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Research Paper

Isoquercitrin restrains the proliferation and promotes apoptosis of human osteosarcoma cells by inhibiting the Wnt/β-catenin pathway

Zhun Wei [a,1], Di Zheng [a,1], Wenfeng Pi [b,1], Yonglong Qiu [a], Kezhou Xia [a,*], Weichun Guo [a,*]

[a] Department of Orthopaedics, Renmin Hospital of Wuhan University, Hubei Province, Wuhan 430060, China
[b] Department of Orthopaedics, Chinese Traditional Medicine of Daye Hospital, Hubei Province, Daye 435100, China

HIGHLIGHTS

- Isoquercitrin inhibits proliferation and invasion of osteosarcoma both *in vitro and vivo*.
- The role of isoquercitrin in inhibiting osteosarcoma progression is achieved by inhibiting the Wnt/β-catenin signaling pathway.
- Enhancing β-catenin activity rescues the anti-OS effect of ISO.
- We are the first to study the mechanism of action of ISO in osteosarcoma.

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ABSTRACT

Currently, chemotherapeutic drugs are widely used for the treatment of osteosarcoma. However, many of these drugs exhibit shortcomings such as poor efficacy, high toxicity, and tolerance. Isoquercitrin (ISO) is a traditional Chinese medicine that has been proved to exert good therapeutic effects on various tumors; however, its role in osteosarcoma has not been reported. Here, we observed that ISO exerted a marked inhibitory effect on the occurrence and development of osteosarcoma in a time- and dose-dependent manner. First, we determined that ISO significantly inhibited proliferation, induced EMT-related migration and invasion and induced apoptosis of osteosarcoma cells *in vitro*. Concurrently, we also observed that both β-catenin and its downstream genes (c-Myc, CyclinD1, and Survivin) were significantly down-regulated. To verify if the anti-tumor effect of ISO was related to the Wnt/β-catenin signaling pathway, we altered the protein expression level of β-catenin using recombinant lentivirus, then we observed that the effects of ISO on the proliferation, metastasis, and apoptosis of osteosarcoma cells were significantly reversed. Additionally, we used a nude mouse xenograft model and observed that ISO significantly inhibited the growth of osteosarcoma and improved the survival rate of the animal models. In conclusion, this study demonstrates that ISO can exert anti-tumor effects in part by inhibiting the Wnt/β-catenin signaling pathway, thus providing a new potential therapeutic strategy for the treatment of osteosarcoma.

1. Introduction

Osteosarcoma is the most common malignant primary bone tumor in children and adolescents, often manifests as local swelling and pain during the early stages and is prone to pulmonary metastasis during the late stages [1]. Current primary treatments include complete surgical resection of all detectable tumor sites and neoadjuvant chemotherapy. However, the treatment effects of patients with metastatic or recurrent osteosarcoma remains poor with an overall survival rate of <20 % [2]. This may be a result of the poor targeting of current chemotherapy drugs to tumor cells [3].

In regard to the treatment of tumors in the past few years, an increasing number of traditional Chinese medicines such as peiminine, cinobufagin, and sinomenine have been observed to improve treatment efficacy and to prevent and treat postoperative metastasis and recurrence when combined with radiotherapy and chemotherapy, and these medicines can also be used as an independent method for the treatment of advanced cancer [4–7]. ISO is an herbal medicine that is extracted from *Bidens pilosa* L. that exerts a number of potential biochemical effects, including anti-inflammatory, antioxidant, and anti-tumor effects

* Corresponding authors at: Department of Orthopaedics, Renmin hospital of Wuhan university, 238 Jiefang Road, Wuhan 430060, China.
E-mail addresses: xiakz0617@163.com (K. Xia), guoweichun@aliyun.com (W. Guo).
[1] These authors have contributed equally to this work and share the first authorship.

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[8,9]. The therapeutic effects of ISO on tumors had been extensively studied in recent years. Wu *et al.* [10] reported that ISO could induce apoptosis in bladder cancer cells via mitochondrial-mediated and death receptor-mediated signaling pathways that are mediated through Caspase-3/8/9 and p53 phosphorylation by AMPK. Huang *et al.* [11] demonstrated that ISO can inhibit the proliferation, block the transformation of the cell cycle, and induce the apoptosis of human hepatoma cells *in vitro*. Based on the above results, we speculate that ISO also plays an important role in the occurrence, development, and metastasis of osteosarcoma.

Our current study aimed to study the effect of ISO on the biological activity of osteosarcoma and to determine its molecular mechanism. We used ISO to treat 143B and U2OS cells. The results revealed that ISO significantly inhibited the proliferation and metastasis of osteosarcoma cells *in vivo* and *in vitro*, and this was partially depended on Wnt/β-catenin signaling pathway.

2. Materials and methods

2.1. Drugs and antibodies

ISO (99.95 % purity) was obtained from MCE (Cas:21637-25-2) and dissolved in dimethyl sulfoxide (DMSO) to obtain a suitable concentration. The antibodies that were used in our study included cleaved-Caspase-3, bcl-2, Bax, E-cadherin, vimentin, MMP9, MMP2, cyclinD1, β-catenin, c-myc, survivin, and GAPDH. More detailed information regarding these antibodies is provided in Table 1.

2.2. Cell culture

The human osteosarcoma cell lines 143B and U2OS and human osteoblast cell line hFOB1.19 were obtained from the China Centre for Type Culture Collection (Wuhan, China). All 143B and U2OS cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10 % fetal bovine serum (FBS; Tian Hang), and they were propagated in a humidified environment with 5 % CO2 at 37 °C. hFOB1.19 was cultured in DMEM/F12 (Invitrogen) medium supplemented with 10 % fetal bovine serum (FBS; Tian hang) and geneticin (400 μg/ml, Sigma) and was incubated in an incubator with 5 % CO2 at 37 °C.

2.3. Construction of stably transfected cells

To generate stably transfected cell lines, we constructed negative control, upregulation, and downregulation groups using recombinant lentivirus from Shanghai GeneChem Co., ltd (Shanghai, China). The transfected cells were incubated for 48–72 h and then treated with 5 μg/ml of puromycin to screen for stably transfected cells.

Table 1
Details of the antibodies.

Antibodies	Company	Article number	Species	Dilutions
cleaved-Caspase- 3	Wanleibio	WL02117	Rabbit	1:1000
bcl-2	Servicebio	GB113375	Rabbit	1:1000
Bax	Affinity	AF0120	Rabbit	1:1000
E-cadherin	Huabio	ETl607-75	Rabbit	1:1000
Vimentin	Servicebio	GB111308	Rabbit	1:1000
MMP9	Huabio	ET1704-69	Rabbit	1:1000
MMP2	Huabio	ET1606-4	Rabbit	1:1000
cyclinD1	Servicebio	GB13079	Rabbit	1:1000
β-catenin	Huabio	ER0805	Rabbit	1:1000
c-myc	Huabio	RT1149	Mouse	1:1000
surviving	Huabio	ET1602-43	Rabbit	1:1000
GAPDH	Servicebio	GB11002	Rabbit	1:1000

2.4. Cell viability assay

First, cells were evenly seeded onto a 96-well plate at a density of 80 %. When the cells are completely attached, we treated 143B and U2OS cells with ISO at concentrations of 0, 10, 20, 40, 60, 80 μmol/L for 24 h and 48 h, respectively. Prior to testing, 10 μL of CCK-8 solution was added to each well containing a 100 μL mixture of the medium. The plate was incubated at 37 °C with 5 %CO_2 for 2 h. Based on the principle of absorbance measurement, cell viability was estimated using a microplate reader (Bio-Rad Laboratories, Inc.). The OD450 value was used to represent the cell viability.

2.5. Colony formation assay

Osteosarcoma 143B and U2OS cell lines were seeded into a 6-well plate, and when the cell density reached approximately 70 %, the cells were treated with ISO (0,20,40 μmol/L) for 24 h. Then the cells were digested and resuspended, cultured in ISOfree conditions at a concentration of 2×10^3 cells/well, and cultured for another two weeks. Subsequently, the cells were soaked with 5 % paraformaldehyde for 20 min and then crystal violet solution was used to stain the cells for an additional 15 min. Finally, the cells were washed with PBS and dried to compare the number of colonies per well visually.

