Oridonin inhibits the proliferation of human osteosarcoma cells by suppressing Wnt/β-catenin signaling

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Abstract. It has been reported that oridonin (ORI) can inhibit proliferation and induce apoptosis in various types of cancer cell lines. However, the exact mechanism for this function remains unclear. In this study, we investigated the proliferation inhibitory effect of ORI on human osteosarcoma (OS) 143B cells and dissected the possible molecular mechanism(s) underlying this effect. We demonstrated that ORI can inhibit proliferation, induce apoptosis and arrest the cell cycle in 143B cells. Using luciferase reporter assay, we found that the Wnt/β-catenin signaling was inhibited in 143B cells by ORI. Accordingly, the total protein levels and nuclear translocation of β-catenin were reduced by ORI treatment. ORI increased glycogen synthase kinase 3β (GSK3β) activity and upregulated Dickkopf-1 (Dkk-1) expression. We found that Dkk-1 overexpression or β-catenin knockdown can potentiate the proliferation inhibitory effect of ORI in 143B cells, while β-catenin overexpression attenuated this effect. Using the xenograft tumor model of human OS, we demonstrated that ORI effectively inhibited the growth of tumors. Histological examination showed that ORI inhibited cancer cell proliferation, decreased the expression of PNCA and β-catenin. Our findings suggest that ORI can inhibit 143B OS cell proliferation by downregulating Wnt/β-catenin signal transduction, which may be mediated by upregulating the Dkk-1 expression and/or enhancing the function of GSK3β. Therefore, ORI can be potentially used as an effective adjuvant agent for the clinical management of OS.

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Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, mainly arising in the metaphysis of long bones of adolescents and young adults. OS has been characterized by a high propensity for lung metastasis, the second highest cause of cancer-related death in the pediatric age (1). The 5-year survival rate of patients with OS was very low before the 1970s when treatment for OS patients was mainly limb amputation. By now, the standard treatment associates both neoadjuvant and adjuvant chemotherapies and surgical resection of the primary tumor. Most chemotherapy regimens applied for OS are based on methotrexate, cisplatin, doxorubicin and ifosfamide. With well scheduled treatments, long-term survival rate of OS has improved to ~70%, but long-term survival in both localized and metastatic OS has stagnated in the last decades (2). Therefore, it is necessary to explore and develop more effective anticancer drugs for OS. Traditional Chinese Medicine (TCM) plays an increasingly important role in the prevention and treatment of tumors. In particular, the combination of TCM with tranditional chemotherapy agents has greatly improved the prognosis of some cancers.

Oridonin (ORI), a diterpenoid isolated from medicinal herb *Rabdosia rubescens*, has drawn attention of cancer biologists due to its remarkable antitumor activities (3). ORI has been reported to induce apoptosis in a variety of cancer cells, such as lymphoma cells (4), colon cancer cells (5), breast cancer cells (6) and leukemia cells (7). Recently, Jin *et al* reported that ORI can inactivate Akt, extracellular signal-regulated kinase (ERK), activate p38 mitogen-activated protein kinases (MAPK) and c-Jun N-terminal protein kinase (JNK) signaling pathways in human OS cells, resulting in the suppression of proliferation and apoptosis (8). Nonetheless, it remains unknown whether any other molecular mechanisms are involved in the anti-proliferation effect of ORI on OS cells.

Wnt/β-catenin signaling has been identified as one of the critical signalings in development, regulating cell growth, motility and differentiation (9). Aberrant activation of Wnt signaling is a major trait of a variety of bone and soft-tissue

sarcomas (10-13). It was reported that several Wnt ligands, receptors and co-receptors are highly expressed in OS cell lines, whereas Wnt inhibitors are suppressed (14-18). As a result, the Wnt/β-catenin signaling pathway has been considered as a target for developing novel anticancer agents for OS. Although ORI shows a strong antitumor activity in various cancers, it still remains unknown whether the exact mechanism underlaying its anticancer function is associated with Wnt/β-catenin signaling. Recently, it was found that ORI treatment activates GSK3β, a negative regulator of Wnt/β-catenin signaling, by decreasing the phosphorylation level of GSK3β in OS cell lines (8). Additionally, a previous study indicated that ORI can induce apoptosis and senescence in colorectal cancer cells partly through suppressing the expression of c-Myc (19), a downstream target of Wnt/β-catenin signaling pathway. These findings suggest that ORI may exert its antitumor activity though mediating Wnt/β-catenin signaling transduction.

