Melatonin suppresses epithelial-to-mesenchymal transition in the MG-63 cell line

YONGJUN CHEN[1], TAO ZHANG[2], XIONGWEI LIU[1], ZENGYAN LI[3], DONGMING ZHOU[1] and WENSHENG XU[1]

[1]Department of Orthopaedics, The First Affiliated Hospital of Baotou Medical College,
Inner Mongolia University of Science and Technology, Baotou, Inner Mongolia 014010;
[2]Department of Immunology, Basic and Forensic Medicine of Baotou Medical College, Inner Mongolia University of Science
and Technology, Baotou, Inner Mongolia 014060; [3]Department of Nephrology,
The First Affiliated Hospital of Baotou Medical College, Inner Mongolia University of
Science and Technology, Baotou, Inner Mongolia 014010, P.R. China

Abstract. Epithelial-to-mesenchymal transition (EMT) is a major process involved in tumor progression and metastasis. Melatonin is secreted by the pineal gland and has been documented as a potential therapeutic agent for multiple tumors. However, the effects of melatonin on EMT during osteosarcoma (OA) development remain undefined. The present study explored the biological functions and effects of melatonin on EMT induced by transforming growth factor β1 (TGF-β1) and its underlying mechanisms in MG-63 cells. Using western-blotting and immunofluorescence, it was found that the switch in E-cadherin/N-cadherin and vimentin expression was induced by TGF-β1, which was reversed by melatonin through the suppression of Snail and matrix metalloproteinase 9 (MMP-9), through hypoxia-inducible factor 1α (HIF-1α) inhibition. These findings demonstrated that the anticancer effects of melatonin against OA MG-63 cells is through the suppression of EMT via HIF-1α/Snail/MMP-9 signaling.

Correspondence to: Professor Wensheng Xu, Department of Orthopaedics, The First Affiliated Hospital of Baotou Medical College, Inner Mongolia University of Science and Technology, 41 Lin Yin Road, Baotou, Inner Mongolia 014010, P.R. China
mail: wsxu123_9@126.com

Introduction

Osteosarcoma (OA) is a malignant and aggressive bone tumor prevalent in children and young adults, representing 60% of all bone tumors globally (1). Although OA treatment including surgery and systemic chemotherapy has progressed, local infiltration and distant metastasis are frequent. For patients lacking tumor spread and metastasis, the five-year survival rates are 60-80%. For patients with tumor metastasis, the five-year survival rates decrease to 17% (2). A deeper understanding of the key mechanisms promoting OA tumorigenesis and effective therapeutic interventions towards OA are thus essential.

Melatonin is secreted by the pineal gland and plays a cyto-protective role in the regulation of oxidative stress, apoptosis-related factors and signaling pathways (3). Melatonin is beneficial during the treatment of insomnia, obesity, type 2 diabetes and liver fibrosis (4-6) and can inhibit hormone-dependent or hormone-independent tumors (7). Notably, melatonin was found to exert its anticancer activity through various biological processes including chemosensitivity, reduced drug resistance and anti-proliferative effects in ovarian, breast, prostate, oral, gastric and colorectal cancers (8-10). The detailed mechanisms underlying these effects and its antitumor activity remain poorly defined. Epithelial-to-mesenchymal transition (EMT) leads to cytological changes whereby tumor cells become more invasive during metastasis and progression. According to Menéndez-Menéndez *et al* (11), the antitumor effects of melatonin on cell survival, invasion and the metastasis of breast cancer cells occur through EMT regulation, as shown by the increased levels of E-cadherin and loss of vimentin, Snail in cancer stem cells (CSCs) (12). Research has demonstrated that EMT transcription factors are key to OA development (13). Here, we used TGF-β1-induced EMT in OA cells to confirm the role of melatonin and to explore new methods for OA treatment.

Materials and methods

Reagents. Melatonin, trypsin, MTT and Triton X-100 were purchased from Sigma Chemical Co./Merck KGaA. Dulbecco's

Table I. Primer sequences.

Genes	Forward primer	Reverse primer
Snail	AAGGCCTTCTCTAGGCCCT	CGCAGGTTGGAGCGGTCAG
HIF-1α	TTCCTTCTCTTCTCCGCGTG	ACTTATCTTTTTCTTGTCGTTCGC
MMP-9	TTGACAGCGACAAGAAGTGG	CCCTCAGTGAAGCGGTACAT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

HIF-1α, hypoxia-inducible factor 1-α; *MMP-9*, matrix metalloproteinase 9; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

modified Eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Thermo Fisher Scientific, Inc.); TGF-β1 was purchased from (R&D); YC-1 (cat. no. sc-202856) was purchased from Santa Cruz Biotechnology, Inc. Antibodies against MMP-9 (cat. no. sc-13520), E-cadherin (cat. no. sc-52327), N-cadherin (cat. no. sc-8424), vimentin (cat. no. sc-53464), Snail (cat. no. sc-10437), β-actin (cat. no. sc-69879) and HIF-1α (cat. no. sc-53546) were purchased from Santa Cruz Biotechnology, Inc. The ECL kit was purchased from Pierce/Thermo Fisher Scientific, Inc. RIPA buffer and the BCA protein assay kit were purchased from Beyotime. PVDF membranes were purchased from Millipore. All reagents used were trace element analysis grade. All water used was glass distilled.