2.6. Hoechst 33,258 staining

The Hoechst 33,258 staining kit (G1011, Servicebio) was applied for this assay. The 143B and U2OS cell lines were cultured in 6-well plates and incubated for 24 h. After the cells were treated with or without ISO, they were then stained with this solution and observed under a fluorescence microscope (Olympus). Blue nucleus were observed under the microscope. When the nucleus shrinks, the cells undergo apoptosis.

2.7. Flow cytometry

The PE Annexin V/7-AAD Apoptosis Detection Kit (BD Biosciences) was used to detect the extent of apoptosis. After treating 143B and U2OS cells with different concentrations of ISO, the cells were washed twice with PBS and resuspended in 1X binding buffer at a density of 1 × 10 * 6 cells/ml. A total of 5 μL of PE annexin V and 5 μL of 7-AAD were added to 500 μL of this solution (1 × 10 × 5 cells) and incubated for 15 min at RT (25 °C) in the dark. Flow cytometry (Beckman Coulter, USA) was used to analyze the level of apoptosis.

2.8. EdU (5-Ethynyl-2′- deoxyuridine) staining

Firstly, osteosarcoma 143B and U2OS cell lines were seeded into a 6-well plate. ISO (0,20 μmol/L) was added, and the cells were cultured for 24 h until the cell density reached 70 %. Then, EdU storage fluid was added, and the cells were cultured for 2 h based on the manufacturer's requirements. Subsequently, the cells were soaked in 5 % paraformaldehyde for 20 min and stained with an EdU detection kit (G1603, Servicebio,). The nuclei were stained with Hoechst 33,342 for 5 min. EdU-positive cells were observed using an inverted fluorescence microscope (Olympus).

2.9. Wound healing assay

The 143B and U2OS cells were evenly spread onto 6-well plates. When the cell density reached 80 %, a 1 ml sterile pipette tip was used to gently draw a straight line on the surface of the cells. Subsequently, the medium was discarded. The cells were washed three times with PBS, and the cells were then cultured in serum-free 1640-RPMI medium supplemented with varying concentrations of ISO. The wound healing area was observed under an inverted microscope (Olympus).

2.10. Transwell assay

For cell invasion experiments, Transwell chambers (Corning, USA) and Matrigel (Corning, USA) were used. First, Matrigel was added to the serum-free medium at a ratio of 1:8, and 80uL of this solution was then the added to the upper chamber. After the mixture solidified, the cells that were treated with different concentrations of ISO were digested and resuspended in serum-free medium. A 200 μL cell suspension containing 1 × 10*5cells was added to the prepared chamber, and 500 μL of medium containing 20 % FBS was added to the lower chamber. After culturing for 48 h in an incubator, while the lower chamber was soaked with 5 % paraformaldehyde for 15 min and subsequently dyed with crystal violet for 15 min. the number of invading cells was observed under an inverted microscope (Olympus).

2.11. Western blotting assays

Total protein was obtained using radioimmune precipitation assay (RIPA) buffer and then centrifuged for 15 min at 4 °C. Approximately 10–25 μg of total protein was separated by 8 %–12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking, the bands were washed three times with PBS and incubated with the primary antibody at 4 °C for 12 h. Subsequently, the band was removed, washed three times with PBS, and incubated with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Finally, bands were scanned using the ChemiDoc™ Touch Imaging System (Bio-Rad) after treatment with a chemiluminescent substrate, and the expression level of the protein was determined according to the depth of the band color. GAPDH was used as a control. The experiment was repeated 3 times for each group.

2.12. Luciferase assays

First, 143B and U2OS cells were evenly seeded onto a 6-well plate, and when the cell density reached 80 %, the cells were treated with varying concentrations of ISO for 12 h. Subsequently, TCF/LEF-1 luciferase reporter plasmid was added to OS cells. After transfection for 24 h, all cells were lysed, and cellular luciferase was detected using the dual luciferase assay system (Promega, Madison, WI, USA). The experiments were repeated 3 times in each group.

2.13. Immunofluorescence staining

After the cells were treated with different concentrations of ISO, the cells were then fixed with 5 % paraformaldehyde for 15 min and blocked with 1 % bovine serum albumin (BSA) (Servicebio) for 1 h. Subsequently, the samples were incubated with diluted primary antibodies (Bcl-2, E-cadherin, Vimentin, and β-catenin) overnight at 4 °C. After incubation and washing, cells were incubated with the secondary antibody (Servicebio) for 1 h at room temperature. In the end, the nuclei were stained with DAPI. An upright microscope (Olympus) was used for observation and image acquisition.

2.14. Immunohistochemistry staining and TUNEL assay

After fixing the tumor tissue with 5 % paraformaldehyde for 24 h, it was embedded in wax, and the prepared samples were cut into 4 μm slices. After blocking with 1 % BSA for 1 h, the samples were incubated with the corresponding primary antibody overnight at 4 °C. On the second day, the sections were washed thoroughly-three times with PBS and incubated with the secondary antibody for 1 h at room temperature. A DAB kit (Vector Laboratories) was used to detect immunoreactivity. An upright microscope (Olympus) was used for image acquisition.

2.15. Animal experiments

The animal experimental protocol was acknowledged by the Ethics Committee of the Renmin Hospital of Wuhan University. Male BALB/c nude mice (4–6-week-old) were purchased from Beijing HFK Experiment Animal Center and were provided with food and water in a standard laboratory environment. As described above [12], a xenograft tumor model of 143B cells was established in male nude mice. Firstly, 12 nude mice were injected subcutaneously with 143B cells (5×10^6 cells in 0.2 ml normal saline). One week after injection, the injected mice were divided into two groups of six mice each. These groups included group (a) where an equal amount of dimethyl sulfoxide (DMSO) was added to normal saline, and (b) high-concentration group treated with 100 mg/kg of ISO. Nude mice were injected intraperitoneally with ISO or with normal saline every alternate day for 6 weeks. One week after the last drug treatment, all nude mice were sacrificed, and the tumor tissue, livers, lungs, and other tissues of the animals were collected for later experimental analysis.

2.16. Statistical analysis

Our statistical diagrams were obtained using GraphPad Prism (version 8.0). SPSS software (version 6.0) was used for the statistical analysis. All data are presented as mean ± standard deviation (SD), and each experiment were repeated at least three times. The results were considered statistically significant only when the P value was <0.05.

3. Results

3.1. ISO restrains the proliferation of OS cells in vitro

The molecular structure of ISO is presented (Fig. 1A). To determine the anti-OS effect of isoquercitrin, 143B and U2OS cells were treated with various concentrations for 24, 48, and 72 h. Cell viability was measured using a CCK-8 assay. Fig. 1B and C showed that the proliferation of 143B and U2OS cells was inhibited in a time- and concentration-dependent manner (Table 2). The IC_{50} value of isoquercitrin in 143B and U2OS were ~ 20 μM in 24 h and 40 μM in 48 h. Based on the CCK-8 assay, we selected 20 and 40 μM concentrations of ISO for further research. In cloning formation experiments, we observed a significant reduction in cellular colony formation after treatment with ISO (Fig. 1E-G). An EdU assay was performed to evaluate cell proliferation capacity, and we found that the ratio of EdU-positive cells was lower in the ISO group than it was in the control group (Fig. 1H, J, and K). Similarly, the live/dead assay indicated that compared to the control group, ISO destroyed the complete morphology of OS cells, and a higher rate of dead cells was observed (Fig. 1I, L, M). In summary, we can conclude that ISO inhibits the proliferation of OS cells in a time- and concentration-dependent manner.

3.2. ISO inhibits the metastatic capacity of OS cells

Tumor metastasis is closely linked to the migration and invasion capabilities of tumor cells [13]. Based on this, we performed a wound healing assay to detect changes in the migration of OS cells. Fig. 2A-D reveals that the migration capabilities were clearly inhibited after treatment with ISO, and this inhibitory effect varied with the concentration gradient. In transwell assay, we evaluated the invasion ability of OS cells by observing the number of cells entering the lower chamber. The results suggested that ISO significantly weakened the invasion of OS cells in a concentration-dependent manner (Fig. 2E-G).

