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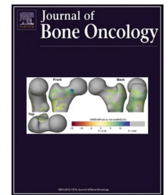

Research Paper

Mometasone furoate inhibits tumor progression and promotes apoptosis through activation of the AMPK/mTOR signaling pathway in osteosarcoma

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HIGHLIGHTS

- This study focuses on the antitumor effect of mometasone furoate on osteosarcoma and the related molecular mechanism.
- After a series of experiments, we found that mometasone furoate can inhibit osteosarcoma proliferation and metastasis and promote osteosarcoma cell apoptosis through the AMPK/mTOR signaling pathway *in vitro* and *in vivo*.
- In addition, mometasone furoate was found to significantly inhibit osteosarcoma progression *in vitro* in a dose-dependent manner, and it had no negative effect on the internal organs in animal models.
- Therefore, we think our study can provide a new rationale for subsequent academic and clinical research on osteosarcoma treatment.

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ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor in adolescents. While treatments for osteosarcoma have improved, the overall survival has not changed for three decades, and thus, new targets for therapeutic development are needed. Recently, glucocorticoids have been reported to have antitumor effects. Mometasone furoate (MF), a synthetic glucocorticoid, is of great value in clinical application, but there are few reports on its antitumor effect. Here, we verified the effect of MF on osteosarcoma *in vitro* and *in vivo*. *In vitro*, cell proliferation, cell cycle progression, apoptosis and cell metastasis were detected using Cell Counting Kit-8 (CCK-8), colony formation, flow cytometry, wound-healing and transwell assays, respectively. *In vivo*, we generated a xenograft mouse model. To examine the potential role of the AMPK pathway, an AMPK-specific inhibitor (dorsomorphin) was used. The expression levels of factors related to the cell cycle, apoptosis and activation of the AMPK/mTOR pathway were assessed by immunohistochemistry and Western blotting. MF inhibited proliferation and metastasis and induced S phase arrest and apoptosis in osteosarcoma cells in a dose-dependent manner. *In vivo*, MF effectively inhibited osteosarcoma cell growth and pulmonary metastasis; however, it had no negative effect on the internal organs. Additionally, MF could activate the AMPK/mTOR pathway in osteosarcoma. Dorsomorphin significantly attenuated MF-induced antitumor activities. In summary, MF can inhibit osteosarcoma proliferation and metastasis and promote osteosarcoma cell apoptosis through the AMPK/mTOR signaling pathway *in vitro* and *in vivo*, which can provide a new rationale for subsequent academic and clinical research on osteosarcoma treatment.

Abbreviations: AMPK, AMP-activated protein kinase; mTOR, the mammalian target of rapamycin; MF, mometasone furoate; ATCC, the American Type Culture Collection; CCK-8, Cell Counting Kit-8; ALL, acute lymphoblastic leukemia; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; CDK2, cyclin-dependent kinase 2; BCA, the bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; IHC, immunohistochemistry; H&E, hematoxylin and eosin; CDKs, cyclin-dependent kinases; AMP, adenosine monophosphate; ATP, adenosine triphosphate; mTORC1, the mechanistic target of rapamycin complex 1.

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1. Introduction

Osteosarcoma, derived from primitive mesenchymal cells, is the most frequent malignant bone and soft tissue tumor in children and adolescents, especially at 15–19 years of age [1]. In addition, osteosarcoma shows a predilection for children and adolescents and arises principally in the metaphysis of long bones, which suggests a possible relationship between osteosarcoma onset and active osteogenic activities. Surgical resection and multiagent chemotherapy are the regular therapeutic interventions in osteosarcoma [1,2]. The 5-year survival rate of localized osteosarcoma patients has reached approximately 67 %, while it still remains at 20 % in metastatic cases [3]. Patient survival has not improved significantly in recent decades [4,5]. Thus, it is urgent to find new therapy methods for osteosarcoma.

Glucocorticoids are the main therapy for inflammatory and autoimmune diseases and are often applied to help cancer patients tolerate treatment [6]. However, its role as a curative agent in cancer is still controversial. Glucocorticoids are routinely used to treat lymphoma and leukemia [7,8]. It was reported that glucocorticoids can also be used to cure some solid tumors, such as colon cancer and prostate cancer [9,10]. Mometasone furoate (MF) is a synthetic glucocorticoid that has a high-potency inhibitory effect on inflammatory reactions. It has been reported that MF is very effective in reducing symptoms in chronic rhinosinusitis, persistent asthma and adenoid hypertrophy [11–13]. Although MF has been proven to have the effect of inhibiting the growth of acute lymphoblastic leukemia (ALL[2]) cells in childhood [14], the role of MF in osteosarcoma cells is still unknown.

Here, we demonstrated that MF could inhibit the proliferation and metastasis of osteosarcoma cells both *in vitro* and *in vivo*. Further molecular mechanisms revealed that MF exerted antitumor activity via the AMPK/mTOR signaling pathway. Thus, our findings suggest that MF may be a novel promising drug candidate for osteosarcoma.

2. Materials and methods

2.1. Cell lines and cell culture

The human osteosarcoma cell lines (MNNG-HOS, MG63 and U2OS) were obtained from the American Type Culture Collection (ATCC[3]) (Manassas, VA, USA). MNNG-HOS and MG63 cells were cultured in Dulbecco's modified Eagle's medium high glucose medium (HyClone, Logan, UT, USA) with 10 % fetal bovine serum (Yeasen, Co., Ltd., Shanghai, China). U2OS cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10 % fetal bovine serum (Yeasen, Co., Ltd., Shanghai, China). They were maintained at 37 °C in a humidified atmosphere of 5 % CO_2.

2.2. Materials and reagents

MF was purchased from Selleck Chemicals, and a stock solution was prepared at a 10 mM concentration in DMSO[4] (Solarbio, Beijing, China) and stored at −80 °C. Dorsomorphin 2HCl (Compound C) was purchased from Selleck Chemicals. A stock solution was prepared at a 10 mM concentration in H_2O and stored at −80 °C. Antibodies against cyclin A2, cyclin E1, cyclin D2, CDK2,[5] Bax, caspase-7, cleaved caspase-3 and the secondary antibodies (anti-rabbit and anti-mouse) were purchased from Proteintech. Antibodies against caspase-9, AMPK, p-AMPK, mTOR and p-mTOR were purchased from Cell Signaling Technology. Antibodies against Bad and Bak were purchased from Abcam. An antibody against β-actin was purchased from Abbkine.

[2] acute lymphoblastic leukemia.
[3] the American Type Culture Collection
[4] dimethyl sulfoxide.
[5] cyclin-dependent kinase 2.

2.3. Cell viability assay

Osteosarcoma cells were seeded in 96-well plates for 24 h and then incubated with different concentrations of MF for 48 h. After treatment, cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay. Then, SPSS and GraphPad software were used to calculate the IC25 and IC50 of MF in osteosarcoma cells.

2.4. Cell proliferation assay

Then, the cells (5000 cells/well) were seeded in 96-well plates for adherence and treated with the IC25 and IC50 of MF. Cell viability was evaluated by CCK-8 assay on Days 1–5. The cell growth curve was described according to the absorbance values.

