating the urgent development of novel therapeutic strategies. Melatonin (MT), a natural hormone with known antitumor activity, exhibits potential anti-GBM effects; however, its underlying molecular mechanisms remain incompletely understood. The present study aimed to investigate whether MT suppresses the malignant phenotype of GBM cells through the sirtuin 1 (SIRT1)/high mobility group box 1 (HMGB1)/Toll-like receptor 4 (TLR4)/NF-κB signaling axis. The effects of MT on GBM cell viability, apoptosis, migration and invasion were assessed using standard *in vitro* functional assays. Network pharmacology, protein-protein interaction (PPI) analysis and molecular docking were performed to identify potential targets and pathways. Western blotting and EX527-mediated SIRT1 inhibition were used to evaluate the involvement of the SIRT1/HMGB1/TLR4/NF-κB axis. MT notably inhibited GBM cell viability, migration and invasion while inducing apoptosis. Network pharmacology and PPI analyses indicated that SIRT1, TLR4 and NF-κB1 were functionally relevant targets, and enrichment analysis suggested the involvement of TLR and NF-κB signaling pathways. Molecular docking further suggested a potential interaction between MT and SIRT1. Mechanistically, MT upregulated SIRT1 protein expression, reduced HMGB1 acetylation, decreased TLR4 expression and suppressed p65 phosphorylation. Conversely, EX527-mediated inhibition of SIRT1 induced molecular changes opposite to those observed after MT treatment, including increased HMGB1 acetylation, TLR4 expression and p65 phosphorylation. The present study suggests that MT suppresses the malignant phenotype of GBM cells, at least partly, in association with increased SIRT1 expression, reduced HMGB1 acetylation and attenuation of TLR4/NF-κB-related signaling. The present findings provide new theoretical support for developing MT as a potential adjuvant therapeutic candidate for GBM.

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Abbreviations: GBM, glioblastoma; MT, melatonin; HMGB1, high mobility group box 1; TLR4, Toll-like receptor 4; CTD, Comparative Toxicogenomics Database; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: melatonin, glioblastoma, high mobility group box 1, acetylation, NF-κB, sirtuin 1

Introduction

Gliomas represent the most prevalent and aggressive primary intracranial tumors, accounting for >40% of all primary brain tumors (1). Among these, glioblastoma (GBM), classified as grade IV glioma in the World Health Organization classification, represents the highest level of malignancy, characterized by highly invasive growth and worse prognosis (2). Despite the incorporation of multimodal treatment strategies, which include maximal safe resection supplemented by temozolomide chemotherapy and radiotherapy, the clinical prognosis for patients with GBM remains dismal. The median survival period is typically <15 months, with the majority of patients experiencing tumor recurrence within 1 year following surgery (3,4). Consequently, the exploration of innovative therapeutic molecules and strategies with the potential to effectively impede GBM progression has emerged as a pivotal scientific imperative in this domain, necessitating prompt and comprehensive resolution.

Melatonin (MT; N-acetyl-5-methoxytryptamine), a naturally occurring indoleamine hormone, is primarily

secreted by the pineal gland and serves a key role in regulating circadian rhythms, sleep-wake cycles and various metabolic processes (5-7). The amphiphilic properties of MT, which are both lipophilic and hydrophilic, enable its diffusion through biological membranes, including the blood-brain barrier, resulting in its distribution throughout tissues and body fluids (8,9). Beyond classical receptor-mediated signaling, MT has been revealed to exert its broad physiological and pharmacological activities through direct, receptor-independent interactions with intracellular targets (10,11). MT has been demonstrated to possess particularly potent neuroprotective effects in central nervous system disorders, including Parkinson's disease, Alzheimer's disease and ischemic brain injury models (12,13). MT has been revealed to possess dual antioxidant properties. In normal cells, such as neurons, MT acts as an effective free radical scavenger and antioxidant enzyme inducer (14,15). However, in several tumor models, MT paradoxically exerts pro-oxidant effects, thereby inhibiting cancer cell proliferation and promoting apoptotic cell death (16-18). The antitumor effect of MT has been suggested in a variety of malignancies, including prostate cancer, colorectal cancer, hepatocellular carcinoma and breast cancer (19). However, the molecular mechanisms underlying the action of MT in GBM, particularly its regulation of key signaling pathways, remain to be elucidated.

High mobility group box 1 (HMGB1) is a nuclear non-histone protein that is widely expressed in eukaryotic cells (20,21). It serves a key role in maintaining nuclear structural homeostasis and regulating gene transcription by modulating chromosome structure and function, as well as sustaining cellular autophagy (22). In conditions of cellular stress, injury or necrosis, HMGB1 can be actively secreted or passively released into the extracellular space (23,24), serving as a key damage-associated molecular pattern molecule. By binding to its receptors [such as Toll-like receptor 4 (TLR4)], it activates downstream pro-survival and pro-inflammatory signaling pathways, such as NF-κB, which ultimately drive tumor cell proliferation, migration, invasion and treatment resistance (25). TLR4 is expressed at high levels in glioma cells and tumor-associated glial cells within the brain. The activation of the NF-κB pathway, triggered by its binding to HMGB1 (26), is considered a pivotal step in promoting the malignant progression of gliomas. However, the potential of MT to inhibit GBM by regulating the HMGB1/TLR4/NF-κB signaling axis remains to be elucidated. Therefore, the present study aimed to investigate the anti-GBM effects of MT and to explore whether the SIRT1/HMGB1/TLR4/NF-kB axis is involved in this process. To address this aim, *in vitro* functional assays were performed to evaluate the effects of MT on GBM cell viability, apoptosis, migration and invasion. In addition, network pharmacology, protein-protein interaction (PPI) analysis and molecular docking were used to identify potential molecular targets and pathways, and western blotting together with EX527-mediated SIRT1 inhibition was performed to assess the involvement of SIRT1/HMGB1/TLR4/NF-κB-related signaling.

Materials and methods

Cell culture. U87MG cells, a commonly used GBM cell line of unknown origin (cat. no. CL-0238), and LN229 human GBM cells (cat. no. CL-0578) were purchased from Procell Life Science & Technology Co., Ltd. The U87MG cell line used in the present study was the American Type Culture Collection version, which is most probably a GBM cell line of unknown origin. The authenticity of the U87MG cell line was verified by short tandem repeat profiling. Both cell types were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. Cell cultures were kept in a humidified incubator at 37˚C under a 5% CO_2 atmosphere. For subculturing, cells were detached using 0.25% trypsin and passaged following standard protocols.