Researches has demonstrated that matrix metalloproteinases (MMPs) play a key role in the degradation of the extracellular matrix during tumor metastasis [14,15]. Therefore, after treatment of 143B and U2OS cells with ISO for 48 h, we measured the protein expression levels of MMP-2 and MMP-9. Our results suggested that with an increased

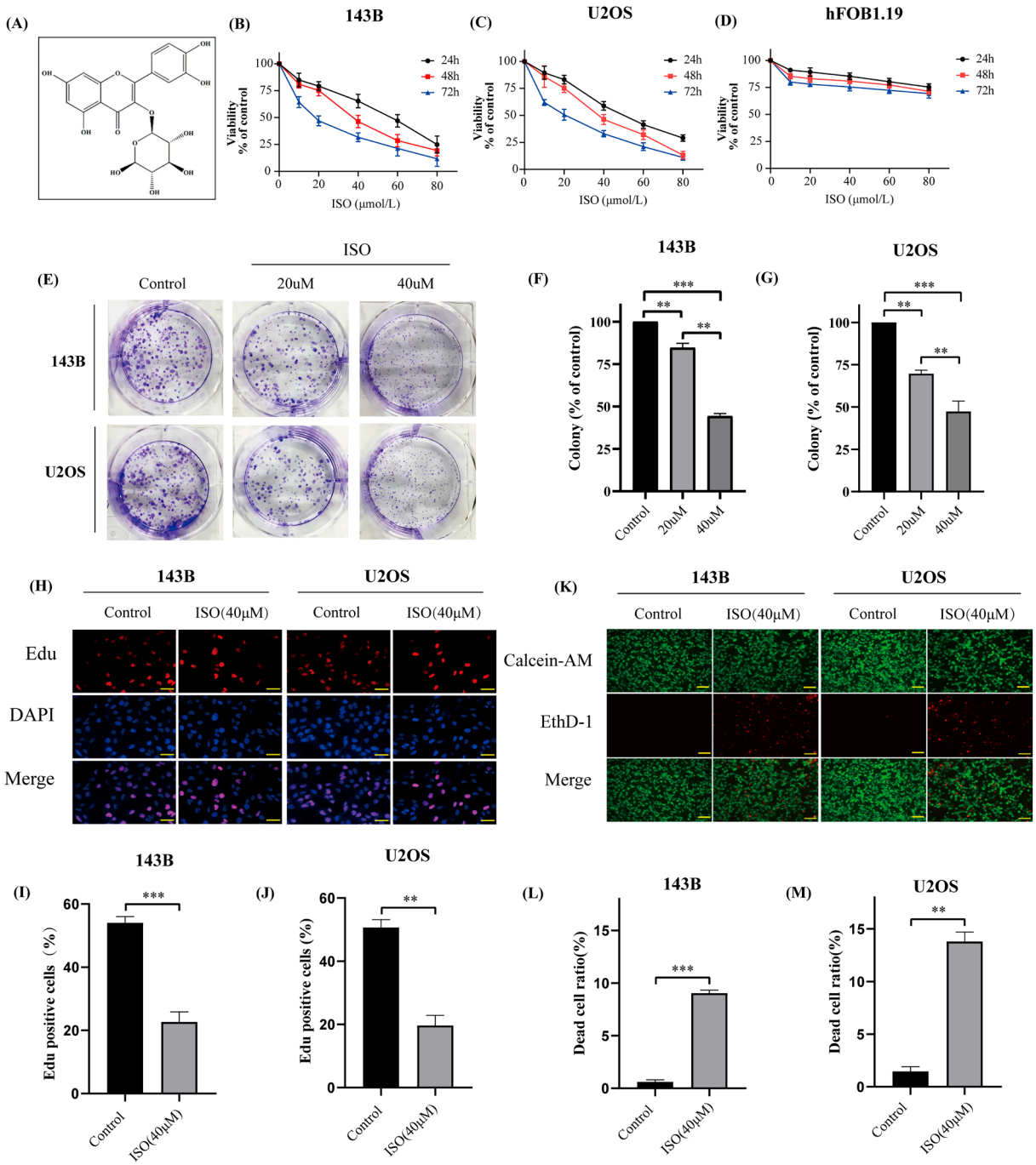

Fig. 1. Isoquercitrin inhibits the proliferation of 143B and U2OS cells *in vitro* **(A)** Schematic diagram of the molecular chemical structure of Isoquercitrin (B, C) After 143B and U2OS cells were treated with different concentrations of isoquercitrin for 24, 48, and 72 h, the cell viability was detected by CCK-8 assay. (D) After normal human osteoblast hFOB1.19 was treated with different concentrations of isoquercitrin, its activity was detected by CCK-8 assay (E) 143B and U2OS cells were treated with 20 and 40 μM isoquercitrin, respectively, then colony-formation assay was performed. (F-G) Quantitative analysis of the colony-formation assay. (H) Edu experiments showed that isoquercitrin significantly inhibited the DNA synthesis of osteosarcoma cells compared with the control group, scale bar: 20 μm. (I) After treatment of osteosarcoma cells with isoquercitrin, viable cells (green) were significantly reduced and dead cells (red) were significantly increased, and varied with concentration, scale bar: 80 μm. (J, K) Quantitative analysis of Edu positive cells in the Edu experiments (L, M) Quantitative analysis of the percentage of dead cells in live/dead assays. All data are from three independent experiments and are shown as mean ± SD, * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration of ISO, the levels of MMP-2 and MMP-9 were significantly downregulated. Additionally, other EMT-related proteins, such as E-cadherin and Vimentin, was also significantly changed. (Fig. 2H-K). Moreover, we detected the expression levels of E-cadherin and Vimentin using immunofluorescence staining, and we observed that ISOtreated OS cells expressed higher levels of E-cadherin and lower levels of Vimentin than did in the control group (Fig. 2L-O). In summary, we can infer that ISO significantly reduces the metastatic potential of OS cells.

Table 2
IC_{50} of Isoquercitrin in osteosarcoma cell lines.

Cell lines	IC_{50}(μM)		
	24 h	48 h	72 h
143B	18 ± 3.1	37 ± 2.4	58 ± 3.3
U2OS	19 ± 1.8	36 ± 3.1	53 ± 4.1

3.3. ISO inhibits the transition of the cell cycle of OS cells from G1 to S phase

As abnormal blockade of each portion of the cell cycle adversely affects cell occurrence and development, flow cytometry analysis was performed to explore the effect of ISO on the cell cycle. As presented in Fig. 3A-C, the proportion of cells in the G0/G1 phase increased significantly in a concentration-dependent manner. Consistent with the results of the flow cytometry analysis, the expression level of Cyclin D1, a key gene in the transition from G1 to G2 phase of the cell cycle, was significantly changed after ISO treatment. (Fig. 3D-F).

3.4. ISO induces apoptosis of OS cells in vitro

Researchers have shown that the anti-tumor effects of many drugs can be achieved by inducing tumor cell apoptosis [16–18]. To prove this, 143B cells were treated with 20 and 40 μM concentrations of ISO. Hoechst 33,258 staining assays revealed that as the concentration of the drug increased, the proportion of nuclear fragmentation also increased. (Fig. 4A, B). Flow cytometry provides an effective method for the detection of apoptosis. Annexin V-FITC/PI staining revealed that ISO treatment induced apoptosis in a dose-dependent manner (Fig. 4C and D). To further examine the biomolecular mechanism underlying ISO-induced apoptosis, the expression levels of apoptosis-related proteins were measured. We found the levels of Bax and cleaved Caspase-3 after treatment with ISO for 24 h were significantly increased, while the anti-apoptotic protein Bcl-2 exhibited the opposite trend according to the results of western blot and immunofluorescence staining (Fig. 4E-J). Thus, we can conclude that ISO induces apoptosis in 143B and U2OS cells.

3.5. ISO inhibits Wnt/β-catenin signaling pathways in OS cells in vitro

Previous research has demonstrated that the Wnt/β-catenin signaling pathway plays an important role in tumor metabolism [19,20]. Based on this, we further explored if the anti-tumor effect of ISO was achieved in part by the inhibition of the Wnt/β-catenin signaling pathway. OS cells were treated with different concentrations of ISO for 48 h, we then detected the expression levels of β-catenin and its downstream genes such as c-Myc, CyclinD1, and Survivin. The results revealed that the expression levels of β-catenin in the nuclear component and cytoplasmic component were markedly decreased in 143B and U2OS cells after treatment with ISO. Downstream target genes such as c-Myc, CyclinD1, and Survivin also showed a downward trend. (Fig. 5A-D). To further verify these results, we assessed the expression levels of β-catenin using immunofluorescence staining. The results revealed that the application of ISO led to the downregulation of Wnt/β-catenin signaling pathway in OS cells (Fig. 5E-F).