2.5. EdU staining assy

After treatment with MF, osteosarcoma cells were incubated with 10 μM EdU working solution for 2 h at 37C. Then, the cells were fixed with 4 % paraformaldehyde for 15 min, incubated with 0.1 % Triton-X100 for 10 min. After that, EdU and Hoechst staining were followed by manufacturer's protocol (Beyotime Biotech, Nanjing, China). The images were taken and quantified to measure the number of EdU positive cells.

2.6. Colony formation assay

Osteosarcoma cells were cultured in 6-well plates for adherence and treated with the IC25 and IC50 of MF for 48 h. Then, the cells were digested and seeded in 6-well plates (1000 cells/well) in 2 mL culture medium. After 10 days, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet. The images were taken and quantified to measure the number of colonies.

2.7. Wound-healing assay

Osteosarcoma cells were cultured in 6-well plates for adherence and treated with the IC25 and IC50 of MF for 48 h. Then, the cells were digested and seeded in 6-well plates (3×10^5 cells/well) in 2 mL culture medium. When the cells grew to 90 %, the cell monolayer was scratched with a sterile 200 μL pipette tip. After washing with PBS,[6] the culture medium was changed to 0.1 % FBS culture medium containing MF. The wound coverage was recorded by microscopy at 0 h, 12 h and 24 h. The migrated cells were quantified by ImageJ software, and the percentage of migration was then calculated.

2.8. Transwell assays

Osteosarcoma cells were cultured in 6-well plates for adherence and treated with the IC25 and IC50 of MF. After 48 h, the cells were digested and made into a cell suspension. For the migration assay, a total of 5×10^4 cells suspended in 200 μL serum-free culture medium were plated into the upper chambers. For the invasion assay, 1×10^5 cells were seeded into the upper chambers, which were coated with 60 μL Matrigel diluted with serum-free culture medium in advance. Then, the upper transwell chamber was placed into a 24-well culture plate, with 800 μL of culture medium in the lower chambers. After incubation, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet.

2.9. Flow cytometry analysis

The cells were placed in a 6-well plate and treated with different concentrations of MF for 48 h. After treatment, the cells were collected,

[6] phosphate buffered saline.

fixed and stained according to the manufacturer's protocol (Beyotime Biotech, Nanjing, China). The gating strategy for apoptotic cells detection was followed by the manufacturer's protocol. Finally, the samples were detected by flow cytometry.

2.10. Western blotting analysis

Cells and tissues were lysed in Protein Extraction Reagent (Thermo Fisher, Waltham, USA) with a cocktail of proteinase and phosphatase inhibitor (Roche Applied Science, Indianapolis, USA) for 30 min, and the lysates were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was collected, and total protein concentrations were measured by a bicinchoninic acid (BCA[7]) protein assay kit (Thermo Fisher, Waltham, USA). Equal amounts of total protein were separated by SDS–PAGE[8] and transferred to polyvinylidene difluoride (PVDF[9]) membranes (Millipore, Billerica, MA, USA). The membrane was blocked and incubated with the primary antibodies and the secondary antibodies. Finally, signals were detected using an enhanced chemiluminescence (ECL[10]) detection system and recorded by a Tanon 5200 automatic chemiluminescence image analysis system.

2.11. ATP assay

Osteosarcoma cells were cultured in 6-well plates for adherence and treated with the IC25 and IC50 of MF. After 48 h, Intracellular ATP concentrations were assayed with an ATP assay kit (Beyotime Biotech, Nanjing, China). The cells were lysed and the proteins were discarded. Then, intracellular ATP contents were detected by chemiluminescence, using an ATP standard to create a calibration curve.

2.12. Animal experiment

All animal rearing and experimental procedures were performed in strict accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) of the Sixth People's Hospital Affiliated to Shanghai Jiaotong University. This study was performed under IACUC (Animal Welfare Ethics acceptance number No:DWLL2022-0435, May 13, 2022) approval. Forty 4-week-old BALB/c female nude mice were used in this experiment. All nude mice were purchased and raised in the specific-pathogen-free (SPF) animal laboratory. For the subcutaneous tumor model, 200 μL of MNNG-HOS cell suspension at a density of 5×10^6/mL was injected subcutaneously into the right scapula of nude mice. When the subcutaneous tumor was visible, the nude mice were randomly divided into 3 groups (8 per group): (1) control group (0.9 % NaCl); (2) MF-treated group (MF: 25 mg/kg/day); and (3) MF-treated group (MF: 50 mg/kg/day). For the lung metastasis model, 100 μL of MNNG-HOS cell suspension at a density of 2×10^7/mL was injected into nude mice through the tail vein. After one week, the nude mice were randomly divided into 2 groups (8 per group): (1) the control group (0.9 % NaCl) and (2) the MF-treated group (MF: 50 mg/kg/day). The body weight and tumor volume were measured every 3 days, and the nude mice were euthanized on the 27th/33th day of administration. The subcutaneous tumor/lung was weighed and fixed with 4 % paraformaldehyde for H&E[11] staining or immunohistochemistry. Part of the tumor tissue was preserved in liquid nitrogen to perform Western blotting. The heart, liver, spleen, lung and kidney were fixed and stained with H&E.

[7] the bicinchoninic acid.
[8] sodium dodecyl sulfate polyacrylamide gel electrophoresis.
[9] polyvinylidene difluoride.
[10] enhanced chemiluminescence.
[11] hematoxylin and eosin

2.13. Statistical analysis

All data are presented as the mean ± SEM from at least three independent experiments. The statistical significance of differences between the control and MF-treated groups was analyzed by Student's *t* test or analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Statistical analyses were performed with SPSS version 26.0 software.

3. Results

3.1. MF reduces osteosarcoma cell viability and inhibits osteosarcoma cell proliferation in a dose-dependent manner

The CCK-8 assay was conducted to detect the impact of MF on the viability of osteosarcoma cell lines (MNNG-HOS, MG63, U2OS). The three cell lines were treated with MF at a series of concentrations for 48 h. As shown in Fig. 1A, MF caused a significant decrease in osteosarcoma cell viability in a concentration-dependent manner. The IC50 and IC25 were calculated and chosen for the subsequent experiments. The IC50 and IC25 for MNNG-HOS cells were 30.26 μM and 25.974 μM, respectively. The IC50 and IC25 for MG63 cells were 36.86 μM and 26.423 μM, respectively. The IC50 and IC25 for U2OS cells were 20.92 μM and 16.909 μM, respectively (Fig. 1E). Then, to evaluate cell proliferation, the CCK-8 assay, EdU staining and the colony formation assay were performed. The CCK-8 results showed that MF significantly inhibited the growth of osteosarcoma cells (Fig. 1B). Incorporation of EdU indicated that MF could inhibit the proliferation of osteosarcoma cells (Fig. 1C,D). Furthermore, the colony formation assay results showed that compared with the control group, the number of colonies decreased in groups treated with IC25 and IC50 of MF (Fig. 1F, G). More importantly, the IC50 of MF exhibited a stronger antitumor effect than the IC25 of MF. Thus, these results demonstrated that MF exhibited a suppressive effect in osteosarcoma cells in a concentration-dependent manner *in vitro*.