Reagents and antibodies. MT and the sirtuin 1 (SIRT1) inhibitor EX527 were purchased from Beyotime Biotechnology and were dissolved in dimethyl sulfoxide (DMSO) to prepare storage solutions for use. The primary antibodies used in the present study and their dilution ratios are as follows: Anti-Bax (1:2,000; cat. no. 50599-2-Ig), anti-Bcl-2 (1:2,000; 68103-1-Ig), anti-cadherin CDH1 (1:2,000; 20874-1-AP), anti-CDH2 (1:2,000; 22018-1-AP), anti-MMP3 (1:2,000; 17873-1-AP), anti-Vimentin (1:2,000; 10366-1-AP), anti-SIRT1 (1:2,000; 13161-1-AP), anti-TLR4 (1:2,000; 66350-1-Ig), anti-p65 (1:2,000; 66535-1-Ig) and anti-phosphorylated-p65 (p-p65; 1:2,000; 82335-1-RR) were all purchased from Proteintech Group, Inc. Anti-HMGB1 (1:1,000; YM4697) and anti-acetylated-HMGB1 (acHMGB1; 1:1,000; YK0060) were purchased from Immunoway Biotechnology Company and anti-β-actin (1:10,000; Proteintech Group, Inc.; 81115-1-RR) was used as an internal control. All antibodies were used according to the manufacturer's recommended conditions.

Cell viability assay. The assessment of cell viability was conducted by employing the Cell Counting Kit-8 (CCK-8; Biosharp Life Sciences). In summary, cells were seeded into 96-well plates. Following cell attachment, fresh DMEM containing 10% FBS and MT at various concentrations (0, 1, 3 and 5 mM) was added, and cells were cultured for 24 and 48 h. At each designated time point, the CCK-8 reagent was added to each well (representing 10% of the medium volume). The plates were then incubated at 37˚C in the dark for a period of 2 h. Subsequently, the absorbance was measured at 450 nm by means of a microplate reader.

Calcein AM/propidium iodide (PI) assay for cell viability and cytotoxicity. The viability and cytotoxicity of cultured cells were determined by Calcein-AM/PI double-staining (Beyotime Biotechnology). U87MG and LN229 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. Following the completion of treatments, the medium was discarded, and the cell monolayer was washed twice with pre-cooled PBS. Subsequent to the addition of the Calcein-AM/PI working solution, incubation proceeded at 37˚C for 30 min, protected from light. Observation under a fluorescence microscope distinguished viable cells, characterized by green fluorescence, from non-viable cells, which were labeled with red fluorescence.

Western blot analysis. After treatment with 1 mM MT or 5 μM EX527 for 48 h, cells were washed twice with pre-chilled PBS and lysed for 30 min on ice using RIPA lysis buffer (containing

1% protease inhibitor and 1% phosphatase inhibitor). Cell lysates were collected, centrifuged at 4˚C and 12,000 g for 20 min, and the supernatant was collected. Following protein quantification with a BCA kit, 25 μg of total protein was loaded per lane. samples were diluted in 5X SDS loading buffer and heat-denatured at 100˚C for 5-10 min. The proteins were separated via SDS-PAGE on a 10% gel and then transferred to a PVDF membrane. After blocking with 5% non-fat milk at room temperature for 2 h, the membrane was incubated with respective primary antibodies overnight at 4˚C. Subsequent to washing with TBST containing 0.1% Tween-20, the membrane was exposed to an HRP-conjugated secondary antibody, including HRP-conjugated goat anti-rabbit IgG (cat. no. SA00001-2; Proteintech Group, Inc; 1:10,000) and HRP-conjugated goat anti-mouse IgG (cat. no. SA00001-1; Proteintech Group, Inc; 1:10,000), for 2 h at room temperature. Final detection was achieved using a chemiluminescence substrate (BeyoECL Plus; cat. no. P0018S; Beyotime Biotechnology), and band intensities were quantified based on grayscale values analyzed with ImageJ (v1.53; National Institutes of Health).

Wound healing assay. To assess cell migration capacity, U87MG and LN229 cells were seeded at high density in 6-well plates. Once the monolayer exhibited >90% confluence, a linear incision was made across the monolayer using a 200 μl sterile pipette tip. The cell debris was then washed away twice with PBS, and the cells were then cultured in serum-free DMEM containing the indicated treatments. The images were obtained from the same location with an inverted microscope at two distinct time points: 0 and 24 h post-scratch.

Transwell invasion assay. Cell invasion assays were performed using Matrigel-coated Transwell inserts with an 8.0 μm pore size. Briefly, Matrigel was added to the upper surface of the Transwell membrane and allowed to solidify at 37˚C for 1 h before cell seeding. U87MG and LN229 cells were serum-starved overnight in serum-free DMEM, harvested and seeded into the upper chamber at a density of $1x10^5$ cells/well in serum-free DMEM containing 1 mM MT. The lower chamber contained DMEM supplemented with 10% FBS as a chemoattractant. After incubation at 37˚C in a humidified atmosphere with 5% CO_2 for 48 h, non-invading cells on the upper surface of the membrane were removed. The invaded cells on the lower surface were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.1% crystal violet at room temperature for 20 min. The number of invaded cells was counted in three randomly selected fields under an inverted light microscope.

Network pharmacology, PPI network and molecular docking analysis. To predict potential targets and mechanisms of MT against GBM, potential targets of MT were obtained from the Comparative Toxicogenomics Database (CTD; https://ctdbase.org/) (27) and disease-related targets were retrieved from the GeneCards database (https://www.genecards.org/) (28) using 'Glioblastoma' as the keyword. The intersection of these two target sets yielded common interaction targets. These shared targets underwent Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the DAVID online database (version 2021 update; https://david.ncifcrf.gov/). The GO and KEGG databases were accessed through DAVID for functional annotation and pathway enrichment analysis (http://geneontology.org/; https://www.kegg.jp/). Enrichment results were visualized using the 'ggplot2' package (version 4.0.3; https://CRAN.R-project.org/package=ggplot2) in RStudio software (2025.09.2+418; Posit Software, PBC).

To further explore the relationships among the overlapping targets, a PPI network was constructed using the STRING database (https://string-db.org/) with *Homo sapiens* as the species. The PPI data were imported into Cytoscape software (version 3.10.0; Cytoscape Consortium; https://cytoscape.org/) for network visualization and topological analysis. Core targets were ranked according to degree centrality, and the top 35 nodes were selected for further visualization.

Molecular docking was performed to evaluate the potential interaction between MT and SIRT1. The three-dimensional structure of SIRT1 was obtained from the Protein Data Bank (PDB; https://www.rcsb.org/), and the chemical structure of MT was obtained from PubChem. The docking process was performed using AutoDock Vina (version 1.2.7; Center for Computational Structural Biology, Scripps Research), while Discovery Studio (version 2019; BIOVIA, Dassault Systèmes S.E.) was employed to generate two-dimensional interaction maps illustrating hydrogen bonds, hydrophobic contacts and π-π overlap interactions.