In this study, we investigated the anti-proliferation effect of ORI in OS cells, and unveiled the possible mechanism responsible for the proliferation inhibitory effect of ORI in OS cells. Our results indicate that ORI can inhibit the OS cells proliferation, and this effect may be mediated by down-regulating Wnt/β-catenin signaling transduction through upregulating the expression of Dkk-1 and/or increasing the function of GSK3β.

Materials and methods

Chemicals and drug preparations. ORI was purchased from Hao-xuan Bio-tech Co., Ltd. (Xi'an, China). OS cell line 143B was purchased from American Type Culture Collection. ORI was dissolved in dimethyl sulfoxide (DMSO) for *in vitro* test, or prepared with 0.4% carboxymethylcellulose sodium (CMC-Na) as suspension for *in vivo* experiments. Antibodies were purchased from Santa Cruz Biotechnology. Cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37˚C in 5% CO_2.

Crystal violet viability assay. Crystal violet assay was conducted as described (20). Briefly, 143B cells were seeded in 24-well plates and treated with different concentrations of ORI. At the scheduled time-points, cells were washed carefully with cold (4˚C) phosphate-buffered saline (PBS) and stained with 0.5% crystal violet formalin solution at room temperature to visualize the cell viability. For quantification, crystal violet in the stained cells was extracted with 1 ml 20% acetic acid at room temperature for 20 min with shaking. A total of 100 μl was taken and added to 1 ml ddH_2O. Absorbance at 570 nm was measured. Each assay was done in triplicate.

Construction of the recombinant adenovirus. Recombinant adenoviruses expressing Dkk-1 (Ad-Dkk-1), β-catenin (Ad-BC) and small interfering RNA (siRNA) fragments targeting β-catenin (Ad-siBC) were generated previously using the AdEasy technology, as described (21-23).

Flow cytometric analysis for apoptosis and cell cycle arrest. Sub-confluent 143B cells were seeded in 6-well plates. For apoptosis assay, cells were treated with different concentrations of ORI or DMSO for 48 h. Then, cells were collected and washed with cold (4˚C) PBS, followed by incubating with Annexin V-EGFP and propidium iodide (PI) as described in the instructions of the kit (KeyGen Biotech, Nanjing, China). The stained cells were analyzed by fluorescence activated cell sorting (FACS). For cell cycle analysis, 143B cells were treated with different concentrations of ORI for 24 h. Then, cells were harvested, washed with PBS, fixed with cold (4˚C) 70% ethanol, washed with 50 and 30% ethanol, and PBS finally; stained with 1 ml of 200 mg/ml PI containing RNase (10 mg/ml) in PBS for 30 min followed by FACS for cycle analysis. Each assay was done in triplicate.

Annexin V-EGFP staining. Sub-confluent 143B cells were seeded in 24-well plates and treated with various concentrations of ORI for 12 h. Cells were washed with PBS twice and incubated with 500 μl of binding buffer and 2 μl of Annexin V-EGFP fusion protein (KeyGen Biotech) each well for 5 min, followed by washing with PBS twice. Green fluorescent protein signal was detected under a fluorescence microscope.

Western blot assay. Sub-confluent 143B cells were seeded in 6-well plates and treated with different concentrations of ORI or DMSO for 24 h. For total protein level assay, cells were washed with cold PBS and lysed in 300 μl lysis buffer. For nucleus fraction protein extraction, the protein was harvested with Nuclear and Cytoplasmic Protein Extraction kit (Thermo, no. 78833) according to the manufacturer's instructions. Cell lysates were boiled for 10 min, and then subjected to SDS-PAGE and transfered to polyvinylidene fluoride (PVDF) membranes. The membranes were immunoblotted with various primary antibodies, followed by incubating with HRP conjugated second antibodies. The proteins of interest were visualized by using the SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA). Each assay was done in triplicate.