3.2. MF reduces the metastatic potential of osteosarcoma cells in vitro

First, the migration and invasion abilities were measured by transwell assays. Following treatment with IC25 and IC50 doses of MF for 48 h, identical numbers of osteosarcoma cells were harvested and seeded into the upper chamber. The results in Fig. 2A and B revealed that when treated with MF, the number of migrated cells had significantly dropped with or without Matrigel in the upper chamber. This indicated that MF could suppress the migration and invasion of osteosarcoma cells. Similarly, findings from the wound healing assay also showed that compared with the control group, the MF treatment groups had a lower wound healing rate, illustrating that MF impaired the metastatic potential in osteosarcoma cells (Fig. 2C, D). In addition, the group with a higher concentration of MF obtained a more obvious antimetastatic effect. In summary, MF reduces the metastatic potential of osteosarcoma cells *in vitro*.

3.3. MF induces cell cycle arrest and promotes cell apoptosis in vitro

Next, we examined the cell cycle distribution and apoptosis initiation changes in response to MF treatment. Flow cytometry was used to evaluate cell cycle distribution. The results showed that exposure to MF resulted in a marked increase in the proportion of cells in the S phase. This means that MF can delay cell cycle progression by accumulation of cells in S phase (Fig. 3A, C). Correspondingly, the expression of cyclin A2, cyclin E1, cyclin D2 and CDK2, S phase-related proteins, decreased after treatment with MF, as analyzed by Western blotting (Fig. 3E). When treated with MF, the number of floating cells was observed to increase in a dose-dependent manner. Thus, whether MF could induce cell apoptosis was detected by flow cytometry. The findings revealed that MF induced higher apoptosis rates, and the Western blotting results

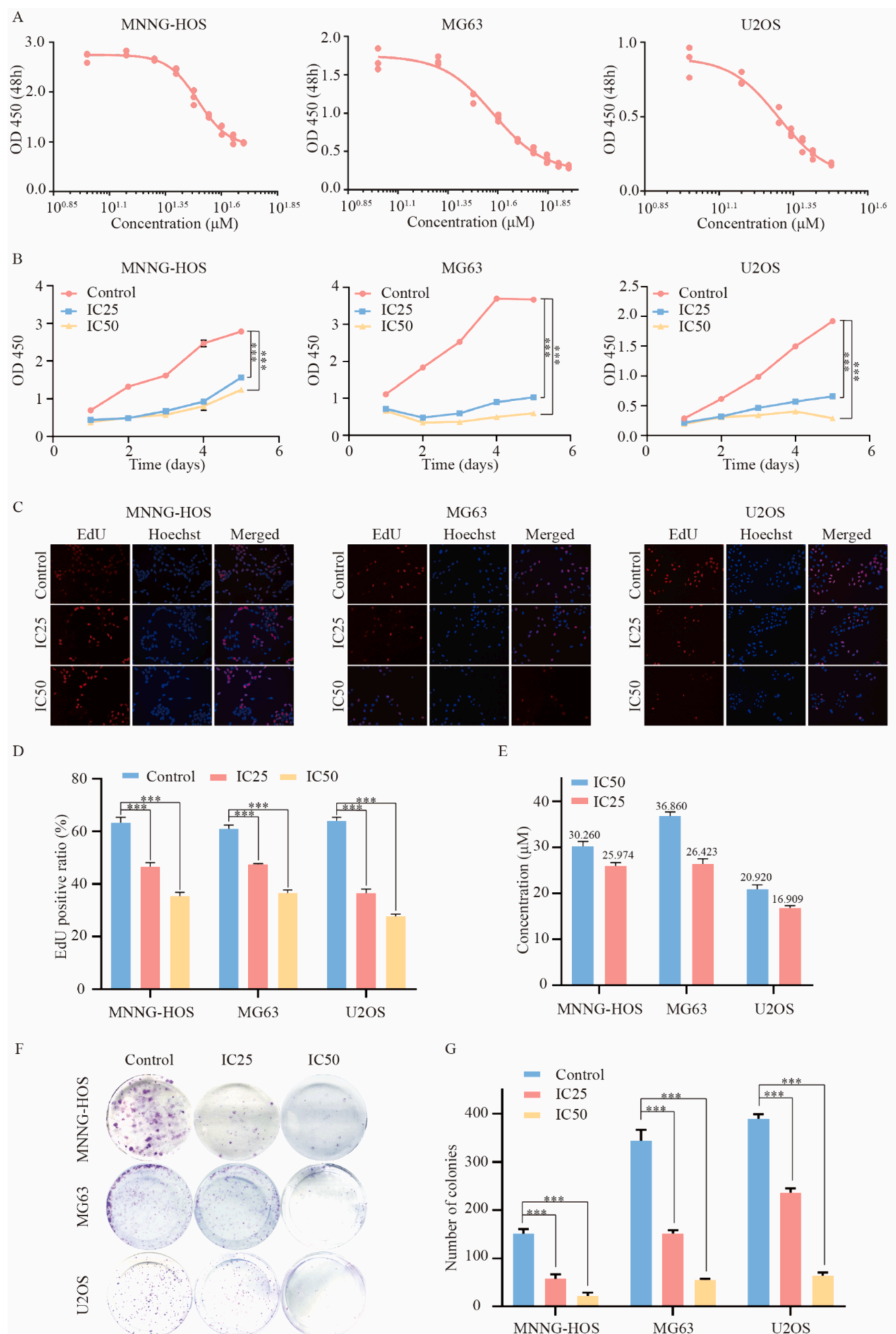

Fig. 1. MF inhibited the proliferation of osteosarcoma *in vitro*. (A) Osteosarcoma cells were treated with MF at various concentrations for 48 h. Cell viability was assessed by CCK-8 assay. (B) Proliferation of osteosarcoma cells was detected by CCK-8 assay. (C) Representative images showing EdU staining in osteosarcoma cells treated with MF. (D) The ratio of EdU-positive osteosarcoma cells was analyzed. (E) The IC50 and IC25 values of MF for 48 h in three osteosarcoma cell lines. (F) A colony formation assay of osteosarcoma cells was performed after MF treatment. (G) The number of colonies in different groups was analyzed. Statistical analysis was performed using ANOVA. Error bars represent the SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