Statistical analysis. Data analysis and visualization were performed using GraphPad Prism 8.0 (Dotmatics). Data are presented as mean ± SD from at least three independent biological replicates. Comparisons between two groups were performed using an unpaired Student's t-test. Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MT inhibits the viability of GBM cells and promotes their apoptosis. To elucidate the direct effect of MT on GBM cell viability, cells were first treated with varying concentrations of MT. CCK-8 assay results indicated that GBM cell viability decreased in a concentration-dependent manner after treatment with 1, 3 and 5 mM MT for 24 and 48 h (Fig. 1A and B). To further validate the effect of MT on cell survival, Calcein-AM/PI dual staining was performed. Fluorescence microscopy revealed a notable reduction in green-fluorescent viable cells and a corresponding increase in red-fluorescent dead cells in MT-treated groups, indicating MT markedly decreased the relative survival rate of GBM cells (Fig. 1C and D). Subsequently, it was investigated whether MT modulates cell survival by regulating apoptosis. Western blot analysis suggested that MT treatment significantly upregulated the expression of the pro-apoptotic protein Bax while markedly suppressing the expression of the anti-apoptotic protein Bcl-2 (Fig. 1E and F).

MT inhibits the migratory and invasive capabilities of GBM cells. The effects of MT on GBM cell migration and invasion were evaluated using a series of functional assays.

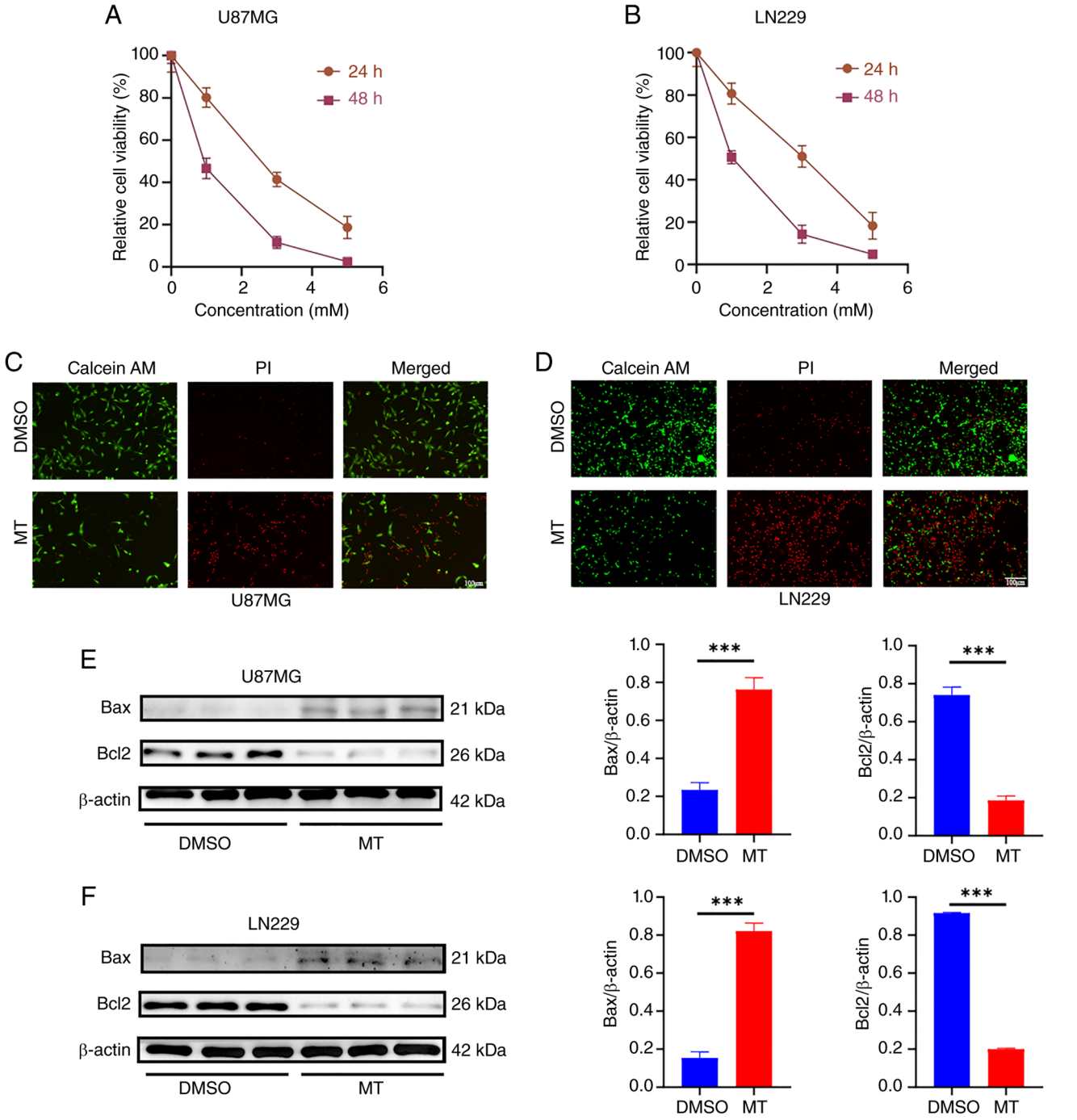

Figure 1. MT inhibits the viability of glioblastoma cells and promotes their apoptosis. (A) U87MG and (B) LN229 cells were treated with different concentrations of MT (1, 3 and 5 mM) for 24 and 48 h, and cell viability was expressed as a percentage relative to the control group. Representative Calcein-AM/PI double-staining images of (C) U87MG and (D) LN229 cells after treatment with 1 mM MT for 48 h. Live cells are presented in green, and dead cells are presented in red. Representative western blot bands and quantitative analysis of the apoptosis-related proteins Bax and Bcl-2 in (E) U87MG and (F) LN229 cells after treatment with 1 mM MT for 48 h. Data are presented as mean ± SD from three independent biological replicates (n=3). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test for multiple-group comparisons or an unpaired Student's t-test for two-group comparisons, as appropriate. ***P<0.001. MT, melatonin; PI, propidium iodide.

Transwell assays demonstrated that MT treatment reduced the number of invaded U87MG and LN229 cells after 48 h (Figs. 2A and S1A). Wound healing assays further indicated that treatment with MT for 24 h significantly reduced the relative migration of both GBM cell lines (Figs. 2B and S1B). To investigate the molecular changes associated with the effects of MT on migration and invasion, the expression levels of epithelial-mesenchymal transition and associated invasive proteins were examined. Western blot analysis revealed that MT treatment significantly upregulated the expression of the epithelial marker CDH1. Conversely, the expression of the mesenchymal markers CDH2 and vimentin, as well as the matrix metalloproteinase MMP3, were significantly downregulated (Fig. 2C and D). These molecular changes collectively suggest the functional role of MT in inhibiting GBM cell migration and invasion.