Cell culture. OS MG-63 cells were purchased from the Shanghai Cell Bank (Shanghai, China). The cells were treated with DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 with 95% humidity. Cells were passaged at ~80% confluency.

Cell viability assays. MG-63 cells were seeded into 96-well plates at a density of $2x10^4$ cells/well and exposed to 0-1,000 nmol/l melatonin for 24 h. MTT reagent (10 μl) was added to each well and incubated for 4 h at 37°C. Reaction products were extracted with DMSO (150 μl) and absorbances were recorded at ~450 nm on a microplate reader (Bio-Rad Laboratories, Inc.).

Western blot analysis. MG-63 cells were lysed in RIPA buffer and BCA assays performed. Proteins (10 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% milk in TBS (containing 0.5% Tween-20) and probed with primary antibodies at 4°C overnight. The antibodies included: Anti-β-actin (dilution 1:400), anti-HIF-1α (dilution 1:400), anti-E-cadherin (dilution 1:400), anti-N-cadherin (dilution 1:400), anti-vimentin (dilution 1:400), anti-Snail (dilution 1:400), and anti-MMP-9 (dilution 1:400). After washing three times with TBS/0.1% Tween 20, the membranes were labeled with HRP-conjugated secondary antibodies (cat. no. sc-2030; dilution 1:1,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Immunoreactive bands were visualized using ECL. The intensity of the bands was quantified using Image Lab software (version 2.1, Bio-Rad Laboratories, Inc.). All blots were representative of three independent experiments.

Immunofluorescence. MG-63 cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 for 5 min and blocked in 10% AB-serum in 1% bovine serum albumin (BSA) for 30 min. Cells were then washed and stained with anti-E-cadherin primary antibodies (dilution 1:400) for 2 h at 37°C and incubated with TRITC-conjugated fluorescent secondary antibodies (cat. no. BA1089; dilution 1:100) for 30 min at room temperature. Nuclei were stained with Hoechst 33342 for 10 min and cell morphology was examined under an optical microscopy (magnification, x400; Olympus Corporation).

Transient transfections of Snail cDNA. Snail was cloned into pcDNA3.1 (Genechem Co.) and transiently transfected into MG-63 cells using Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific, Inc.). Cells were harvested 48 h post-transfection.

RT-PCR. Total RNA was extracted using TRIzol (Invitrogen/Thermo Fisher Scientific, Inc.) and reverse transcribed using SYBR PrimeScript RT-PCR kits (Takara Inc.) according to the manufacturer's protocol. cDNAs were amplified by polymerase chain reaction (PCR) using the primers shown in Table I. PCR reactions were performed using a Gene Amp PCR system 9700 (PerkinElmer). Amplified products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Images were quantified using FluoroImager SI (GE Healthcare). Representative results were shown (n=3).