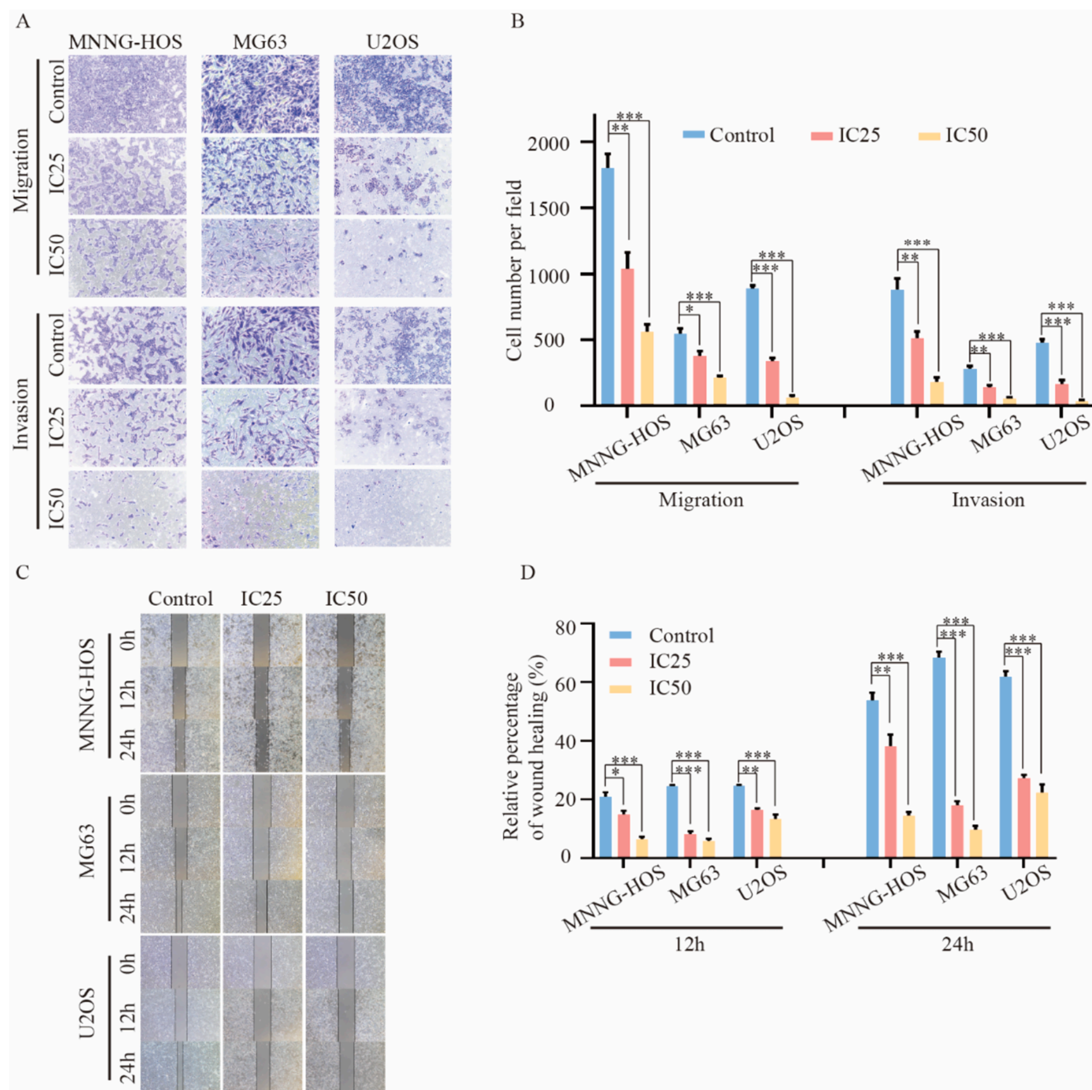

Fig. 2. Metastasis was affected by MF in osteosarcoma. (A) Transwell assay in osteosarcoma cells after treatment with MF (magnification, ×200). (B) The cell number per field in the lower chamber is shown. (C, D) A wound-healing assay was performed at 0, 12, and 24 h after treatment with MF (magnification, ×200), and the wound distance of migration was calculated. Statistical analysis was performed using ANOVA. Error bars represent the SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

showed increased protein expression of caspase-9, cleaved caspase-3, cleaved caspase-7, Bad, Bak and Bax in all three cell lines (Fig. 3B, D and E). Taken together, these findings indicated that MF could induce accumulation of cells in S phase and promote apoptosis in osteosarcoma cells *in vitro*.

3.4. MF inhibits the growth and metastasis of osteosarcoma cells in vivo

To examine the effects of MF on osteosarcoma growth and metastasis *in vivo*, we established a subcutaneous transplantation model and a lung metastasis model by transplanting MNNG-HOS cells into nude mice subcutaneously or injecting them into the lateral tail veins of nude mice. A schematic diagram was used to illustrate the procedures mentioned above (Fig. 4A). The mice were randomized into 3 groups and treated with intraperitoneal injection daily (0.9 % NaCl, Mometasone Furoate: 25 mg/kg/day and 50 mg/kg/day) in the subcutaneous transplantation model. As shown in Fig. 4B and D, tumor volumes were markedly inhibited by MF, and the group treated with 50 mg/kg MF received a more obvious suppressive effect than the group treated with 25 mg/kg MF. In addition, the tumor growth rate in the MF-treated groups was lower than that in the control group (Fig. 4B). The tumor weight in the MF-treated groups was lighter as well (Fig. 4C). A lung metastasis model was established to investigate whether MF could restrain osteosarcoma cell metastasis *in vivo*. The results showed that MF could significantly inhibit lung metastasis with more pulmonary metastatic nodules macroscopically and a higher level of lung weight in the control group (Fig. 4E, G). The histological analysis showed that the MF-treated group had a lower number of lung metastatic nodules (Fig. 4H). Interestingly, we observed a decrease in body weight in the mice treated with MF in the subcutaneous tumor model, while no significant change in body weight was noted in the control group. However, this phenomenon was not observed in the lung metastasis model (Fig. 4F, I). Taken together, we concluded that MF dramatically hindered tumorigenesis and lung metastasis of osteosarcoma cells *in vitro* and *in vivo*.

3.5. MF exerted antitumor effects through activation of the AMPK/mTOR signaling pathway in osteosarcoma

Activation of the AMPK/mTOR signaling pathway is regularly associated with apoptosis in different kinds of tumors [15–18]. AMPK and mTOR are important molecules in tumorigenesis and tumor development, so further experiments were conducted to investigate whether MF induced osteosarcoma cell apoptosis by regulating the AMPK/mTOR signaling pathway. Notably, the Western blotting results indicated that