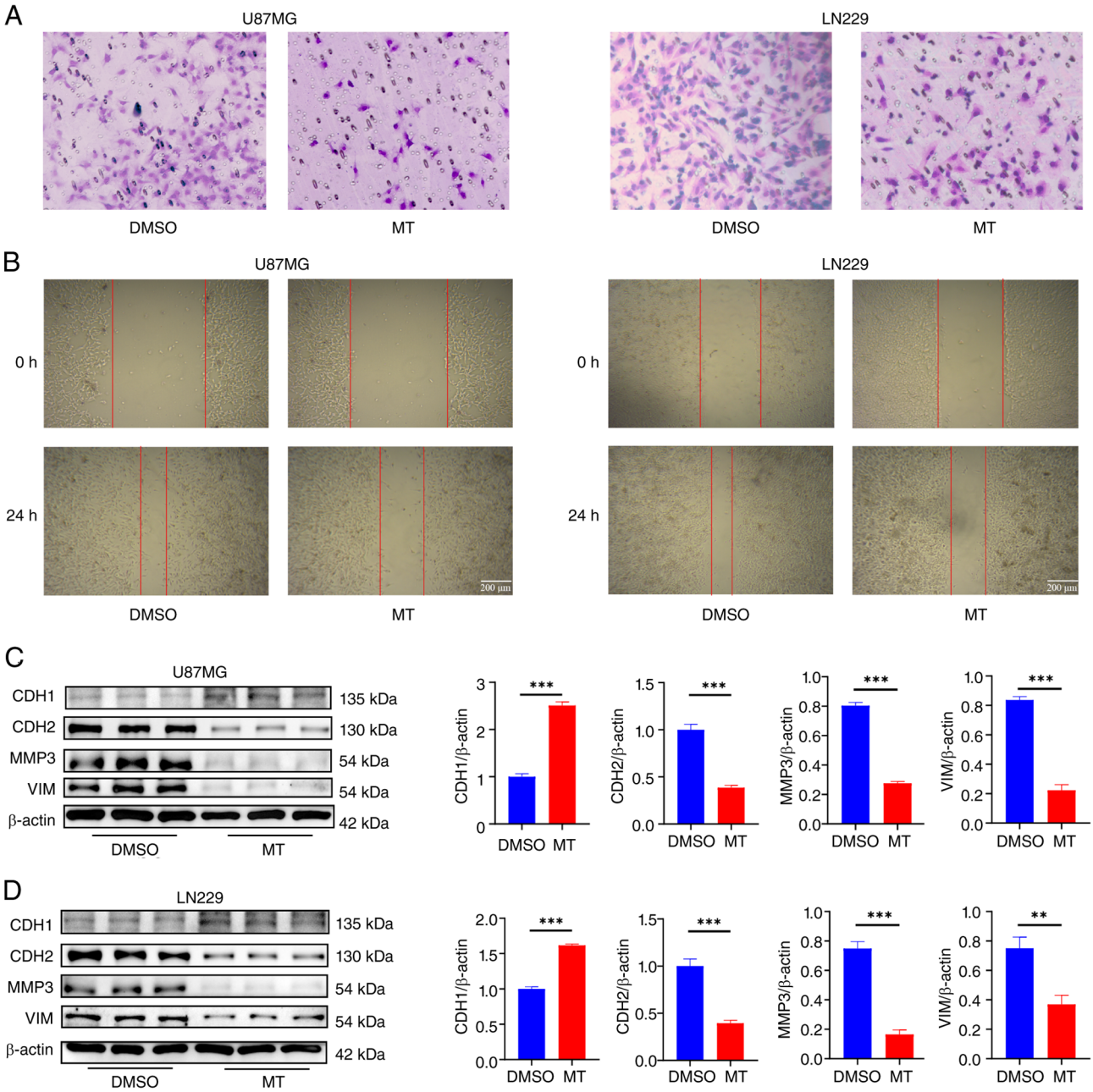

Figure 2. MT inhibits the migration and invasion capabilities of GBM cells. (A) Representative images of the Transwell invasion assay showing the invasive capacity of GBM cells after treatment with 1 mM MT for 48 h. Cells were stained with crystal violet. Original magnification, x50. (B) Representative images of the wound healing assay showing the migratory capacity of GBM cells after treatment with 1 mM MT for 24 h. Scale bars, 200 μm. Representative western blot bands and quantitative analysis of migration- and invasion-related proteins, including CDH1/E-cadherin, CDH2/N-cadherin, vimentin and MMP3, in (C) U87MG and (D) LN229 cells after treatment with 1 mM MT for 48 h. Data are presented as mean ± SD from three independent biological replicates (n=3). Statistical significance was determined using an unpaired Student's t-test. **P<0.01 and ***P<0.001. GBM, glioblastoma; MT, melatonin; VIM, vimentin; CDH, cadherin.

Network pharmacology predicts potential targets and pathways for MT in GBM. To explore the potential mechanisms underlying the anti-GBM effects of MT, network pharmacology, PPI network analysis and molecular docking were performed. MT-related targets were obtained from the CTD, whereas GBM-related targets were retrieved from GeneCards. Intersection of the two datasets identified 388 overlapping targets as potential targets of MT against GBM (Fig. 3A).

GO enrichment analysis indicated that these targets were mainly associated with transcriptional and gene expression regulation, apoptosis, signal transduction and inflammatory responses. The targets were predominantly localized in the cytoplasm, nucleus, plasma membrane, extracellular region and mitochondria and were enriched in molecular functions such as 'protein binding', 'DNA binding', 'ATP binding' and 'DNA-binding transcription factor activity' (Fig. 3B-D). KEGG analysis further revealed enrichment in cancer and inflammation-related pathways, including TLR signaling, NF-κB signaling, glioma-related pathways and apoptosis (Fig. 3E).

A PPI network was subsequently constructed, and degree centrality analysis was performed using Cytoscape. The top 35 hub genes are presented in Fig. 3F. Notably, NF-κB1, SIRT1