In canonical Wnt signaling, the activity of nuclear β-catenin is primarily mediated by the TCF/LEF transcription factor family [21]. Therefore, we used TCF/LEF luciferase reporter analysis to detect the level of the TCF/LEF transcription factor in 143B and U2OS cells that were treated with ISO. As presented in Fig. 5G and H, the activity of transcription factors in the ISOtreated group was markedly decreased in a concentration-dependent manner. Therefore, we can infer that ISO can inhibit the transcriptional level of TCF/LEF transcription factor in OS cells, thereby the inhibition of the Wnt/β-catenin signaling pathway was achieved.

3.6. Enhancing β-catenin activity rescues the anti-OS effect of ISO

Since the Wnt/β-catenin signaling pathway is closely related to the occurrence and progression of various cancers [22], recombinant lentivirus was used to construct negative control and upregulation groups. After ISO treatment of these cells for 24 h, a number of further experiments were performed. As presented in Fig. 6A and B, the overexpression of β-catenin reduced the anti-proliferative effect of ISO on 143B and U2OS cells. In conclusion, the anti-proliferative effect of ISO was achieved in part through downregulation of the Wnt/β-catenin signaling pathway.

Additionally, colony formation assays indicated that overexpression of β-catenin significantly alleviated the inhibitory effect of ISO on 143B and U2OS cell proliferation (Fig. 6C-E). The migration and invasion capabilities were measured by wound-healing assays (Fig. 6F-I) and transwell experiments (Fig. 6J-L). Both of these were markedly increased by the overexpression of β-catenin We also detected related protein expression levels using western blot analysis. We observed that the overexpression of β-catenin inhibited the pro-apoptotic effect of ISO as evidenced by an increased ratio of Bcl-2/Bax. Meanwhile, the upregulation of the Wnt/β-catenin signaling pathway led to decreased levels of E-cadherin and increased level of Vimentin (Fig. 6M-O). Taken together, upregulation of the Wnt/β-catenin signaling pathway can significantly alleviate anti-OS effects induced by ISO.

3.7. ISO inhibits OS cells growth in vivo

To further examine the anti-tumor effect of ISO *in vivo*, a mouse xenograft model in nude mice bearing 143B cells was established. The data presented in Fig. 7A-C indicate that ISO significantly inhibited tumor volume and tumor weight in a concentration-dependent manner. Additionally, to further identify its mechanism *in vivo*, western blot analysis, immunohistochemistry, and TUNEL assays were performed (Fig. 7D-F). Fig. 7D indicates dramatically increased expression of apoptosis and a decreased level of proliferation as measured by TUNEL and Ki-67 staining. Consistent with the western blot analysis, immunohistochemistry revealed that β-catenin was significantly decreased and that E-cadherin was increased after ISO treatment. The above results revealed that ISO exerted its anti-OS effect *in vivo* by blocking the process of the cell cycle, inhibiting EMT, and accelerating the process of cell apoptosis partially by inhibiting Wnt/β-catenin signaling pathways.

4. Discussion

Osteosarcoma is the most common malignant bone tumor and exhibits high recurrence and metastasis rates. Although current surgical resection and neoadjuvant chemotherapy have greatly improved the survival rate of many patients, the prognosis is still not optimistic for patients with metastasis or recurrence, and the 5-year survival rate of patients with lung metastases is only 28 % [23,24]. Currently, commonly used chemotherapeutic drugs include doxorubicin, cisplatin, and methotrexate; however, these drugs exhibit a number of side effects such as drug resistance and great damage to the body [3]. Therefore, it is crucial to develop more suitable chemotherapy drugs.

ISO is a Chinese patent medicine exhibiting various pharmacological effects and that is extracted from *Bidens pilosa* L. [25]. In recent years, ISO has been observed to restrain the occurance and progression of tumor cells in human bladder, breast, ovarian, colon, and pancreatic cancers, and it can also induce tumor cell apoptosis, epithelial-mesenchymal transition, and autophagy [8,9,26,27]. In this study, we used the commonly used osteosarcoma cell lines 143B and U2OS to examine the antitumor effect of ISO. We thoroughly investigated how ISO inhibits the proliferation and metastasis of osteosarcoma cells *in vitro* and *in vivo*. To our knowledge, this study is the first to investigate

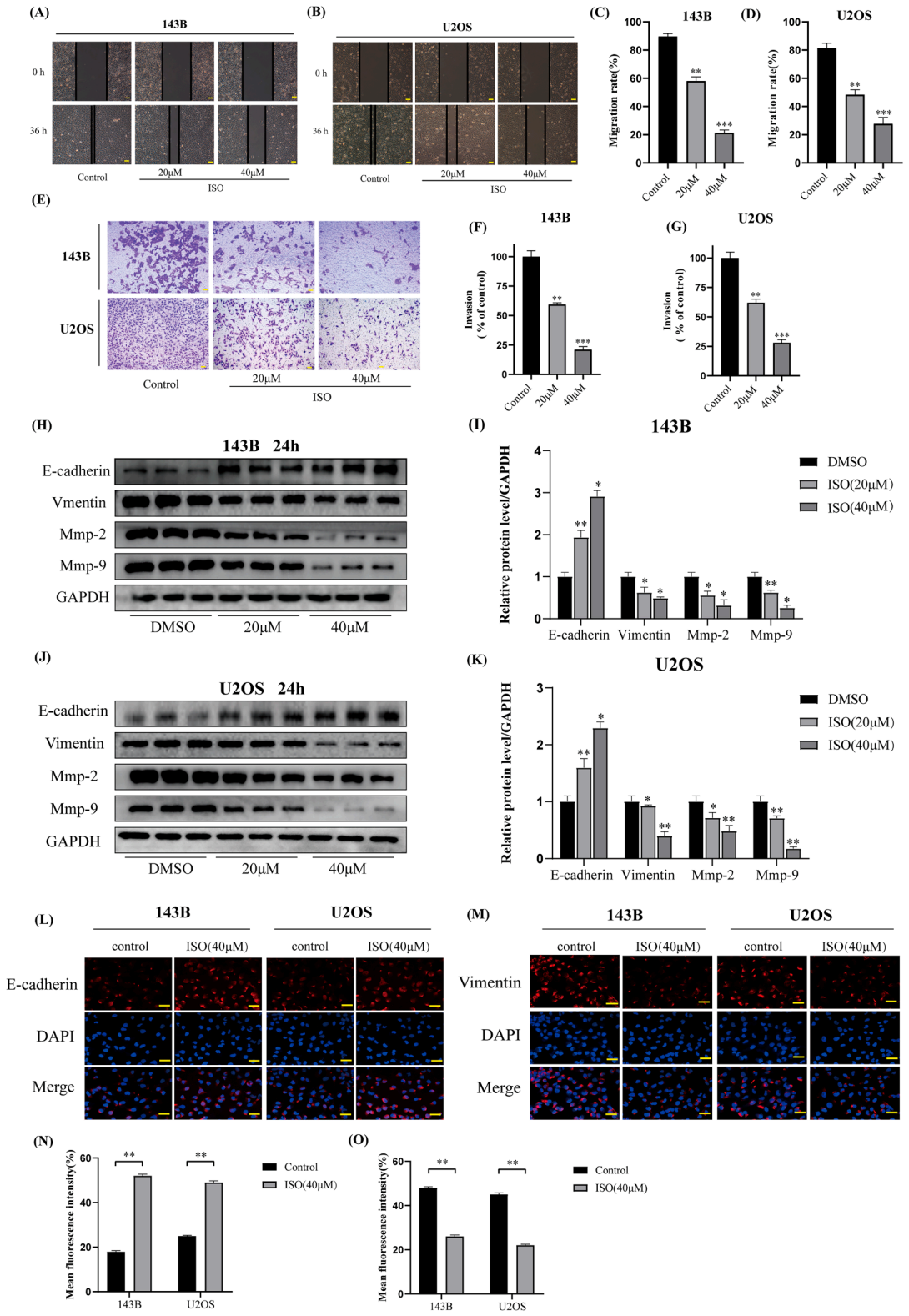

Fig. 2. Isoquercitrin inhibits the metastatic potential of OS cells *in vitro* (A, B). Effects of isoquercitrin treatment on the migration of 143B and U2OS cells measured by wound-healing assay, scale bar: 80 μm. (C, D) Quantitative analysis of migration rate in wound-healing assay. (E) Effects of isoquercitrin treatment on the invasion of 143B and U2OS cells measured by transwell assay, scale bar: 80 μm. (F, G) Quantitative analysis of the percentage of invasion cells in transwell assay. (H, J) Relative expression levels of EMT-related proteins including E-cadherin, Vimentin, MMP-2, MMP-9 after 143B and U2OS cells were treated with or without isoquercitrin, GAPDH is used as an internal reference. (I, K) Quantitative analysis of the related-protein expression level. (L, M) Immunofluorescence was used to compare the expression level of E-cadherin and Vimentin after ISO treatment, scale bar: 20 μm. (N, O) Relative quantification of E-cadherin and Vimentin in immunofluorescence analysis. All data are from three independent experiments and are shown as mean ± SD, * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