Statistical analysis. All statistical analyses were performed using SPSS (version 19.0; IBM Corp.). Data are represented as the mean ± SD. One-way ANOVA test was used for statistical comparisons. If multigroup comparisons were made, then ANOVA was used together with Scheffe post-hoc test (n=5). $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGF-β1-mediated EMT in MG-63 cells. EMT is key to cancer progression and can be induced by TGF-β [14]. MG-63 cells were cultured with TGF-β1 (20 ng/ml) to assess its ability to induce EMT in OA cells [15] through the expression of known EMT markers including E-cadherin, vimentin and N-cadherin by western blot analysis (Fig. 1A) and RT-PCR (Fig. 1B). E-cadherin was downregulated, while N-cadherin and vimentin were significantly induced by TGF-β1 in a
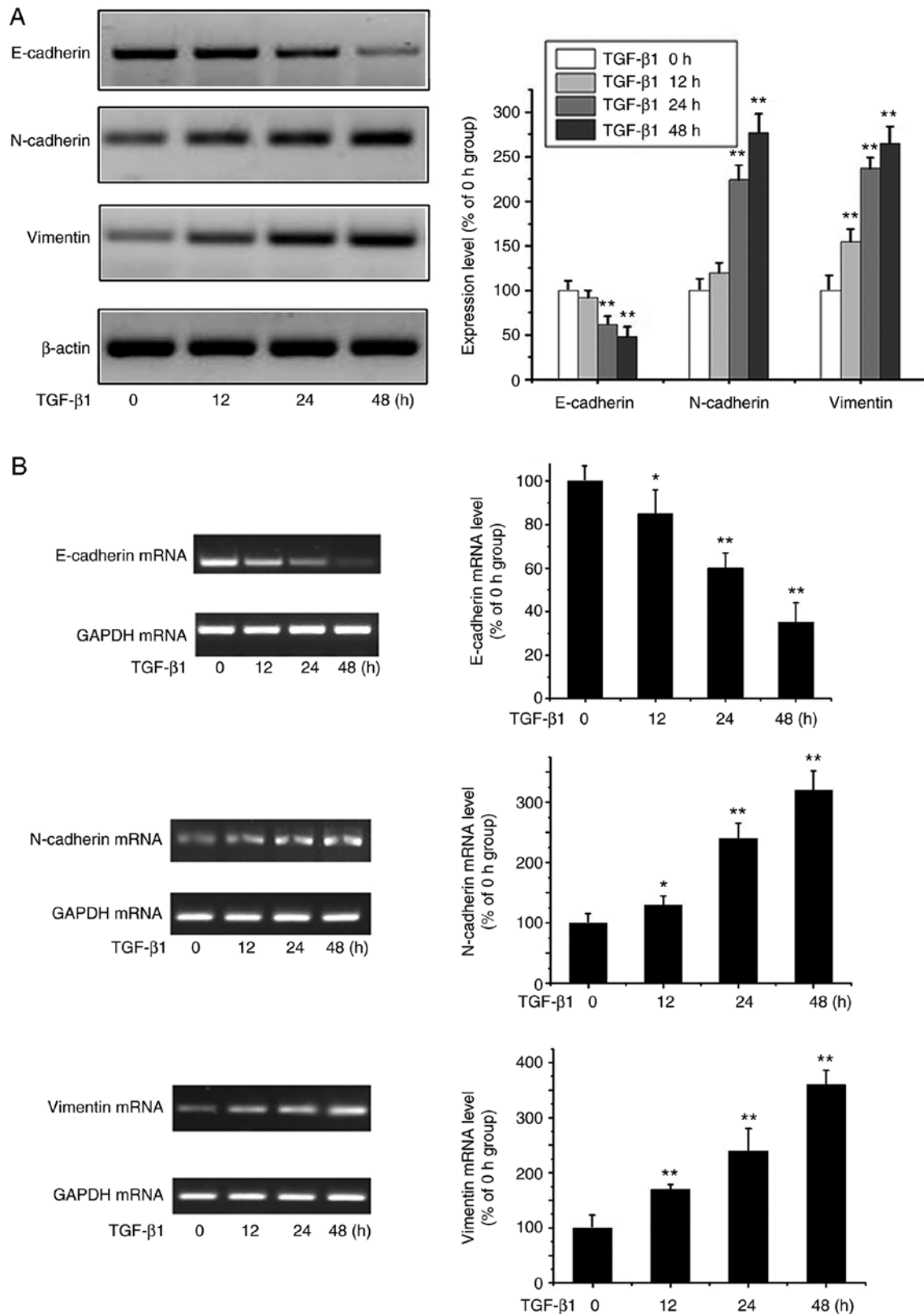
Figure 1. EMT is triggered by TGF-β1. (A) OA MG-63 cells were treated with TGF-β1 (20 ng/ml) for 0, 12, 24 and 48 h, and E-cadherin, N-cadherin, vimentin and β-actin were analyzed by western blot analysis. Data are presented as means ± SD of 3 independent experiments. β-actin was used as the loading control. **P<0.01 vs. the 0 h group. (B) OS MG-63 cells were treated with TGF-β1 (20 ng/ml) for 0, 12, 24 and 48 h, and the levels of E-cadherin, N-cadherin, vimentin were detected by RT-PCR. Data are presented as means ± SD (n=3). GAPDH was used as the loading control. *P<0.05 and **P<0.01 vs. the 0 h group. OA, osteosarcoma; EMT, epithelial-to-mesenchymal transition; TGF-β1, transforming growth factor β1.

time-dependent manner. These data suggested that TGF-β1 triggers EMT in OA cells.