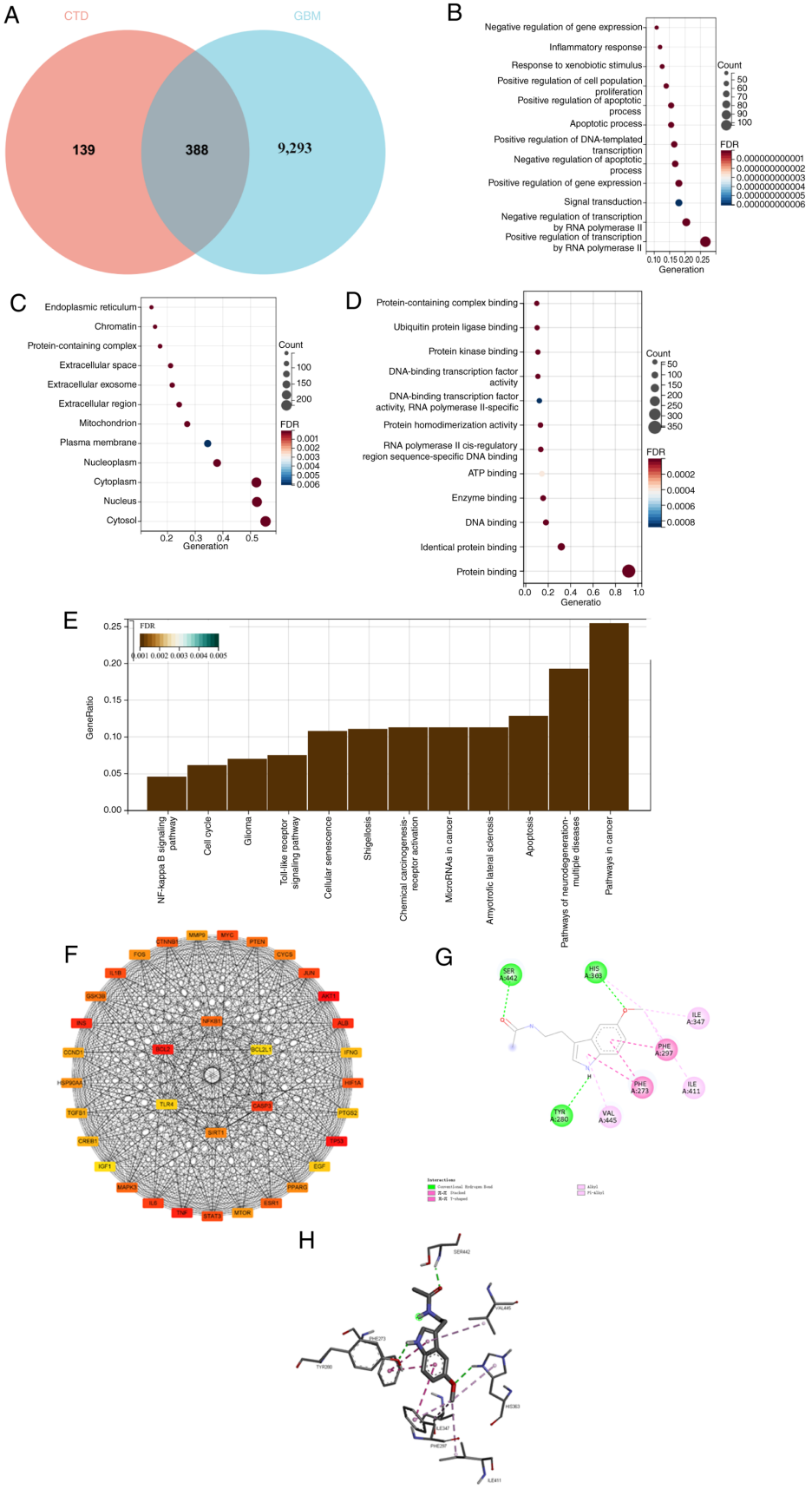

Figure 3. Network pharmacology, PPI network and molecular docking analyses of potential melatonin-related targets in GBM. (A) Venn diagram showing 388 overlapping targets between melatonin-related targets obtained from the CTD and GBM-related targets obtained from GeneCards. Gene Ontology enrichment analyses of the overlapping targets, including (B) biological processes, (C) cellular components and (D) molecular functions. (E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the overlapping targets. (F) Network of the top 35 hub genes identified by degree centrality analysis of the PPI network using Cytoscape. (G) Two-dimensional interaction diagram showing the predicted interactions between melatonin and key amino acid residues of SIRT1. (H) Three-dimensional view of melatonin within the predicted SIRT1 binding pocket. CTD, Comparative Toxicogenomics Database; GBM, glioblastoma; FDR, false discovery rate; PPI, protein-protein interaction; SIRT1, sirtuin-1.