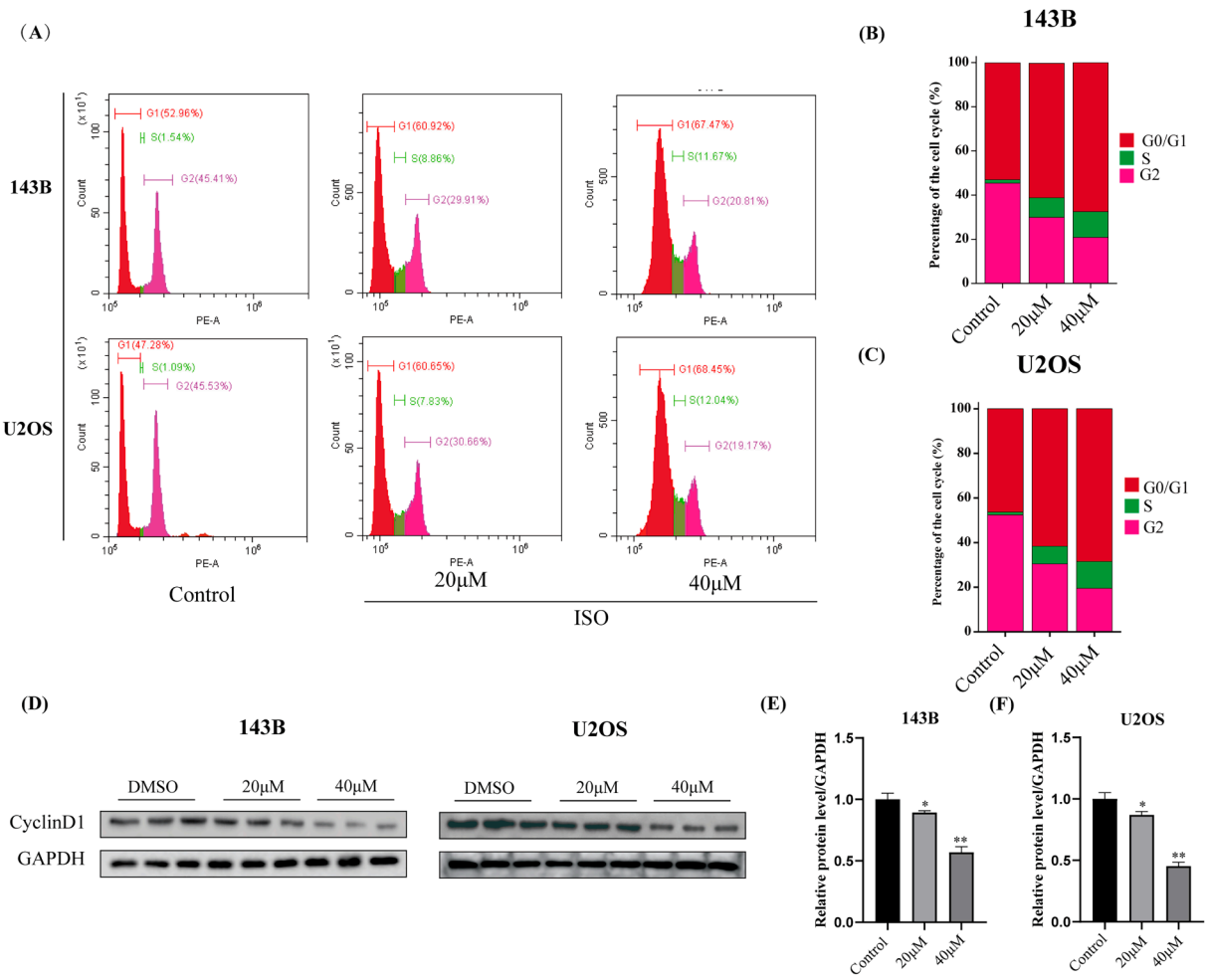

Fig. 3. Isoquercitrin induces G0/G1 phase arrest in OS cells *in vitro* (A)The changes of cell cycle were detected by flow cytometry after treatment of 143B and U2OS cells with different concentrations of isoquercitrin. (B, C) Relative quantification of the cells in G0/G1 phase after treatment with different concentrations of isoquercitrin. (D) The effects of different concentrations of isoquercitrin on the protein expression level of CyclinD1. (E, F) Quantitative analysis of the expression level of CyclinD1. All data are from three independent experiments and are shown as mean ± SD, $*P < 0.05$, $**p < 0.01$, $***p < 0.001$.

the specific function and mechanism of the effect of ISO on osteosarcoma.

First, we performed CCK-8 and colony formation assays to demonstrate that ISO can significantly inhibit the proliferation of osteosarcoma cells. Since cell proliferation is closely related to the cell cycle [28], flow cytometry analysis then confirmed that ISO weakened the proliferation of osteosarcoma cells by blocking the G0/G1 phase of the cell cycle, which was further vertified *in vivo*. These results indicate that ISO inhibits the proliferation of osteosarcoma cells by impeding the transition of the cell cycle from G1 to S phase in a manner that is closely related to the suppression of CyclinD1.

Apoptosis is the autonomous and orderly death of cells that is controlled by genes and involves the activation, expression, and regulation of a series of specific genes [29]. The results of the flow cytometry analysis revealed that ISO significantly induced apoptosis in OS cells in a concentration-dependent manner. Similarly, in the Hoechst 33,258 staining experiment, we observed that ISOtreated OS cells clearly possessed shrunken bright blue nuclei when viewed under the fluorescence microscope, and this is a biological indicator of apoptosis. To further explore the specific molecular mechanism by which ISO induces apoptosis in osteosarcoma cells, we measured the expression levels of apoptosis-related proteins in osteosarcoma cells. Activation of the caspase family, particularly Caspase-3 that is known to act downstream of Bax/Bcl-2, is a key mechanism that leads to apoptosis [30,31]. Following ISO treatment, we observed that the ratio of Bax/Bcl-2 was significantly increased, and this eventually led to the activation of the caspase family. Based on the above results, we can conclude that ISO can significantly induce apoptosis in osteosarcoma cells in a concentration-dependent manner.

EMT is a process in which epithelial cells gain the characteristics of mesenchymal cells during the process of tumorigenesis, and it is closely related to the processes of tumor migration and invasion [32]. To clarify the effect of ISO on the invasion and migration of osteosarcoma cells, wound healing and transwell assays were performed, and the results demonstrated that ISO significantly reduced the invasiveness and migration of osteosarcoma cells *in vitro*. Western blot analysis was then performed, and we observed that the expression levels of indicators of EMT such as Vimentin, MMP-2, and MMP-9 were significantly increased and that E-cadherin was downregulated after ISO treatment. These results were supported by the results of our experiments utilizing *in vivo* models. Based on these data, we can conclude that ISO inhibits the invasiveness and migration of osteosarcoma cells by inhibiting EMT.