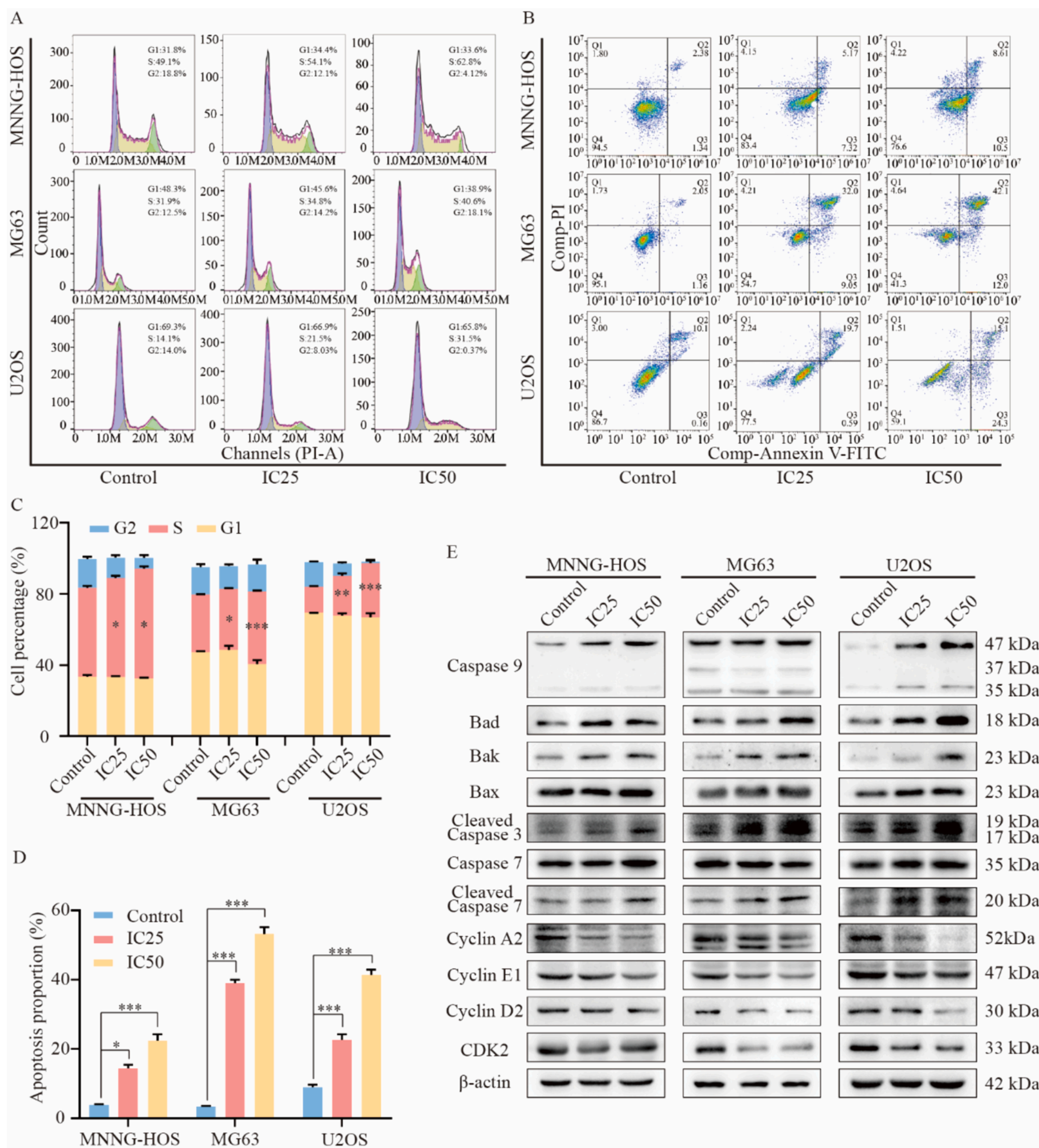

Fig. 3. MF induced cell cycle arrest and promoted apoptosis of osteosarcoma cells.(A, C) MF caused accumulation of cells in S phase as revealed by flow cytometry. (B, D) The proportion of apoptotic cells is shown. (E) Western blotting analysis of caspase-9, cleaved caspase-3, cleaved caspase-7, Bad, Bak, Bax, cyclin A2, cyclin E1, cyclin D2 and CDK2 expression in each group. β-actin was used as a control. Statistical analysis was performed using ANOVA. Error bars represent the SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

MF significantly increased the phosphorylation of AMPK and decreased the phosphorylation of mTOR without affecting the expression of AMPK and mTOR (Fig. 5A). To explore the mechanism by which MF activates the AMPK/mTOR pathway, we measured the ATP levels after MF treatment and interestingly found a significant reduction in ATP levels across three osteosarcoma cell lines (Fig. 5B). Moreover, we used the AMPK inhibitor dorsomorphin (2 μM) to determine the role of the AMPK/mTOR signaling pathway in MF-mediated antitumor activity. The CCK-8 assay showed that the proliferation of osteosarcoma cells increased in the MF group after treatment with dorsomorphin (Fig. 5C). Similarly, flow cytometry showed that apoptosis was reduced in the MF group after treatment with dorsomorphin (Fig. 5D and E). When dorsomorphin was combined with MF, the expression of p-AMPK, p-mTOR and caspase-9 was significantly restored (Fig. 5F). Thus, MF exerted an antitumor effect through activation of the AMPK/mTOR signaling pathway in osteosarcoma.