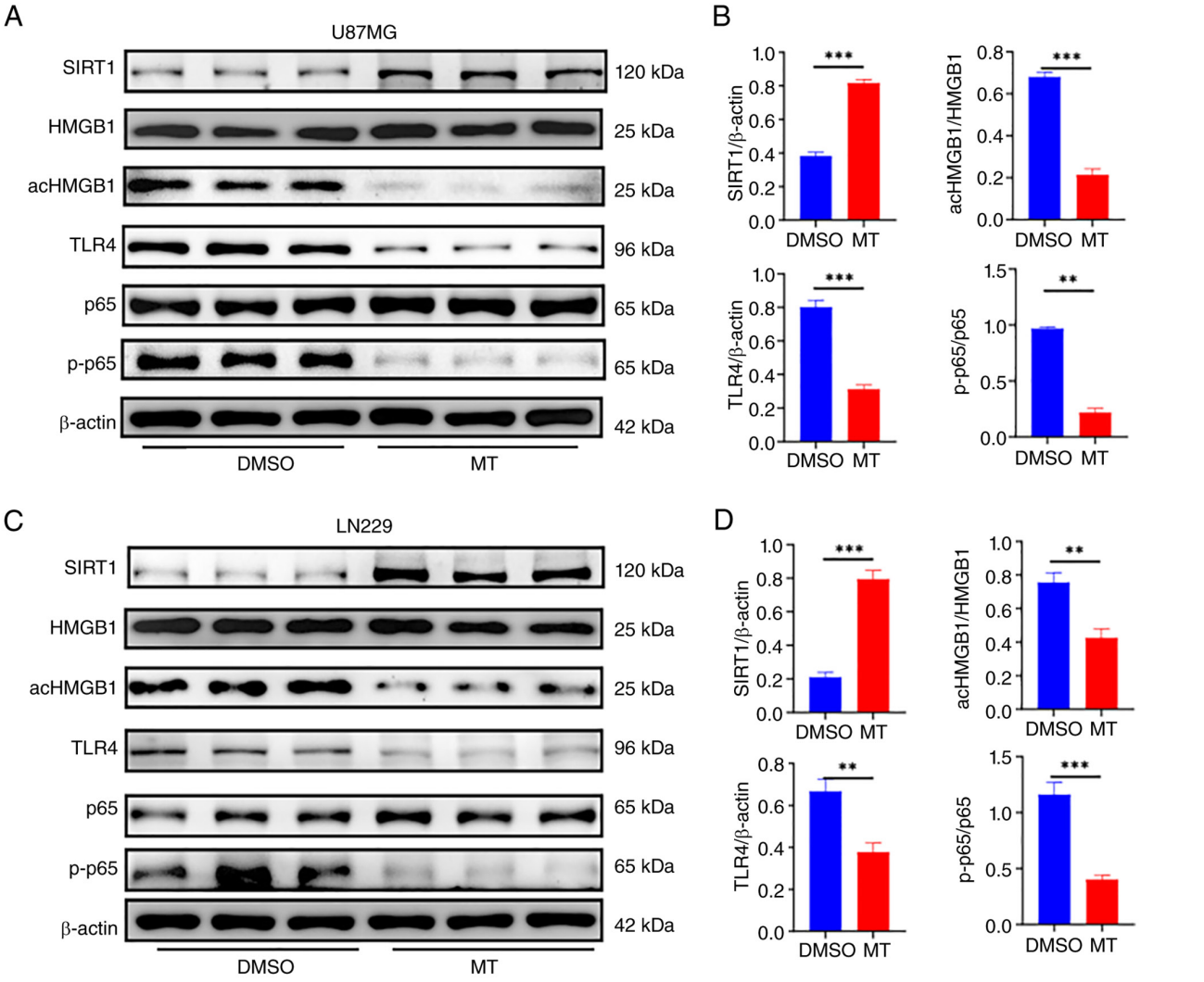

Figure 4. MT increases SIRT1 expression and attenuates HMGB1/TLR4/NF-κB-related signaling in glioblastoma cells. (A) Western blot bands showing the expression of SIRT1, HMGB1, acHMGB1, TLR4, p65 and p-p65 in U87MG cells after treatment with 1 mM MT for 48 h. (B) Semi-quantitative analysis of SIRT1, acHMGB1/HMGB1, TLR4 and p-p65/p65 protein levels in U87MG cells. (C) Western blot bands showing the expression of SIRT1, HMGB1, acHMGB1, TLR4, p65 and p-p65 in LN229 cells after treatment with 1 mM MT for 48 h. (D) Semi-quantitative analysis of SIRT1, acHMGB1/HMGB1, TLR4 and p-p65/p65 protein levels in LN229 cells. Data are presented as mean ± SD from three independent biological replicates (n=3). Statistical significance was determined using an unpaired Student's t-test. **P<0.01 and ***P<0.001. MT, melatonin; SIRT1, sirtuin-1; HMGB1, high mobility group box 1; TLR4, Toll-like receptor 4; acHMGB1; acetylated HMGB1; p-p65, phosphorylated p65.

and TLR4 were included among these hub genes, providing a network-level basis for further investigation of SIRT1- and TLR4/NF-κB-related signaling.

Molecular docking suggested that MT could be accommodated within the binding pocket of SIRT1 and interact with surrounding amino acid residues (Fig. 3G and H), supporting SIRT1 as a plausible upstream candidate target of MT. Collectively, the PPI topology, pathway enrichment and docking results suggest that MT may regulate TLR4/NF-κB-related signaling through SIRT1. Due to the deacetylase activity of SIRT1 and the mechanistic relevance of HMGB1 acetylation to TLR4/NF-κB signaling (29,30), the SIRT1/HMGB1/TLR4/NF-κB axis was selected for subsequent experimental validation.

MT increases SIRT1 expression and attenuates HMGB1/TLR4/NF-κB-related signaling in GBM cells. To elucidate the antitumor regulatory mechanism of MT, western blotting was performed to analyze the effects of MT treatment on the expression of proteins involved in relevant signaling pathways. Previous studies have suggested that MT can upregulated the expression of SIRT1 (31-33). The present experimental results demonstrated that MT treatment significantly upregulated the protein expression level of SIRT1 in U87MG cells (Fig. 4A and B) and concurrently markedly reduced the levels of acHMGB1, its key receptor TLR4 and p-p65 (Fig. 4A and B). To validate the universality of this regulatory relationship, the experiment was repeated using another cell line, LN229, with results consistent with those observed in U87MG cells: MT similarly significantly increased SIRT1 expression in LN229 cells (Fig. 4C and D) while suppressing the protein levels of acHMGB1, TLR4 and p-p65 (Fig. 4C and D). Collectively, the data suggests that MT may inhibit the activity of the HMGB1/TLR4/NF-κB signaling pathway by activating SIRT1 expression. This synergistic effect suggests that MT attenuates HMGB1/TLR4/NF-κB-dependent signaling by enhancing SIRT1 expression.