The Wnt/β-catenin signaling pathway regulates various biological activities such as cell growth, apoptosis, and drug resistance to maintain the normal body function [33,34]. Accordingly, it is speculated that inhibition of Wnt signaling could provide a new therapeutic method for treating and preventing human tumors. In our study, we first verified that treatment with ISO can significantly downregulate the expression levels of downstream genes in the Wnt pathway, including c-Myc, CyclinD1, and Survivin. Inhibition of this pathway is likely the reason

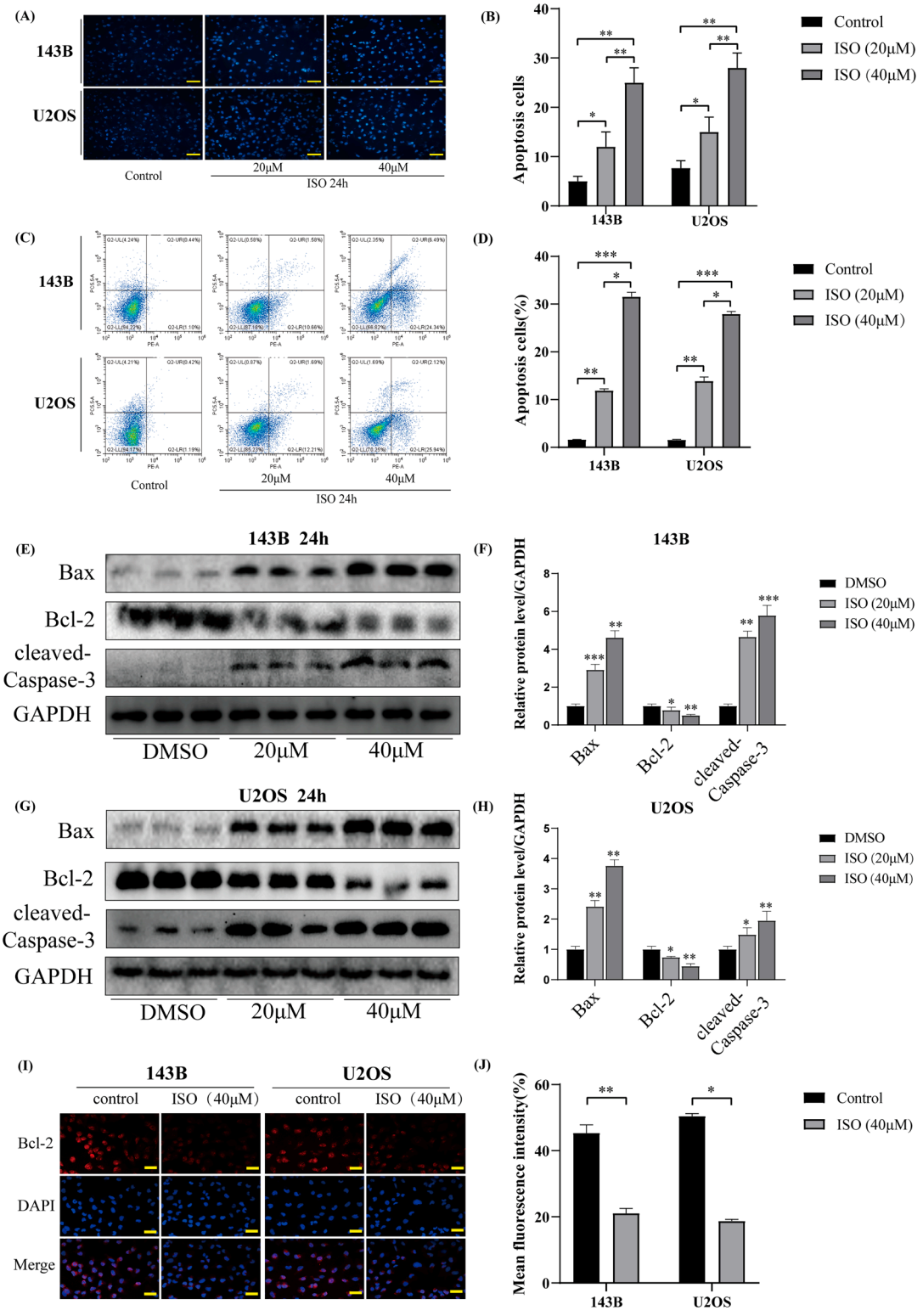

Fig. 4. Isoquercitrin induces OS cells apoptosis *in vitro* (A) After 143B and U2OS cells were treated with 20 μM and 40 μM isoquercitrin, respectively, the cells were stained with hochest33258, and cells with bright blue or shrunken nuclei were considered apoptotic cells, scale bar: 80 μm. (B) Relative quantification of apoptosis cells stained with hochest33258. (C) Apoptotic cells were stained with PE Annexin V and 7-AAD and detected by flow cytometry after cells were treated with or without isoquercitrin for 48 h. (D) Quantitative analysis of apoptosis cells in flow cytometry analysis. (E, G) Expression level of apoptosis-related proteins, including Bax, Bcl-2 and Caspase-3 after isoquercitrin treatment, GAPDH is used as an internal reference. (F, H) Quantitative display of apoptosis-related proteins. (I) Immunofluorescence images of Bcl-2 in each group after isoquercitrin treatment, scale bar: 20 μm. (J) Relative quantification of Bcl-2 in immunofluorescence analysis. Each bar represents the mean ± SD of three independent experiments, $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

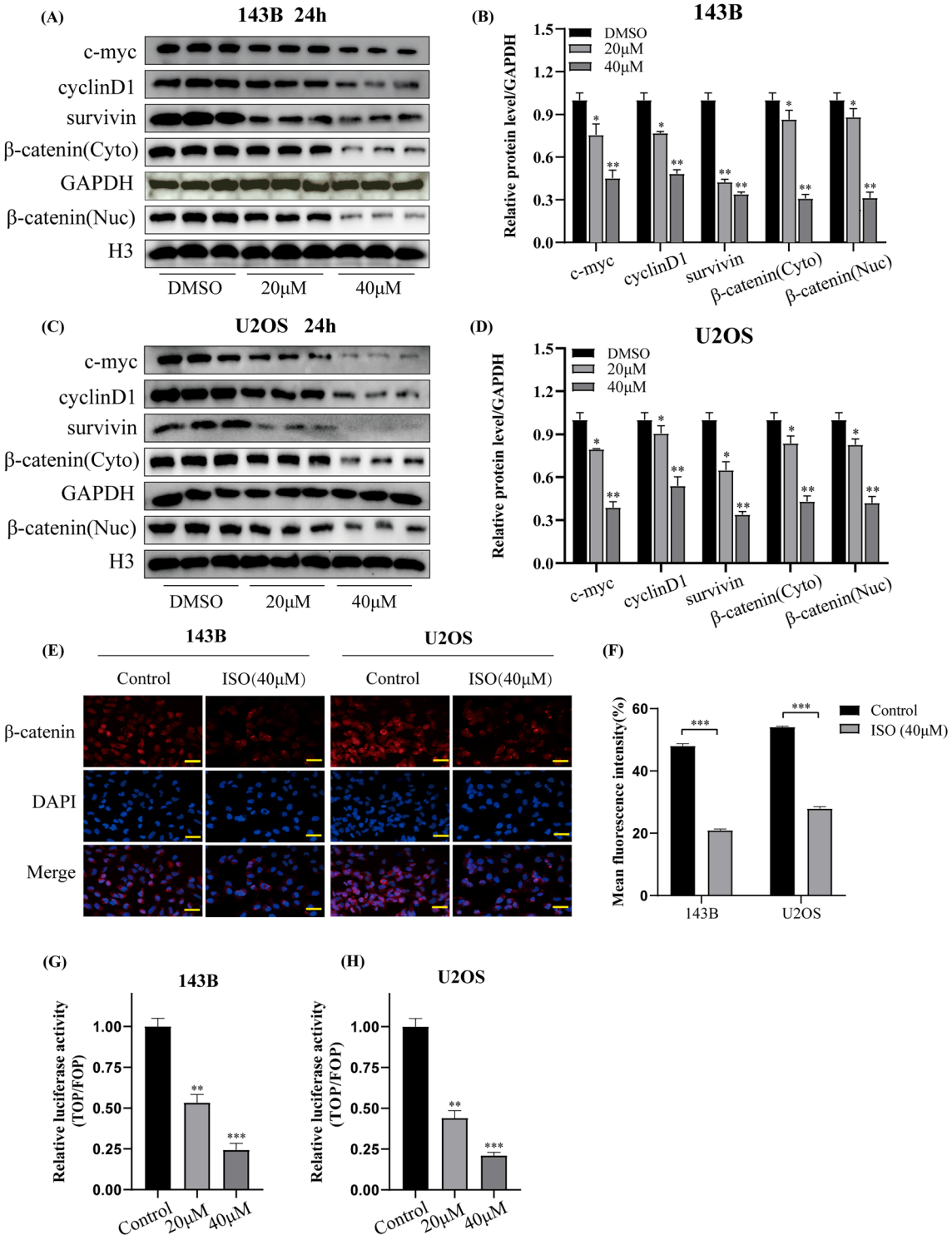

Fig. 5. Wnt/β-catenin signaling pathways was significantly repressed after treatment with ISO in OS cells *in vitro* (A-C) Expression levels of Wnt/β-catenin signaling pathway-related proteins, including β-catenin, c-myc, cyclinD1 and survivin after isoquercitrin treatment. GAPDH is used as an internal reference. (B, D) Quantitative analysis of Wnt/β-catenin signaling pathway-related protein. (E)After isoquercitrin treatment, the expression level of Bcl-2 in each group was measured by immunofluorescence, scale bar: 20 μm. (F) Relative quantification of Bcl-2 in immunofluorescence assay. (G, H) The transcriptional activity of Wnt/β-catenin signaling pathway was measured by TOP/FOP luciferase reporter assay after isoquercitrin treatment. All data are from three independent experiments and are shown as mean ± SD, $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