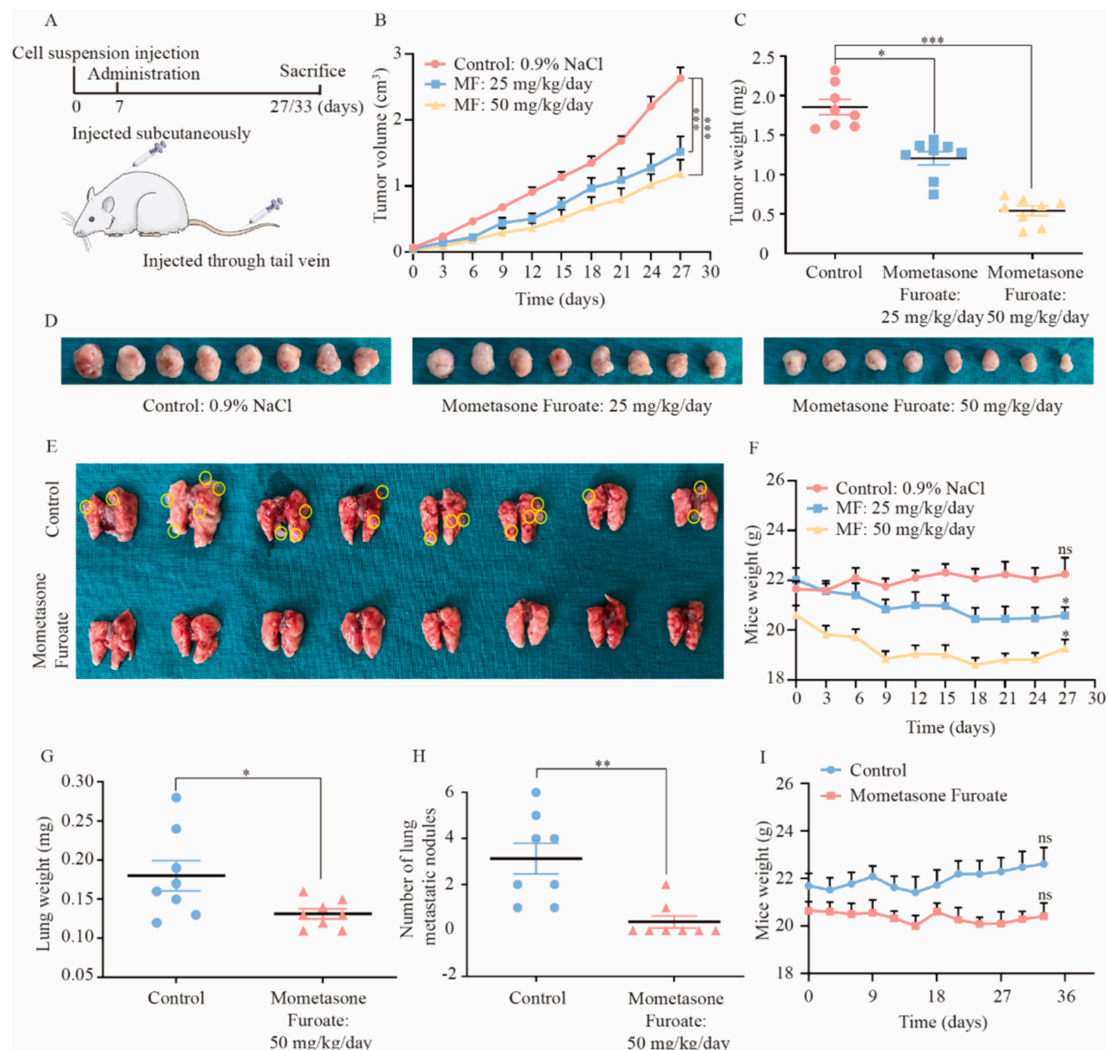

Fig. 4. MF suppressed the proliferation and metastasis of osteosarcoma *in vivo*. (A) Schematic diagram of the xenograft tumor model in mice. (B) Tumor volume was measured every 3 days after MF treatment. (C) Tumor weight was recorded when the mice were euthanized. (D) Photograph of tumor specimens in each group. (E) Photograph of lung specimens in the lung metastasis model showing metastatic nodules. (F) Mouse weight was recorded every 3 days in the subcutaneous transplantation model. (G) Lung weight was recorded when the mice were euthanized. (H) The number of lung metastatic nodules was counted. (I) Mouse weight was recorded every 3 days in the lung metastasis model. Statistical analysis was performed using ANOVA and Student's *t* test. Error bars represent the SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.6. *MF induces apoptosis by activating the AMPK/mTOR signaling pathway in vivo*

Finally, Western blotting was performed to verify whether the AMPK/mTOR signaling pathway functioned *in vivo*. The results showed that the protein levels of p-AMPK increased significantly, followed by downregulated expression of p-mTOR. Simultaneously, the apoptosis-related protein Bak was significantly upregulated, and the protein cyclin D2 was downregulated after MF treatment (Fig. 6A). Then, further immunohistochemical staining was conducted to investigate the underlying pathological changes. Similarly, the IHC[12] analysis indicated that the staining of p-AMPK and caspase-9 was higher, and the staining of p-mTOR and cyclin D2 was lower in the MF-treated group than in the control group. Ki-67 immunohistochemical staining was used to evaluate the proliferation status of tumor cells. After MF treatment, the proportion of Ki-67-positive cells decreased substantially (Fig. 6B). In addition, H&E staining results of the heart, liver, spleen, lung, and kidney excised from the mice showed that MF had no negative effect on internal organs compared to the control group (Fig. 6C). Taken together, we concluded that MF exerted antitumor activity through the AMPK/mTOR signaling pathway *in vivo*.

4. Discussion

Osteosarcoma, the most severe malignant bone tumor, is characterized by faster progression, a high rate of metastasis and heterogeneity. The current standard therapy for osteosarcoma ensures that approximately 67 % of patients with localized osteosarcoma survive for 5 years or longer. However, the low survival rate in metastatic cases and the poor prognosis of recurrent patients have remained stagnant for several decades [3,19]. Although a series of chemotherapeutic drugs have been routinely applied in the clinic, there is no completely decisive strategy for each individual due to the high heterogeneity of osteosarcoma. The chemotherapy insensitivity and drug resistance tendency make the treatment unable to maintain a consistent effect and even lead to irreversible side effects [20]. Therefore, the identification of novel therapeutic strategies is of great importance to ameliorate the prognosis of

[12] immunohistochemistry.

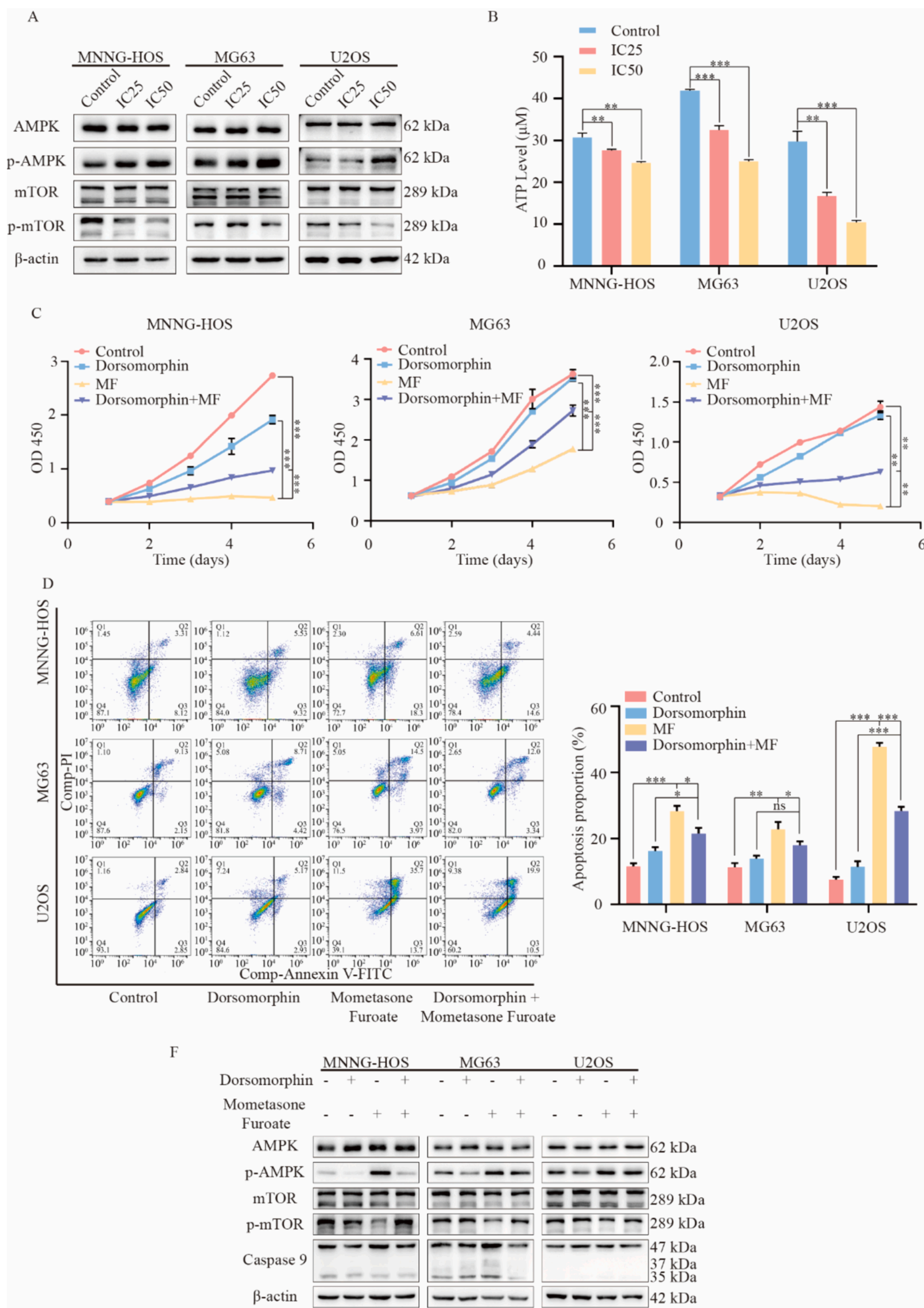

Fig. 5. MF inhibited osteosarcoma *in vitro* by activating the AMPK/mTOR signaling pathway. (A) The expression of AMPK/mTOR signaling pathway proteins in osteosarcoma cells. (B) The ATP levels of osteosarcoma cells after MF treatment. (C) Proliferation of osteosarcoma cells was detected by CCK-8 assay after MF treatment without or with dorsomorphin. (D, E) Apoptotic cells were detected by flow cytometric assay after MF treatment without or with dorsomorphin. (F) The expression of p-AMPK, AMPK, p-mTOR, mTOR and caspase-9 was tested with Western blotting after MF incubation without or with dorsomorphin for 48 h. Statistical analysis was performed using ANOVA. Error bars represent the SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