Reverse transcription and polymerase chain reaction analysis (RT-PCR). Sub-confluent 143B cells were seeded in T25 flasks and treated with different concentrations of ORI or DMSO for 24 h. Total RNA was isolated using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA templates by RT reaction. Then, the cDNAs were used as templates for detecting the expression level of interesting genes by PCR. The primers used were as following: GAPDH, forward 5'-CAACGAATTTGGCTACAGCA-3', reverse 5'-AGGGGAGATTCAGTGTGGTG-3'; Dkk-1, forward 5'-CCT TGGATGGGTATTCCAGA-3', reverse 5'-GGCAAGACAG ACCTTCTCCA-3'; β-catenin, forward 5'-CCCACTAATG TCCAGCGTTT-3', reverse 5'-AACGCATGATAGCGTGT CTG-3'. Each assay was done in triplicate.

Luciferase reporter assay. Sub-confluent 143B cells were seeded in T25 flask and transfected with 2 μg per flask of β-catenin/Tcf4 luciferase reporter (pTop-luc) (21-23) with Lipofectamine (Invitrogen), replacing the medium 4 h later with fresh complete medium. After incubating for 12 h, cells were seeded in 24-well plates and then treated with different concentrations of ORI or DMSO. At 24 h after treatment, cells

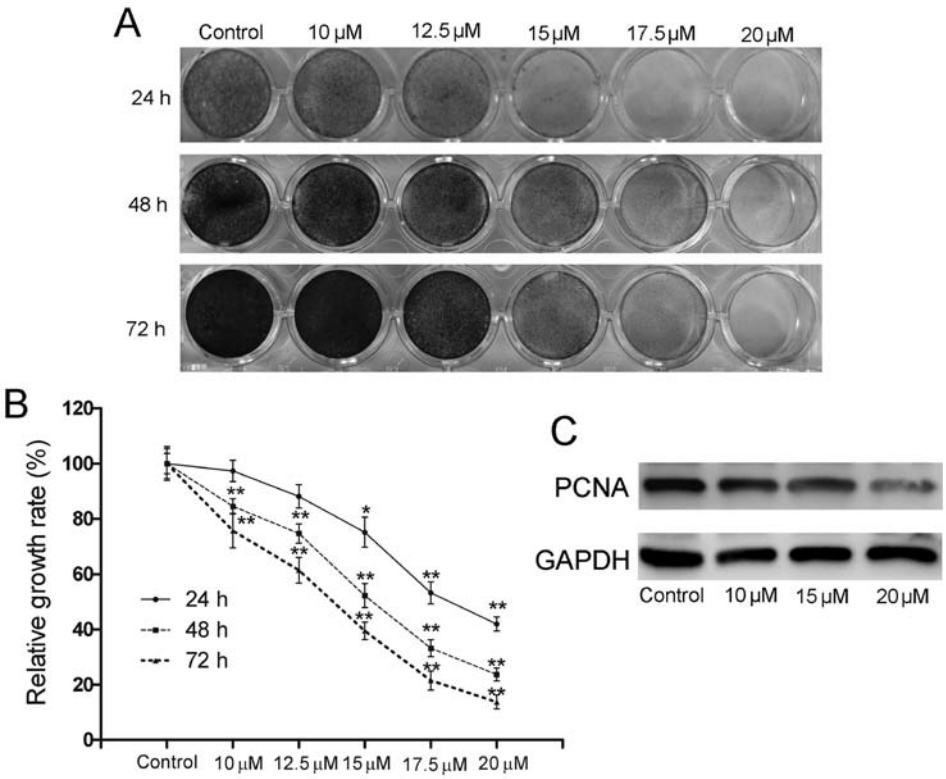

Figure 1. The effect of ORI on the proliferation in OS cells. (A) The crystal violet staining result in 143B cells. Sub-confluent 143B cells were seeded in 24-well plates and treated with different concentrations of ORI or DMSO, followed by crystal violet staining at the indicated time-points. (B) Quantitative results of crystal violet staining in 143B cells. The quantification of crystal violet staining results was processed as described in Materials and methods. The assay was performed in triplicate. $^{*}p<0.05$, compared with control; $^{**}p<0.01$, compared with control. (C) Western blot assay results show the PCNA protein level in 143B cells. GAPDH was used as loading control.

were lysed and subjected to luciferase assays using luciferase assay kit (Promega, E1500). Each assay was done in triplicate.