why osteosarcoma cell proliferation and metastasis are inhibited and apoptosis is promoted. Given the importance of the Wnt signaling pathway in regard to the development of human tumors, we hypothesized that the antitumor effect of ISO may be attributed in part to its effect on this pathway. To test our hypothesis, we constructed recombinant lentiviruses that regulate the Wnt/β-catenin signaling pathway. We observed that the effects of ISO on apoptosis, proliferation, EMT, and other phenotypes in osteosarcoma cells were significantly reversed when the Wnt/β-catenin signaling pathway was upregulated, which demonstrated that the inhibitory mechanism of ISO on

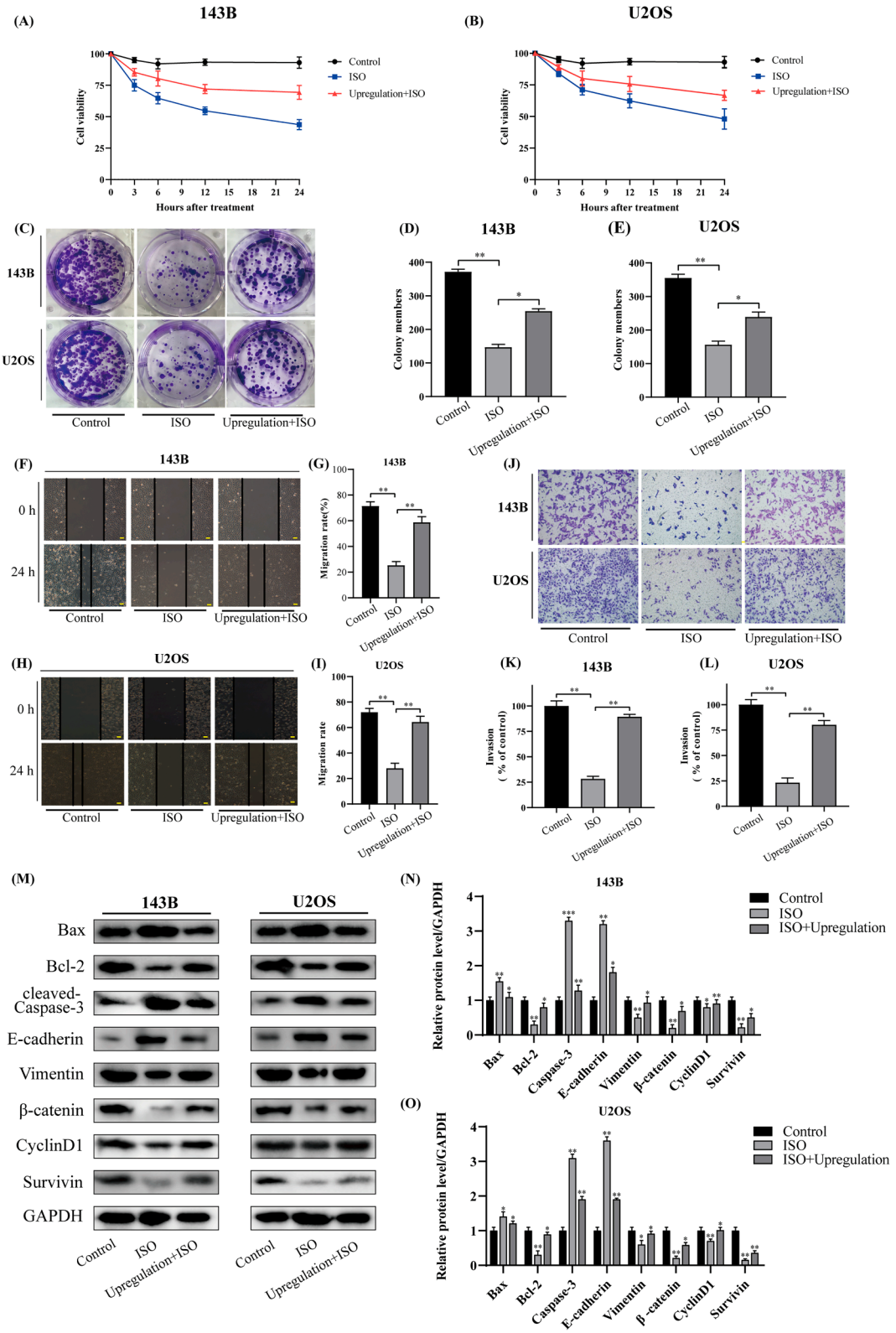

Fig. 6. Enhancing β-catenin activity rescues the anti-OS effect of isoquercitrin (A, B) The viability of stably transfected 143B and U2OS cells were detected by CCK-8 assay after treated by isoquercitrin (40 μM). (C) Upregulation of β-catenin significantly reduced the inhibitory effect of isoquercitrin on 143B and U2OS cells proliferation, while downregulation of β-catenin showed the opposite trend. (D, E) Quantitative analysis of colony formation assays. (F-G) Upregulation of β-catenin significantly reduced the inhibitory effect of isoquercitrin on migration of 143B and U2OS cells, while downregulation of β-catenin showed the opposite trend, scale bar: 80 μm. (H, I) Relative quantification of the wound-healing assay. (J) The invasion ability of 143B and U2OS cells in control group, β-catenin upregulation group and β-catenin downregulation group after isoquercitrin treatment were measured by transwell assay, scale bar: 80 μm. (K, L) Quantitative analysis of invasion cells in transwell assay. (M) Effects of up-regulation and down-regulation of β-catenin on the expression levels of some major proteins. (N) Relative quantification of some major proteins. All data are from three independent experiments and are shown as mean ± SD, * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