Melatonin suppresses EMT in MG-63 cells. Previous studies have reported that melatonin inhibits tumor invasion through
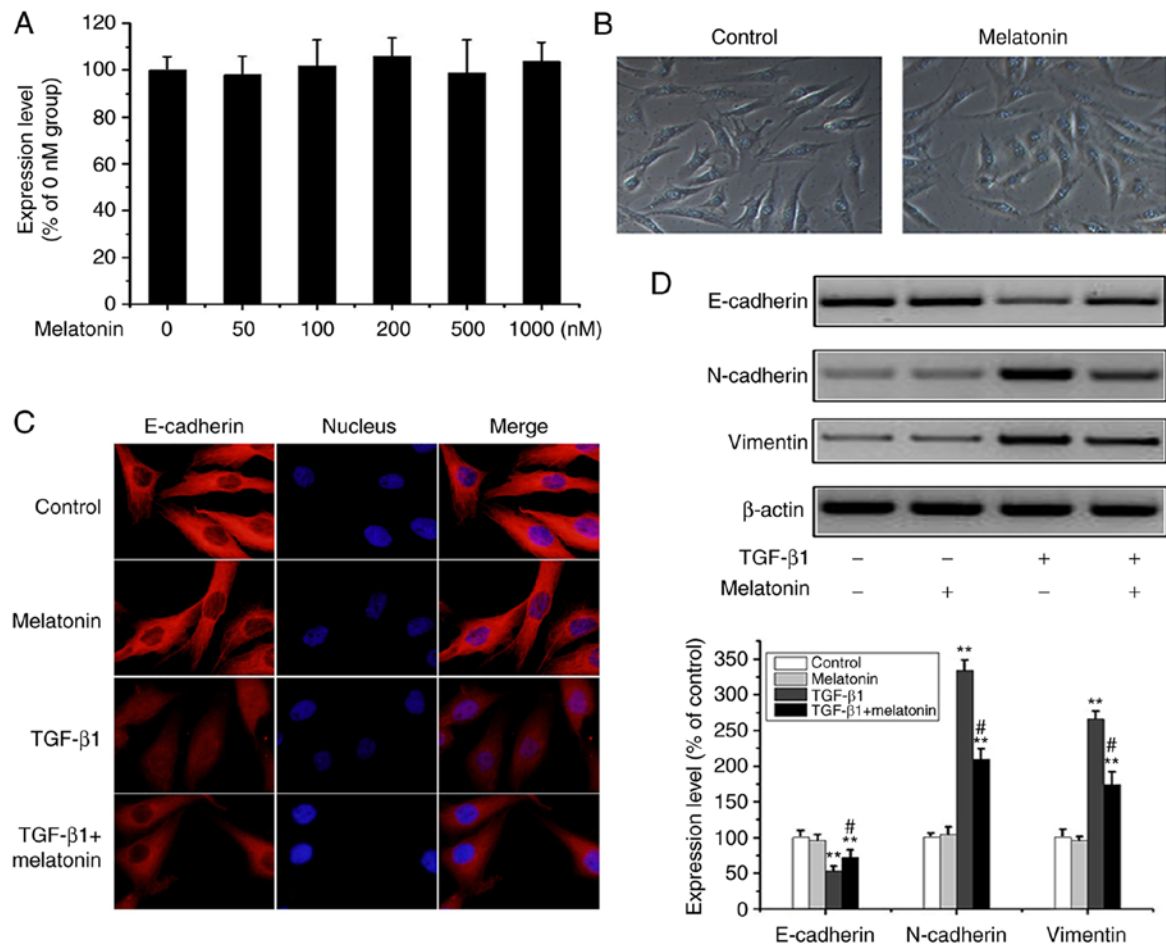
Figure 2. Melatonin reverts TGF-β1-mediated EMT in MG-63 cells. (A) OA MG-63 cells were treated with various doses of melatonin (0-1,000 nM) for 24 h, and cell viability was examined by MTT assay. Data are presented as means ± SD (n=3). (B) OS MG-63 cells were treated with 200 nM melatonin for 24 h, and cell morphology was observed under bright-field microscopy (magnification, x100). (C) MG-63 cells were cultured with 20 ng/m TGF-β1 in the presence or absence of 200 nM melatonin for 24 h, and E-cadherin was detected by fluorescence microscopy (magnification, x400). (D) Cells were treated as above, and E-cadherin, N-cadherin, vimentin and β-actin were detected by western-blot analysis. The results were representatives of three independent experiments. β-actin was used as loading control. **P<0.01 vs. the control group; #P<0.01, the TGF-β1 group vs. the TGF-β1 + melatonin group). OA, osteosarcoma; EMT, epithelial-to-mesenchymal transition; TGF-β1, transforming growth factor β1.

EMT inhibition (16) but its effects on OA cells are unclear. Through MTT assays, no significant changes were observed in cell survival rates for the different concentrations (0, 50, 100, 200, 500 and 1,000 nM) of melatonin (Fig. 2A). In melatonin-containing media, the morphology of the MG-63 cells was unchanged, and no apoptosis occurred (Fig. 2B). In subsequent experiments, 200 nM (intermediate concentration) of melatonin was used which had minimal effect on MG-63 cell survival. However, immunofluorescence and western blot analysis suggested that melatonin partially reversed the loss of E-cadherin expression and increase in N-cadherin and vimentin expression in response to TGF-β1 (Fig. 2C and D). These results show for the first time that melatonin can reverse EMT processes in OA cells.

Melatonin suppresses TGF-β1-mediated EMT through the downregulation of Snail/MMP-9 and HIF-1α. Extensive research indicates that the Snail/MMP-9 signaling plays a vital role in EMT and tumor metastasis (17). To further explore the underlying mechanism of the inhibitory effects of melatonin on TGF-β1-mediated EMT, Snail/MMP-9 signaling were analyzed using western blot analysis. Fig. 3A and B shows that the levels

of Snail and MMP-9 were upregulated in response to TGF-β1 in a time-dependent manner. In addition, TGF-β1 activated Snail/MMP-9 signaling while melatonin alone had no effects on Snail/MMP-9 activation. The addition of melatonin to TGF-β1-stimulated cells reversed the activation of Snail/MMP-9 signaling (Fig. 3C and D). Similarly, the effects of melatonin on HIF-1α expression suggested that melatonin attenuated TGF-β1 signaling through HIF-1α (Fig. 3E and F). Snail expression in response to TGF-β1 was markedly downregulated in cells pretreated with melatonin. Taken together, these data indicate that melatonin exerts its inhibitory effects in part by antagonizing Snail/MMP-9 and HIF-1α pathways in OA cells.