Xenograft tumor model of human OS. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University. Athymic nude mice (female, 4-6-week old, 5/group) were ordered from the Animal Centre of Chongqing Medical University (Chongqing, China). 143B cells were collected and resuspended in cold PBS to a final density of $2x10^7$ cells/ml. Cells in 50 μl of cold PBS were injected into the proximal tibia of athymic mice. At 3 days after injection, animals were treated with either different doses of ORI (50 and 100 mg/kg) or solvent by intragastric administration once a day. Five weeks after injection, the animals were sacrificed and the tumor samples were retrieved for histological evaluation.

Histological evaluation and immunohistochemical staining. Retrieved tumor masses were fixed in 10% formalin and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (24). For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated manner. The deparaffinized slides were subjected to antigen retrieval and probed with an anti-proliferating cell nuclear antigen (PCNA) antibody or anti-β-catenin antibody, or isotype IgG as control, followed by incubation with biotinylated secondary antibodies and streptavidin conjugated horseradish peroxidase. The proteins of interest was visualized by DAB staining and examined under a microscope as described (24).

Statistical analysis. All quantitative experiments were performed in triplicate. Data are expressed as mean ± SD. Statistical significance between vehicle treatment versus drug treatment was determined by the Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results

ORI inhibits the proliferation in OS cells. Using crystal violet staining, we firstly explored the proliferation inhibitory effect of ORI on 143B cells to validate whether ORI can be used as a novel chemotherapeutic agent for human OS. It was shown that ORI effectively inhibited the proliferation of 143B cells in a time- and concentration-dependent manner (Fig. 1A and B). As shown in Fig. 1C, ORI also significantly suppressed the expression of proliferating cell nuclear antigen (PCNA), a marker for the assessment of OS growth (25).

ORI induces apoptosis in OS cells in vitro. We investigated whether ORI can induce OS cells to undergo apoptosis. The 143B cells were treated with indicated concentrations of ORI or DMSO for 24 or 48 h. Then, cells were subjected with FACS analysis (Fig. 2A), or were lysed and subjected to western blot analysis for detecting caspase-3 protein level (Fig. 2B). The results showed that ORI can induce apoptosis in 143B cells,