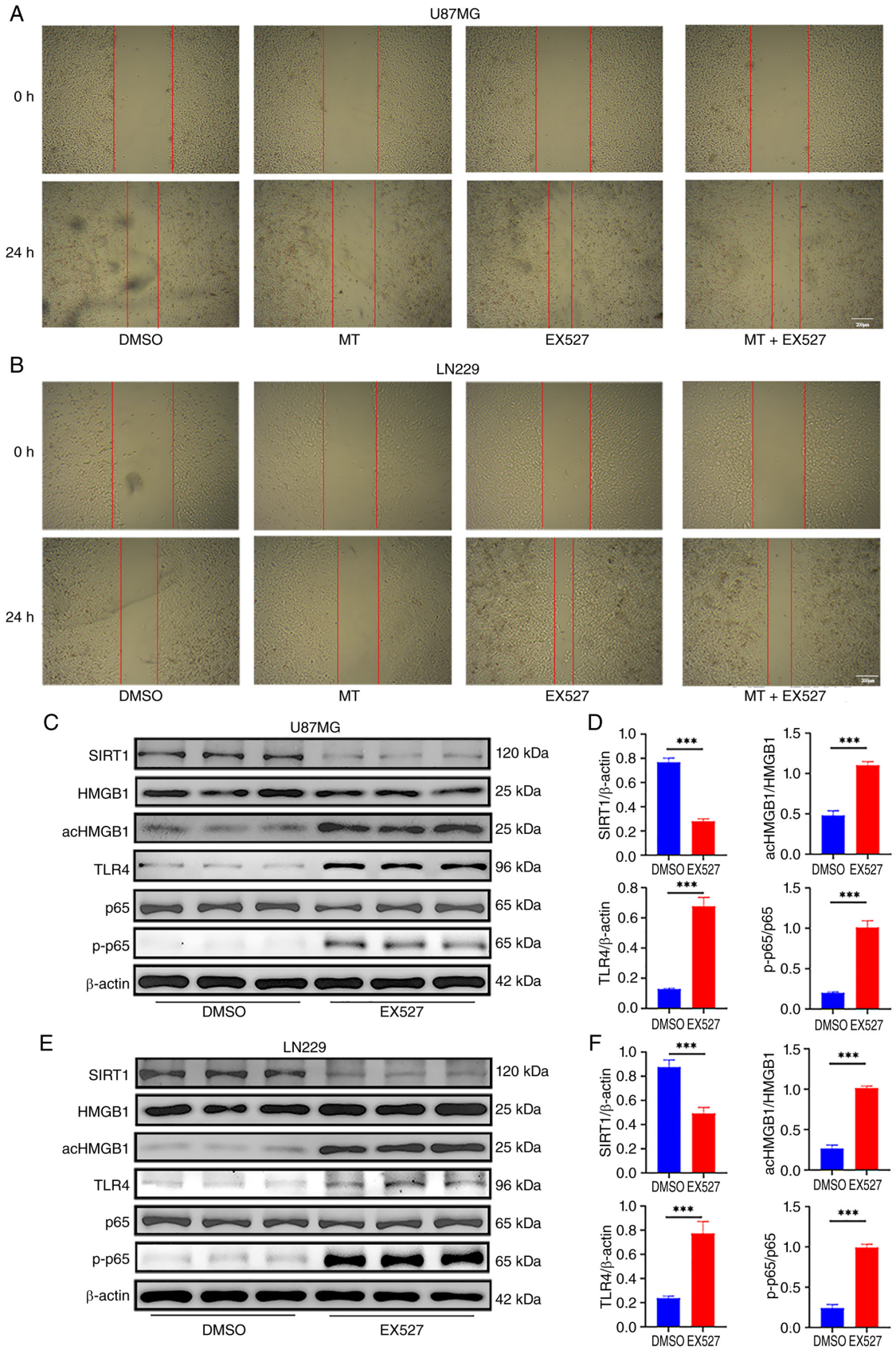

Figure 5. Inhibition of SIRT1 enhances the HMGB1/TLR4/NF-κB signaling pathway in glioblastoma cells. Representative wound healing images of (A) U87MG and (B) LN229 cells treated with DMSO, 1 mM MT, 5 μM EX527 or the combination of MT and EX527 for 24 h. Images were captured at 0 and 24 h after scratching. Scale bars, 200 μm. (C) Western blot bands showing the expression of SIRT1, HMGB1, acHMGB1, TLR4, p65 and p-p65 in U87MG cells after treatment with EX527 for 48 h. (D) Semi-quantitative analysis of SIRT1, ac-HMGB1/HMGB1, TLR4 and p-p65/p65 protein levels in U87MG cells. (E) Western blot bands showing the expression of SIRT1, HMGB1, acHMGB1, TLR4, p65 and p-p65 in LN229 cells after treatment with EX527 for 48 h. (F) Semi-quantitative analysis of SIRT1, acHMGB1/HMGB1, TLR4 and p-p65/p65 protein levels in LN229 cells. Data are presented as mean ± standard deviation from three independent biological replicates (n=3). Statistical significance was determined using an unpaired Student's t-test. ***P<0.001. MT, melatonin; SIRT1, sirtuin-1; HMGB1, high mobility group box 1; TLR4, Toll-like receptor 4; acHMGB1; acetylated HMGB1; p-p65, phosphorylated p65.

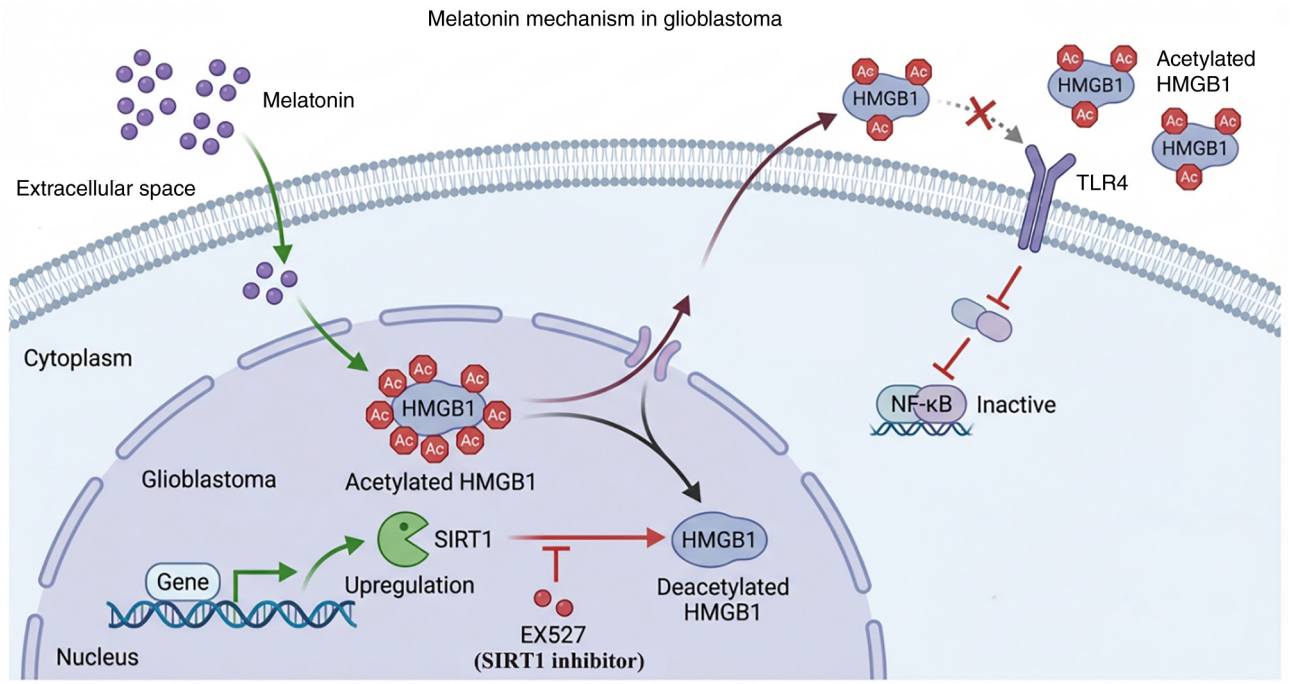

Figure 6. Proposed model of melatonin-associated regulation of the SIRT1/HMGB1/TLR4/NF-κB signaling axis in glioblastoma cells. SIRT1, sirtuin-1; HMGB1, high mobility group box 1; TLR4, Toll-like receptor 4; ac, acetylation.

Pharmacological inhibition of SIRT1 enhances GBM cell migration and HMGB1/TLR4/NF-κB-related signaling. The aforementioned results demonstrated that MT upregulated SIRT1 expression and suppressed the HMGB1/TLR4/NF-κB signaling pathway. To further determine whether SIRT1 contributed to the anti-migratory effect of MT, U87MG and LN229 cells were treated with DMSO, MT, EX527 or MT combined with EX527. Wound healing assays indicated that MT markedly reduced the relative migration of both GBM cell lines, whereas EX527 markedly enhanced cell migration. Importantly, the relative migration rate in the MT+EX527 group was notably higher than that in the MT-alone group, indicating that pharmacological inhibition of SIRT1 partially reversed the inhibitory effect of MT on GBM cell migration (Figs. 5A and B and S1C). Western blot analysis further suggested that EX527 treatment decreased SIRT1 expression in both U87MG and LN229 cells. This reduction was accompanied by increased levels of acHMGB1, TLR4 and p-p65 (Fig. 5C-F). These findings indicate that pharmacological inhibition of SIRT1 induces molecular changes opposite to those observed after MT treatment and suggest that SIRT1 may negatively regulate HMGB1 acetylation and downstream TLR4/NF-κB pathway activity in GBM cells. Together, these results support the involvement of SIRT1 in the MT-associated regulation of the HMGB1/TLR4/NF-κB signaling axis.