Snail overexpression prevents melatonin-mediated EMT suppression in MG-63 cells. The data obtained to this point suggested that Snail/MMP-9 signaling regulates EMT. To further investigate the effects of melatonin on Snail/MMP-9 signaling, Snail was overexpressed in MG-63 cells (Fig. 4A). Snail overexpression was coupled to a marked reduction in E-cadherin and increased expression of vimentin/N-cadherin. The melatonin-mediated suppression of EMT in MG-63 cells was attenuated through Snail overexpression (Fig. 4B).
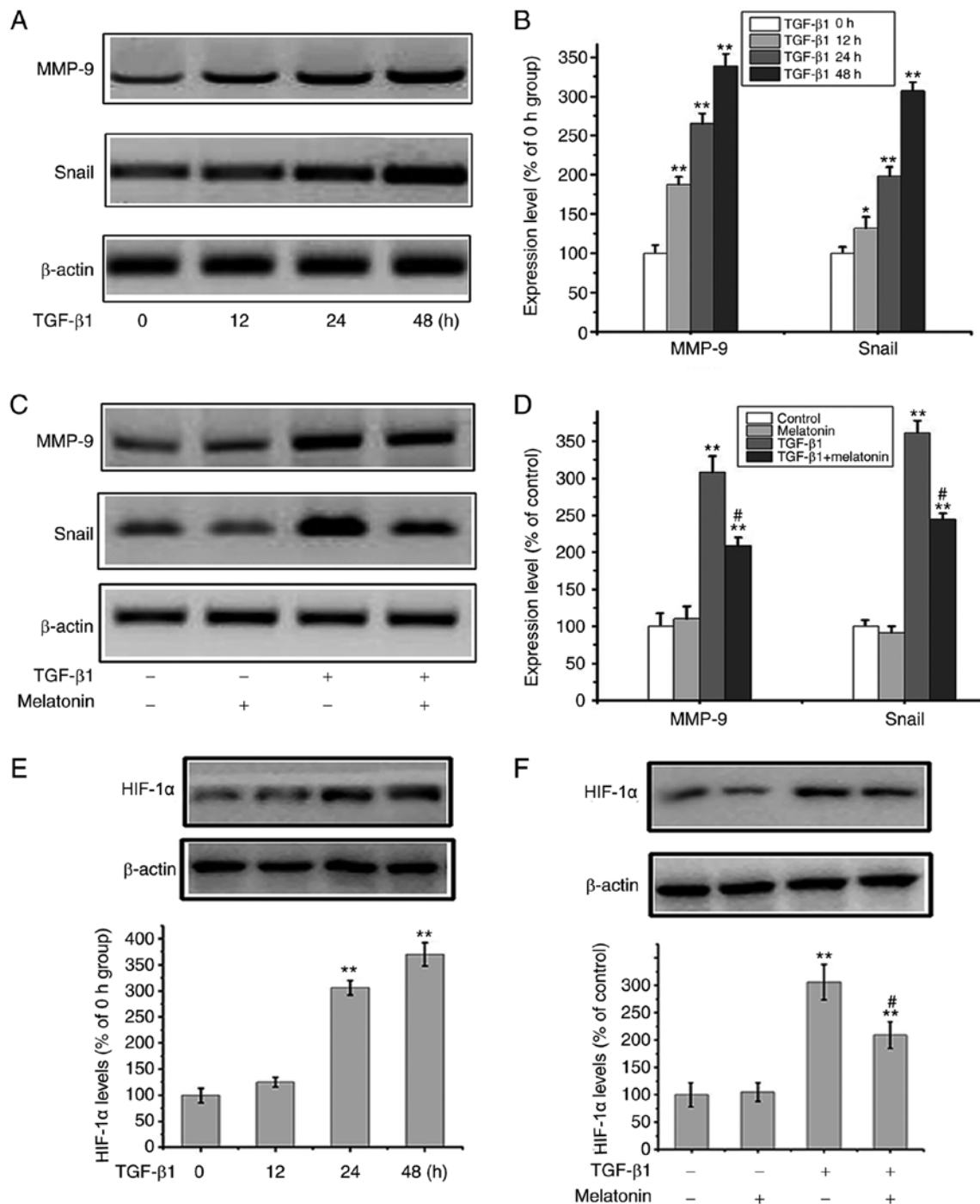
Figure 3. Melatonin suppresses the Snail/MMP-9 and HIF-1α pathway. (A and B) OA MG-63 cells were exposed to TGF-β1 (20 ng/ml) for 0, 12, 24 and 48 h, and then MMP-9, Snail and β-actin were assessed by western blot analysis. In B: Data are presented as means ± SD of 3 independent experiments. β-actin was used as the loading control. *P<0.05, **P<0.01 vs. the 0 h group. (C and D) MG-63 cells were cultured with 20 ng/m TGF-β1 in the presence or absence of 200 nM melatonin for 24 h, and MMP-9, Snail and β-actin were detected by western blot analysis. In D: Data are presented as means ± SD of 3 independent experiments. β-actin was used as the loading control. **P<0.01 vs. the control group; #P<0.01, TGF-β1 group vs. the TGF-β1 + melatonin group. (E) MG-63 cells were exposed to TGF-β1 (20 ng/ml) for 0, 12, 24 and 48 h, and HIF-1α and β-actin were assessed by western blot analysis. Data are presented as means ± SD of 3 independent experiments. **P<0.01 vs. the 0 h group. (F) MG-63 cells were cultured with 20 ng/m TGF-β1 in the presence or absence of 200 nM melatonin for 24 h, and HIF-1α and β-actin were detected by western blot analysis. Data are presented as means ± SD of 3 independent experiments. **P<0.01 vs. the control group; #P<0.01, TGF-β1 group vs. the TGF-β1 + melatonin group. OA, osteosarcoma; HIF-1α, hypoxia-inducible factor 1α; MMP-9, matrix metalloproteinase 9; TGF-β1, transforming growth factor β1.