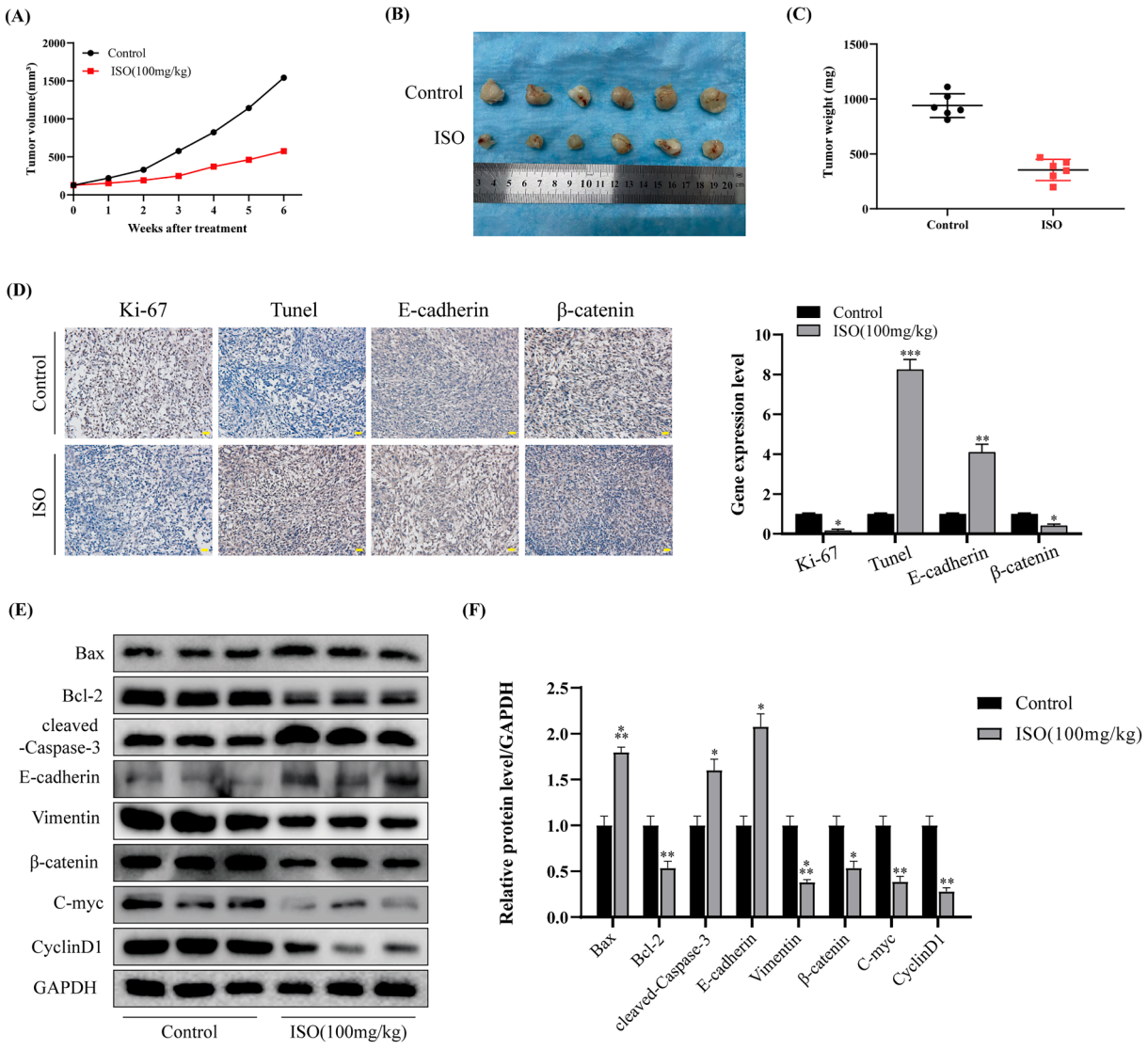

Fig. 7. ISO inhibits OS cells *in vivo* (A) Tumor volume was calculated after treated with or without isoquercitrin (B) Comparison of xenograft tumor size in nude mice with and without isoquercitrin treatment *in vivo*. (C)Tumor weight was calculated after treated with or without isoquercitrin (D) Immunohistochemical of Ki-67, TUNEL, E-cadherin and β-catenin in dissected xenograft tumor after treatment with or without isoquercitrin, scale bar: 40 μm. (E) The effect of isoquercitrin treatment on the expression level of related proteins in animal experiments. (F) Quantitative analysis of some major proteins. All data are from three independent experiments and are shown as mean ± SD, $*P < 0.05$, $**p < 0.01$, $***p < 0.001$.

osteosarcoma cells is inseparable from the Wnt/β-catenin signaling pathway.

In nude mouse xenograft models, we observed that ISO can significantly inhibit the growth and proliferation of osteosarcoma and mice treated with high concentrations of ISO exhibited smaller tumors and longer survival rates than did the control group. According to the TUNEL experiment and the Ki-67 staining results, it can be confirmed that the tumor suppressor effect of ISO is achieved by inhibiting the proliferation and inducing the apoptosis of osteosarcoma cells. Meanwhile, western blot analysis suggested that after ISO treatment, the expression levels of β-catenin and its target genes (c-Myc and CyclinD1) in animal models was decreased significantly, thus suggesting that the deeper mechanism of the tumor suppressor effect of ISO may occur through the inhibition of Wnt/β-catenin signaling pathway.

5. Conclusion

In conclusion, we observed that ISO can inhibit proliferation, induce apoptosis, and inhibit metastasis of osteosarcoma cells in an *in vitro* model, and we determined that these processes are likely related to the Wnt/β-catenin pathway (Fig. 8). Subsequently, our *in vitro* experiments revealed that ISO, as a therapeutic drug, can significantly inhibit tumor growth and prolong the survival rate of animal models and that its mechanism is directly related to the Wnt pathway. Therefore, future studies should focus on the specific targets of ISO that act on the Wnt/β-catenin signaling pathway. Nevertheless, our study revealed that ISO can inhibit osteosarcoma in part by inhibiting the Wnt/β-catenin signaling pathway, thus providing the possibility for a new therapeutic drug for the treatment of osteosarcoma.

CRediT authorship contribution statement

Zhun Wei: Conceptualization, Methodology, Investigation, Writing - review & editing. **Di Zheng:** Investigation, Writing - original draft, Formal analysis, Validation. **Wenfeng Pi:** Investigation. **Yonglong Qiu:** Investigation. Ke**zhou Xia:** Conceptualization, Resources, Project administration. **Weichun Guo:** Conceptualization, Resources, Project administration, Funding acquisition.

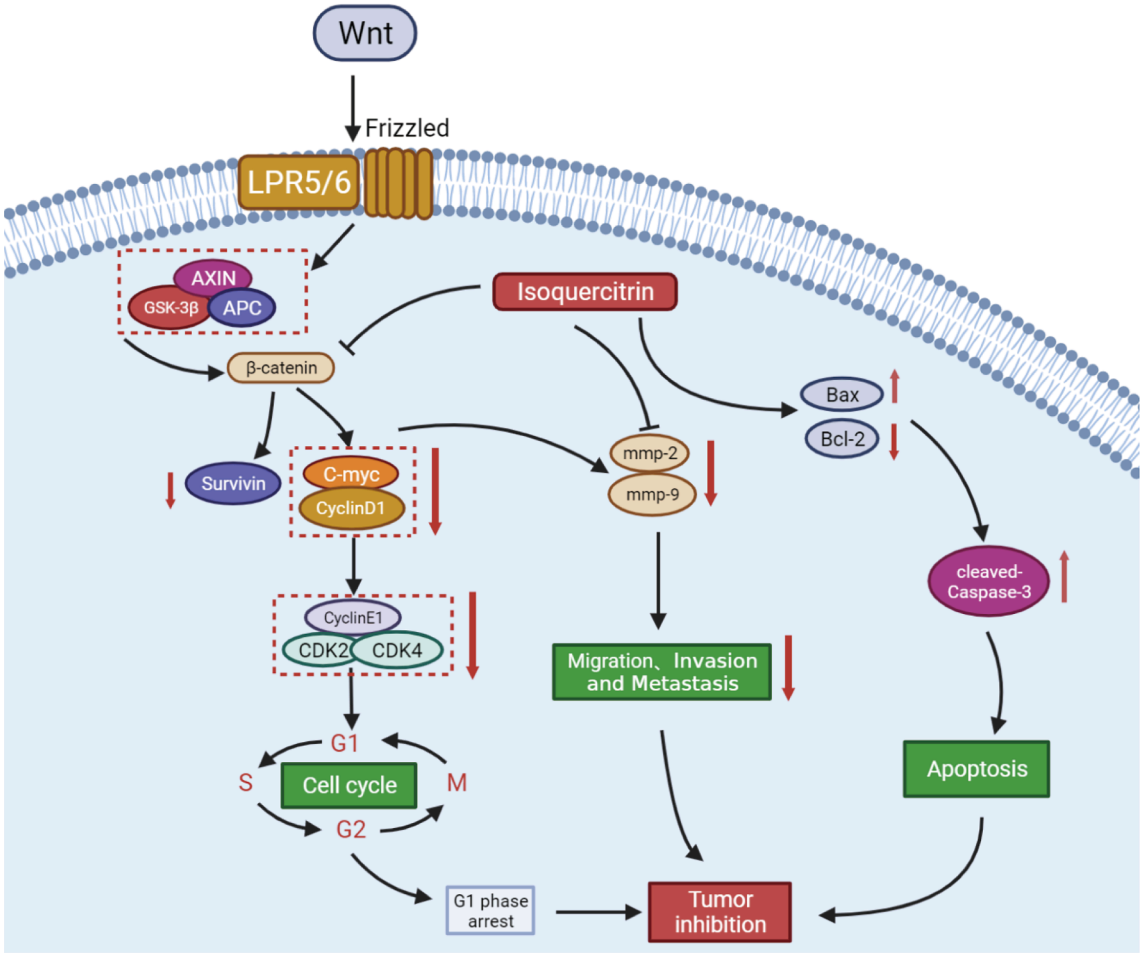

Fig. 8. A diagram depicting the mechanism of isoquercitrin on OS cells.

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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.

Data availability statement

All original data supporting the conclusions of this article are provided by the authors and without improper reservation.

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