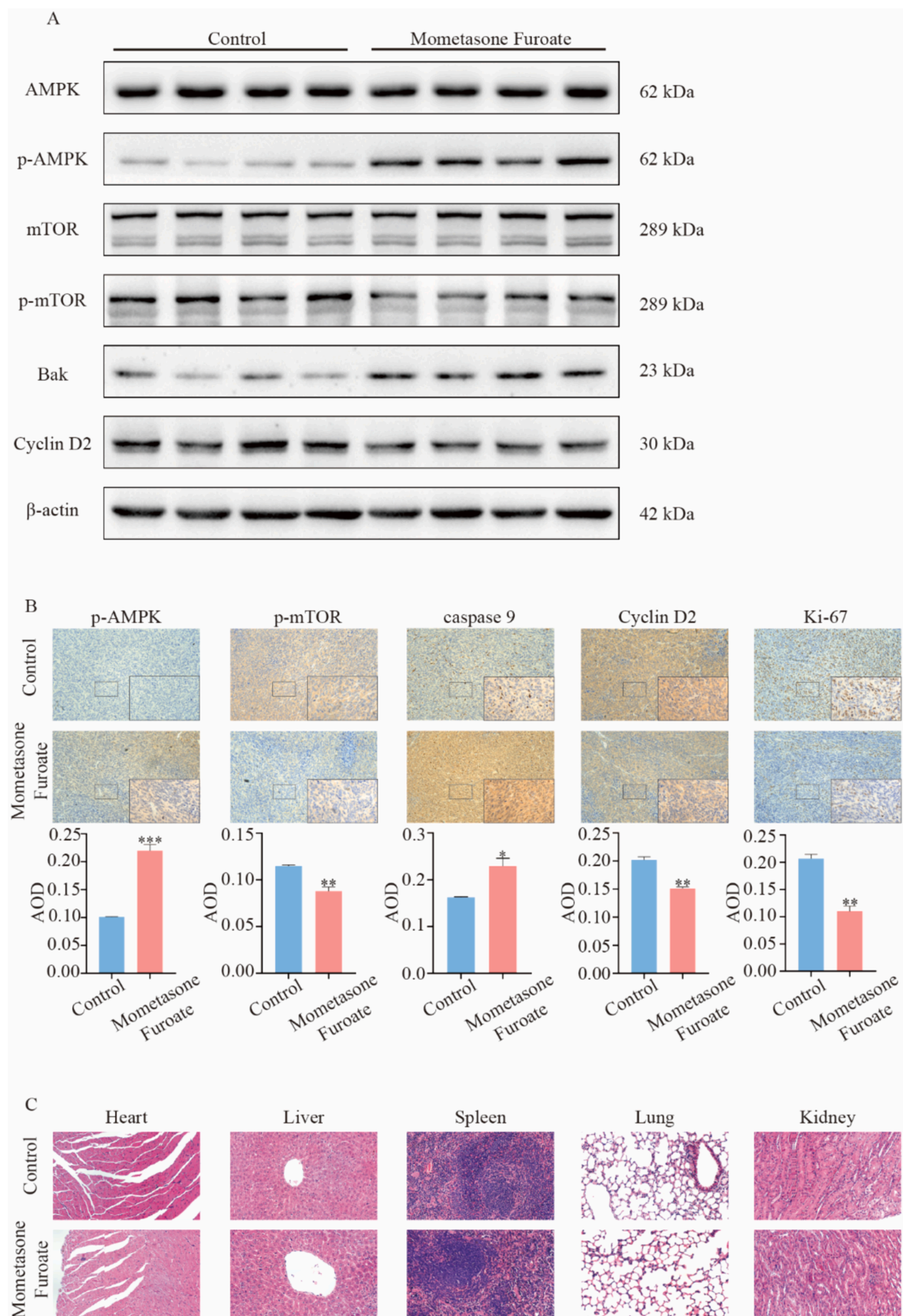

Fig. 6. MF suppressed the growth of osteosarcoma *in vivo* via the AMPK/mTOR signaling pathway. (A) The expression of p-AMPK, p-mTOR, and cycle- and apoptosis-related proteins in tumor tissues. (B) Representative immunohistochemistry images of p-AMPK, p-mTOR, caspase-9, cyclin D2 and Ki-67 in the tumors (magnification, low ×100, high ×200) and corresponding *AOD* analysis. (C) Pathological analysis of the heart, liver, spleen, lung, and kidney of mice (magnification, ×100).

osteosarcoma.

Glucocorticoids are widely applied in the clinic as anti-inflammatory and anti-immune agents. It is also known as a palliative method to relieve the discomfort of cancer patients in the course of treatment. In addition, glucocorticoids also play an active role in the treatment of cancer. The role as a chemotherapeutic was first learned from the treatment of childhood leukemia, and the underlying mechanism is frequently related to apoptosis. A similar mechanism can also explain how glucocorticoids induce cell death in multiple myeloma [6]. Glucocorticoids were proven to inhibit cell growth and induce apoptosis in colon cancer and prostate cancer [9,10]. In a recent study, when treated with glucocorticoids, the expression of genes involved in the cell cycle