These data further confirmed that melatonin suppresses Snail/MMP-9 signaling to inhibit EMT in OA cells.

HIF-1α inhibition reverses the TGF-β1-induced upregulation of Snail/MMP-9. HIF-1α can induce EMT and metastasis in cancer cells (18). Next, it was ascertained whether a loss of HIF-1α negatively affects the Snail/MMP pathways. As shown in Fig. 5, the HIF-1α inhibitor YC-1 not only downregulated HIF-1α expression, but markedly inhibited the upregulation of Snail and MMP-9 in response
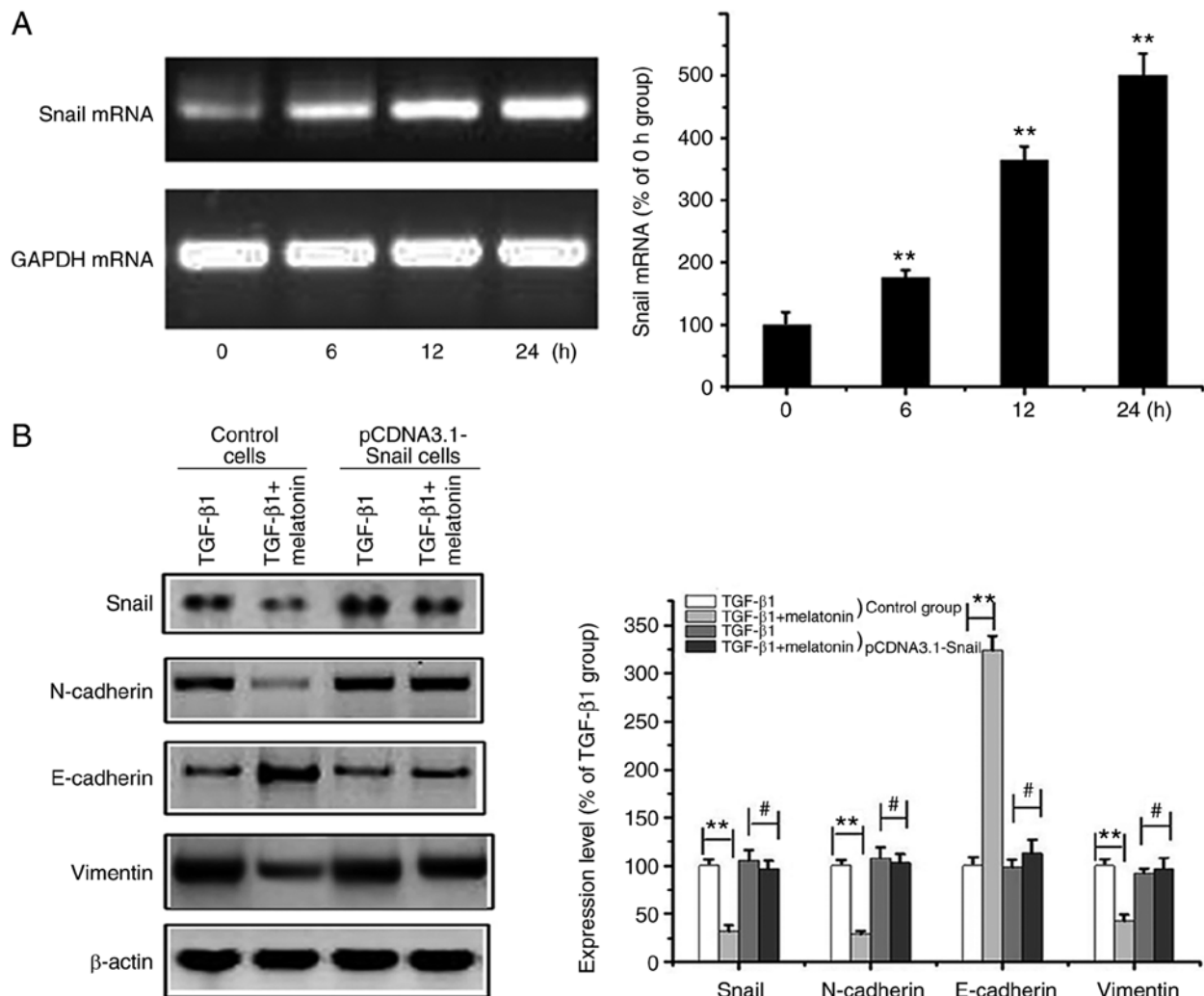
Figure 4. Overexpression of Snail reverses melatonin-mediated suppression of EMT in MG-63 cells. (A) OA MG-63 cells were transfected with the Snail-pcDNA3.1 plasmid and the Snail mRNA level at different time intervals (0, 6, 12, 24 h) was determined by RT-PCR. GAPDH was used as the loading control. Data are represented as mean ± SD (n=5). **P<0.01 vs. the 0 h group. (B) Control and Snail-overexpressing cells (pCDNA3.1-Snail) (after transfection for 24 h) were exposed to 20 ng/m TGF-β1 in the presence or absence of 200 nM melatonin for 24 h, and Snail, E-cadherin, N-cadherin, vimentin and β-actin were measured by western blot analysis. Data are presented as means ± SD of 3 independent experiments. β-actin was used as the loading control. **P<0.01 control cells: TGF-β1 group vs. the TGF-β1 + melatonin group; #P>0.05 pcDNA3.1-Snail cells: TGF-β1 group vs. the TGF-β1 + melatonin group). OA, osteosarcoma; EMT, epithelial-to-mesenchymal transition; TGF-β1, transforming growth factor β1.