Discussion

The present study provides evidence that MT may suppress the malignant phenotype of GBM cells and suggests the involvement of the SIRT1/HMGB1/TLR4/NF-κB signaling axis. MT inhibited GBM cell viability, migration and invasion and promoted apoptosis. These effects were accompanied by increased SIRT1 expression, reduced HMGB1 acetylation, decreased TLR4 expression and suppressed p65 phosphorylation (Fig. 6).

GBM is the most aggressive malignant tumor in the central nervous system, accounting for ~49.1% of all primary brain tumors (34). The incidence rate is ~3.2 cases per 100,000 people annually, and it is projected to continue rising over the coming decades, posing a notable public health challenge (35,36). Despite comprehensive treatment approaches including surgery, radiotherapy and chemotherapy, patients with GBM continue to face an unfavorable prognosis, with a median survival of only ~12 months and an extremely low 5-year survival rate (37). Furthermore, the high recurrence and mortality rates of GBM, coupled with its resistance to the first-line chemotherapeutic agent temozolomide and high recurrence rates, further underscore the urgent need to develop novel therapeutic strategies.

MT is an endogenous indoleamine primarily synthesized and secreted by the pineal gland under circadian regulation. It has attracted considerable attention because of its antioxidant, neuroprotective and broad-spectrum anticancer properties (38,39). The anticancer mechanisms of MT involve multiple levels, including inducing autophagy, modulating mitochondrial function, triggering endoplasmic reticulum stress and regulating various forms of cell death (40). The molecular effects of MT vary across types of tumors. For example, MT induces tumor wasting through the peroxisome proliferator-activated receptor γ coactivator 1-α/uncoupling protein 1 pathway in renal cell carcinoma (41) and disrupts folate metabolism in head and neck squamous cell carcinoma (42). SIRT1 has emerged as an important mediator of several biological effects of MT. Previous studies have demonstrated that MT can alleviate endoplasmic reticulum

stress and apoptosis by increasing SIRT1 expression (31) and modulate inflammatory or neuroprotective signaling through SIRT1-related pathways (32). These findings suggest that SIRT1 may serve as a key hub for the biological functions of MT.

HMGB1 is a highly conserved nucleoprotein that serves a key role in maintaining genomic stability (43). During cellular stress, injury or death, HMGB1 can be actively secreted or passively released from the nucleus into the extracellular space, where it functions as a damage-associated molecular pattern molecule (44). Extracellular HMGB1 activates downstream signaling cascades, including NF-κB, by binding to receptors such as the advanced glycation end-product receptor or TLRs, thereby promoting tumor growth, metastasis and the formation of an immune-inflammatory microenvironment (45,46). MT also affected EMT-like and invasion-related proteins in GBM cells. In the present study, MT increased CDH1/E-cadherin expression while decreasing CDH2/N-cadherin, vimentin and MMP3 expression, which was consistent with the observed reduction in migration and invasion. These findings suggest that MT may suppress the invasive phenotype of GBM cells by modulating EMT-like and matrix degradation-related proteins.

The biological effects of MT are highly dependent on cellular context. In normal or non-transformed cells, MT frequently exerts antioxidant, mitochondrial-protective and cytoprotective effects (14,15), whereas in tumor cells it may inhibit proliferation, disturb tumor metabolism and promote apoptosis (16). The effect of MT on immune cells may also differ from those observed in tumor cells and may involve the regulation of cytokine production, inflammatory signaling, immune-cell recruitment and cell-cell interactions (47). Of particular relevance, Lai *et al* (33) reported that MT increased SIRT1 expression in GBM cells and reduced C-C motif chemokine ligand 2-mediated monocyte adhesion by downregulating the adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule 1, indicating that MT may modulate interactions between GBM cells and infiltrating monocytes within the tumor microenvironment. However, as the present study did not include immune-cell co-culture or direct analysis of tumor-associated microglia/macrophages, these potential microenvironmental effects remain to be validated. Previous mechanistic studies have established the biological link between TLR4 signaling and p65 nuclear translocation (48,49). In particular, the cytokine-active disulfide form of HMGB1 has been revealed to bind the myeloid differentiation factor 2 (MD-2)/TLR4 complex and induce NF-κB activation, including increased nuclear accumulation of the p50 and p65 subunits (48). Genetic deletion or silencing of MD-2 markedly attenuated HMGB1-dependent TLR4 signaling. In addition, studies have demonstrated that TLR4 activation induces NF-κB nuclear translocation, with MyD88 serving as a major upstream mediator of this process (48,49). These findings provide a mechanistic basis for the proposed connection between HMGB1/TLR4 signaling and downstream p65 activation. In the present study, MT reduced HMGB1 acetylation and attenuated TLR4/NF-κB-related signaling raises the possibility that this pathway may also influence damage-associated inflammatory signaling and myeloid-cell responses in the GBM microenvironment.

The present findings support the involvement of SIRT1 in the regulation of the HMGB1/TLR4/NF-κB-related signaling axis. MT increased SIRT1 expression while reducing HMGB1 acetylation, TLR4 expression and p65 phosphorylation. Importantly, EX527 partially restored the migratory capacity of MT-treated GBM cells, providing functional evidence that SIRT1 contributes to the anti-migratory effect of MT. EX527 treatment alone also decreased SIRT1 expression and increased acHMGB1, TLR4 and p-p65 levels, further supporting a negative regulatory role of SIRT1 in this pathway. Nevertheless, several limitations should be acknowledged. First, the present study was limited to two established GBM cell lines, and validation in patient-derived GBM cells and *in vivo* models is warranted. Second, NF-κB-related signaling was evaluated mainly through p65 phosphorylation without direct assessment of p65 nuclear translocation. In addition, molecular docking only predicted a potential interaction between MT and SIRT1 and did not directly demonstrate physical binding. Future studies using patient-derived models, orthotopic GBM models, p65 immunofluorescence or nuclear/cytoplasmic fractionation, and more complete molecular rescue experiments will be required to further validate the proposed mechanism and its therapeutic relevance.

In conclusion, the present study suggests that MT inhibits GBM cell viability, migration and invasion and promotes apoptosis, at least partly, through upregulation of SIRT1, reduction of HMGB1 acetylation and attenuation of TLR4/NF-κB-related signaling. PPI analysis and molecular docking provided a computational basis for selecting this candidate pathway, whereas the EX527 experiments provided additional evidence supporting the regulatory involvement of SIRT1. These findings expand the current understanding of the anti-GBM mechanisms of MT and support further investigation of the SIRT1/HMGB1/TLR4/NF-κB axis as a potential therapeutic target. However, *in vivo* validation and more complete functional rescue experiments are required before MT can be considered a clinically applicable adjunctive treatment for GBM.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BS, CZ, YW and SH contributed to the conception and design of the present study. BS, CZ, YW, SH and NL analyzed the data. CZ, YW, SH, NL and LZ drafted the manuscript and

performed the experimental operations. BS, YW, CZ and LZ contributed to the critical revision of the manuscript. BS and LZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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