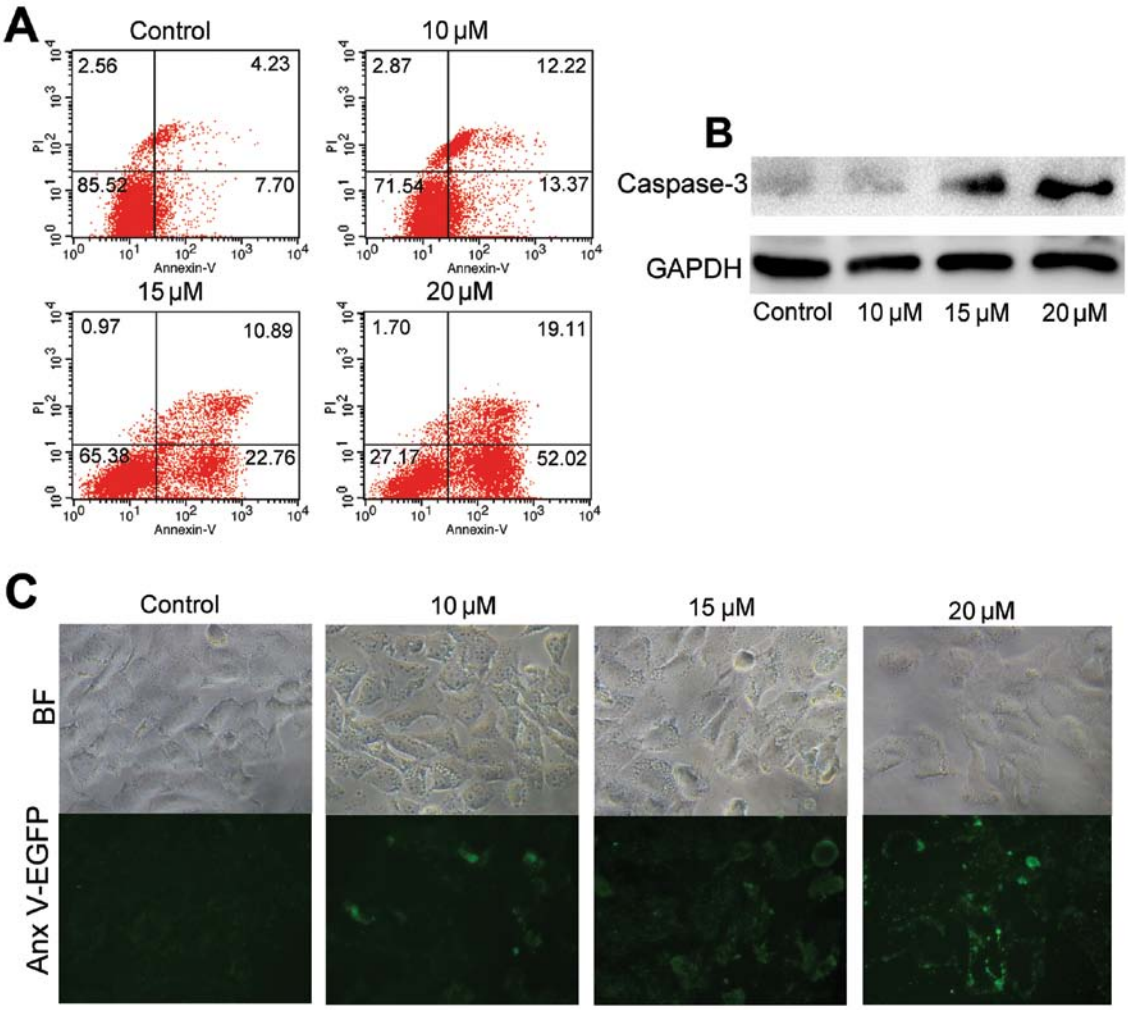

Figure 2. The effect of ORI on apoptosis in human OS cells. (A) FACS analysis showed apoptosis induced by ORI. Cells were seeded in 6-well plates, and treated with the indicated concentration of ORI or DMSO for 48 h, then collected and stained for FACS. (B) Western blot assay showed the protein level of caspase-3. Cells were seeded in 6-well plates and treated with the indicated concentration of ORI or DMSO for 24 h, and then cells were harvested and subjected to western blot assay using an antibody against caspase-3. GAPDH was used as loading control. (C) Annexin V-EGFP staining results show the apoptosis induced by ORI. 143B cells were seeded in 24-well plates and treated with ORI at the indicated concentrations for 12 h. Cells were washed with PBS twice, and incubated with binding buffer and Annexin V-EGFP in each well for 5 min, followed by washing with PBS twice. Green fluorescent protein signal was detected under a fluorescence microscope.

and the protein expression of caspase-3 increased at 24 h in a concentration-dependent manner. Furthermore, 143B cells were also stained with Annexin V-EGFP fusion protein after treatment with different concentrations of ORI for 12 h. We found that ORI induced EGFP staining in a concentration-dependent manner (Fig. 2C), indicating that ORI can effectively induce the translocation of phosphatidylserines in cell membrane phospholipids from the inner surface to the outer surface during the early stages of apoptosis. Taken together, these results suggest that ORI can induce apoptosis in OS cells.

ORI arrests the cell cycle at G1 phase and inhibits the Wnt/β-catenin signaling in human OS cells. To explore the mechanism of ORI-induced proliferation inhibition and apoptosis in human OS cells, we tested whether these functions were associated with the cell cycle arrest. It was shown that the cells in G1 phase of ORI treated groups increased compared to that of the control group concentration-dependently (Fig. 3A), suggesting that ORI can arrest the cell cycle at G1 phase in human OS cells. Cell cycle control is a pivotal event controlled by many essential signaling pathways. Wnt/β-catenin signaling is one of these pathways (26). Therefore, we investigated whether ORI can target Wnt/β-catenin signaling to exert its anticancer activity in 143B cells. Using luciferase reporter assay, we examined the effect of ORI on the β-catenin/Tcf4-responsive reporter and found that ORI effectively inhibited the reporter activity (Fig. 3B). In addition, we further examined the expression of the known target of Wnt/β-catenin signaling, c-Myc, in response to ORI treatment. The result showed that c-Myc expression was decreased in ORI-treated 143B cells in a concentration-dependent manner (Fig. 3C). Taken together, these results suggest that ORI can inhibit Wnt/β-catenin signaling transduction.