to TGF-β1. These data provide evidence that HIF-1α activates Snail/MMP-9 expression and that inhibition of HIF-1α attenuates EMT in MG-63 cells.

Discussion

Previous studies have confirmed that epithelial-to-mesenchymal transition (EMT) is a key stage in the transdifferentiation of epithelial cells and plays a central role in disease progression, wound healing, fibrosis and cancer (19,20). It is generally believed that the EMT phenomenon only occurs in epithelial-derived cells. However, recent studies have shown that certain mesenchymal cells can also alter EMT-related protein and enhance the metastasis process (21,22). Osteosarcoma (OA) is the most common bone malignant tumor of mesenchymal origin. In an OA cell line, the cells were found to regulate EMT-related protein expression and enhance invasion and metastasis, which suggested that EMT is not only the key step in epithelium-derived tumor cells but also in

mesenchymal cell-derived OA (23,24). Thus, targeting EMT represents a key therapeutic goal for OA treatment (25). In recent years, melatonin has emerged as a key molecule for the prevention and management of cancer due to its limited cytotoxicity and/or side effects. The roles of melatonin in OA however, remain largely uncharacterized.

Melatonin isolated from the bovine pineal has numerous physiological functions including the control of the circadian rhythm, sleep-wake rhythms, body temperature, neuronal protection and immune activation (26-28). Melatonin has strong therapeutic potential for various cancers including prostate, breast and ovarian cancer (29,30). Recent studies have demonstrated that melatonin treatment increases apoptosis in breast cancer cells (31). It has also been reported that in thyroid cancer, melatonin inhibits p65 phosphorylation and subsequent redox stress (32). Melatonin also exerts anticancer effects by indirectly regulating the body's immune system (33). Although an array of mechanisms have been proposed, few studies have evaluated the role of melatonin on
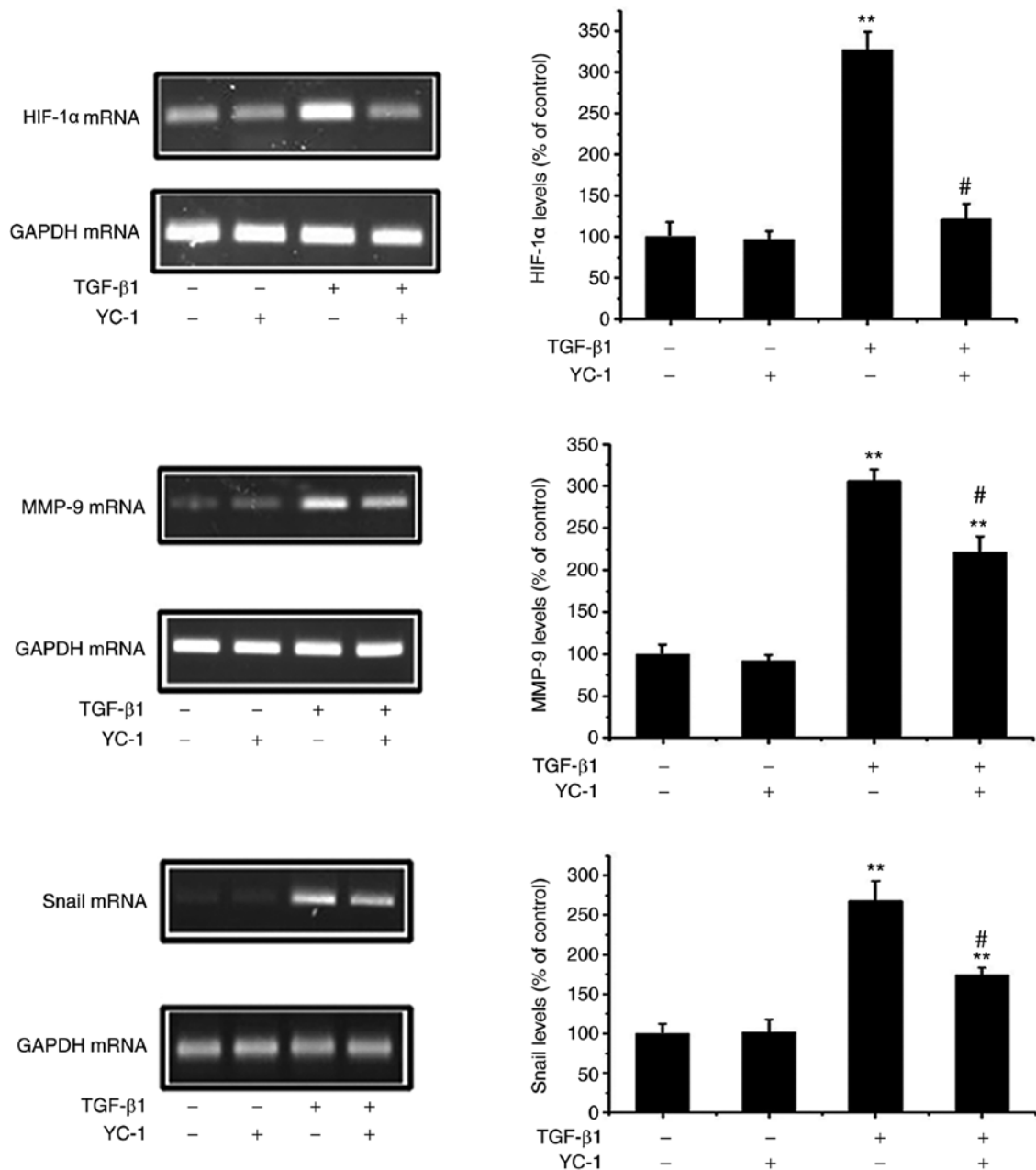
Figure 5. YC-1 reverts TGF-β1-mediated upregulation of MMP-9 and Snail in OA MG-63 cells. Exposure of control cells to TGF-β1 (20 ng/ml) and YC-1 (30 μmol/l) for 24 h and the levels of HIF-1α, MMP-9 and Snail were detected by RT-PCR. GAPDH was used as the loading control. Data are presented as mean ± SD (n=5). **P<0.01 vs. the control; #P<0.01, TGF-β1 group vs. the TGF-β1 + YC-1 group. OA, osteosarcoma; MMP-9, matrix metalloproteinase 9; TGF-β1, transforming growth factor β1.

EMT. Similarly, the anticancer potential of melatonin on OA cells is undefined.