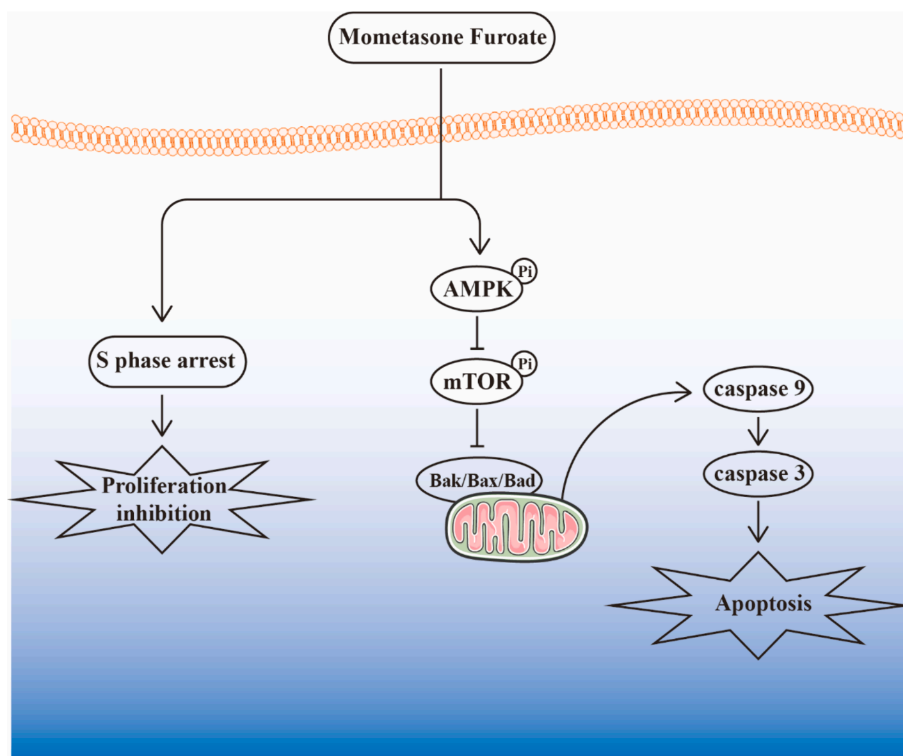

Fig. 7. A schematic diagram of the mechanism of MF-induced proliferation inhibition and apoptosis in osteosarcoma cells.

was downregulated, and the genes in charge of apoptosis were upregulated in U2OS osteosarcoma cell lines. The expression of cell markers related to proliferation and apoptosis was detected to confirm the effect of glucocorticoids on inhibiting proliferation and promoting apoptosis in osteosarcoma [21].

MF, a potent synthetic steroid, is used as a local drug due to its high receptor affinity and low bioavailability. MF is the preferred method to treat inflammatory diseases, such as chronic rhinosinusitis, persistent asthma and adenoid hypertrophy, by decreasing proinflammatory factors and increasing anti-inflammatory factor synthesis. It was reported that MF can reduce radiation dermatitis after receiving radiotherapy [22]. MF was found to be a new therapy in the management of spinal cord injury by modulating inflammatory, oxidative and apoptotic pathways [23]. In addition, a previous study showed that MF can inhibit tumor cell proliferation in a dose-dependent manner and reduce the metastatic potential of acute lymphoblastic leukemia (ALL) cells *in vitro* [14]. This explains why MF has an anticancer effect in pediatric ALL cells. However, the antitumor effect of MF in osteosarcoma has not been reported.

In the present study, MF was found to significantly reduce osteosarcoma cell viability and inhibit the proliferation and metastasis of osteosarcoma cells *in vitro* in a dose-dependent manner. The results of flow cytometry analysis suggested that MF induced accumulation of cells in S phase and promoted apoptosis in osteosarcoma cells. A similar apoptosis-inducing effect has been mentioned in ALL cells. Wang et al. found that MF could induce apoptosis by activating the PI3K pathway and influence the cell cycle with downregulation of cyclin D1 [14]. In addition, the subcutaneous tumor sizes and pulmonary metastatic nodules results revealed that MF could effectively inhibit osteosarcoma cell growth and pulmonary metastasis *in vivo*. S phase arrest and apoptosis were verified by immunohistochemistry in the xenograft mouse model to further validate our findings *in vitro*. The results of H&E staining showed that MF had no negative effect on the internal organs. We also conducted Western blotting to confirm the above findings at the molecular level. S phase entry is principally controlled by cyclin-dependent kinases (CDKs[13]) [24]. Similar to findings by Wang Y et al. where 3-Hydroxyterphenyllin leads to S phase arrest in ovarian cancer cells [25], in our study, MF significantly inhibited Cyclin A2, Cyclin E1, and CDK2, resulting in blocked formation of the Cyclin A2/CDK2 and Cyclin E1/CDK2 complexes, which plays a crucial role in the initiation and progression of the S phase. MF caused S phase arrest by suppressing the expression of Cyclin A2, Cyclin E1, and CDK2. Moreover, we also found that MF decreased the expression of cyclin D2. Previous studies have reported that downregulation of Cyclin D1 and the consequent downregulation of the Cyclin D1/CDK4 complex are markers of S phase arrest [25,26]. Cyclin D2 can also bind CDK4 and exert a similar regulatory effect, thus we believe that downregulation of Cyclin D2 contributes to the S phase accumulation in osteosarcoma cells. Then, the expression of proapoptotic proteins (Bad, Bax and Bax) and caspase-9,7,3 was analyzed by Western blotting. The results revealed that MF could inhibit the proliferation of osteosarcoma cells by inducing apoptosis through the internal pathway. These results indicated that MF may be a promising agent for inhibiting osteosarcoma progression.

AMPK is an important sensor of cellular energy levels, which can be activated by increasing the ratio of AMP[14]/ATP[15] [27,28]. Liu et al. found that MTERF1 knockdown inhibited colorectal cancer cells proliferation by decreasing in ATP levels and thereby activating p-AMPK/mTOR signaling pathway [29]. A study by Daneshmandi et al. showed that induced STAT3 signaling resulted in higher ATP generation, reducing AMPK signalling [30]. Activation of AMPK can inhibit cell growth and proliferation. mTOR is the regulatory center of nutrition and growth factor signaling, stimulating cell growth. AMPK can directly inhibit the mTORC1[16] complex (the mechanistic target of rapamycin

[13] cyclin-dependent kinases.
[14] adenosine monophosphate.
[15] adenosine triphosphate.
[16] the mechanistic target of rapamycin complex 1.

complex 1). AMPK and mTORC1 play opposite roles in the regulation of cell proliferation [28]. The AMPK/mTOR signaling pathway plays a role in many kinds of cancers. The activated AMPK/mTOR signaling pathway inhibits the occurrence and development of cancer by promoting apoptosis [15–18]. In this study, we report for the first time a significant increase in AMPK phosphorylation and a significant decrease in mTOR phosphorylation after MF treatment. This may be related to the reduction in ATP levels in osteosarcoma cells after MF treatment. The stimulation of the AMPK/mTOR signaling pathway can regulate the cell death induced by MF, which is reversed by pretreatment with the AMPK inhibitor dorsomorphin. Taken together, the results suggested that MF could exert antitumor effects through activation of the AMPK/mTOR signaling pathway (Fig. 7).

In conclusion, our study is the first to show that MF can inhibit osteosarcoma *in vivo* and *in vitro*. MF inhibits the proliferation of osteosarcoma cells by blocking the cell cycle and inducing apoptosis. In addition, MF promotes cell death by activating the AMPK/mTOR signaling pathway. These findings indicate that MF not only has antitumor effects but also has the potential to treat osteosarcoma.

Ethics approval

This experiment does not include human experimentation and/or the use of human tissue samples. This study and included experimental procedures were approved by the institutional animal care and use committee of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

CRediT authorship contribution statement

Zhaohui Li: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Xiang Fei:** Visualization, Resources, Methodology, Data curation. **Zhen Pan:** Visualization, Validation. **Yonghui Liang:** Software, Data curation. **Qingcheng Yang:** Supervision, Resources, Project administration, Funding acquisition. **Dongdong Cheng:** Writing – review & editing, Supervision, Project administration, Methodology.

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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