In the present study, the role of melatonin in inhibiting TGF-β1-mediated EMT was investigated and the signaling pathways involved in this regulation were explored. Our findings suggested that melatonin pretreatment provides effective protection against TGF-β1-mediated EMT as evidenced by the downregulation of N-cadherin and vimentin and the increased expression of E-cadherin in MG-63 cells. The mechanisms of these effects were next explored.

Snail regulates EMT and plays a crucial role in tumor invasion and metastasis (34,35). Naber *et al* reported that TGF-β is pro-invasive through its activation of transcriptional repressors (including Slug and Snail) thus inducing

EMT (36). In this study, it was demonstrated that melatonin inhibits TGF-β1-induced Snail expression in MG-63 cells. Melatonin exerted its inhibitory effects in part by antagonizing Snail/MMP-9 signaling in OA cells. Moreover the overexpression of Snail prevented EMT suppression in response to melatonin. Thus, targeting EMT and inhibiting Snail/MMP-9 signaling represents a promising strategy to prevent metastasis and improve the survival of OA patients.

Melatonin suppresses the viability and angiogenesis of cancer cells through the downregulation of HIF-1α/ROS/VEGF in solid tumors containing abundant blood vessels (37). HIF-1α also serves an important role in EMT processes and tumor metastasis (38). Our results demonstrated that melatonin inhibits HIF-1α expression which is stimulated by TGF-β1 in MG-63

cells. We next studied the effects of HIF-1α on Snail/MMP-9 signaling. YC-1 inhibited TGF-β1-mediated EMT in MG-63 cells through its ability to inhibit HIF-1α signaling. This demonstrated that melatonin inhibits Snail/MMP-9 signaling in response to TGF-β1 via inhibiting HIF-1α expression.

In summary, the present study demonstrated that melatonin attenuates TGF-β1-mediated EMT in MG-63 cells by preventing TGF-β1-induced activation of the Snail/MMP-9 and HIF-1α signaling pathways. These findings provide new insight into the mechanisms by which melatonin prevents the development and invasion of OA. These findings also provide experimental evidence for the development of new strategies for OA treatment.

Acknowledgements

Availability of data and materials

The datasets used and/or anlayzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and TZ conceived and designed the study. XL, ZL, DZ, WX and YC performed the experiments. TZ and ZL wrote the paper. YC and WX reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of Baotou Medical College (Baotou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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