ORI inhibits β-catenin expression in human OS cells. Given that stabilization and nucleus translocation of β-catenin are the key events in the transduction of the canonical Wnt/β-catenin signaling, we conducted western blot analysis to

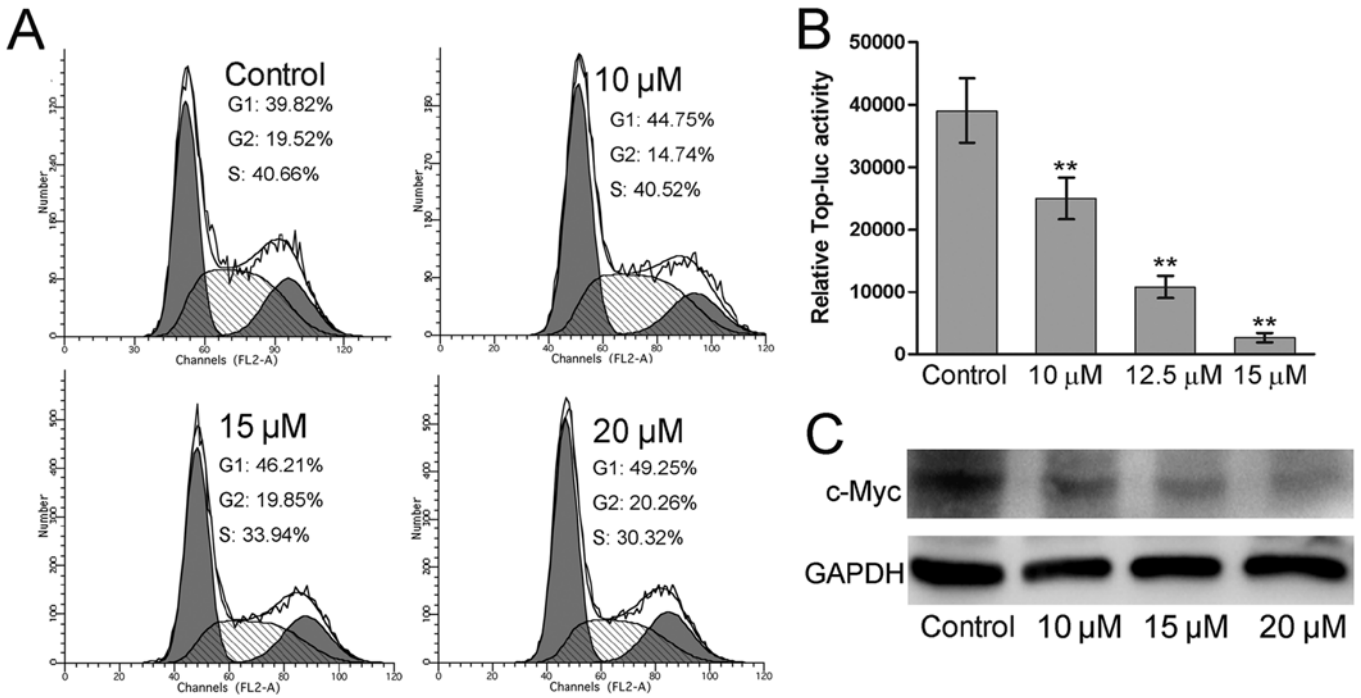

Figure 3. The effect of ORI on the cell cycle and Wnt/β-catenin signaling in human OS cells. (A) Cell cycle analysis showed that ORI induces cell cycle arrest at G1 phase in 143B OS cells. (B) ORI inhibits β-catenin/TCF transcription activity. The 143B cells were seeded in 6-well plates and treated with the indicated concentrations of ORI for 24 h, then harvested for luciferase activity assay. The test was performed in triplicate. $^{**}p<0.01$, compared with control. (C) Western blot result shows the effect of ORI on the protein level of c-Myc. Cells were seeded in 6-well plates and treated with the indicated concentration of ORI or DMSO for 24 h, and then cells were harvested for western blot assay. GAPDH was used as loading control and each assay was performed in triplicate.

explore whether ORI can suppress β-catenin protein level in the cytoplasm, nucleus and the whole cell to investigate how ORI inactivates Wnt/β-catenin signaling. The results indicated that ORI can decrease the β-catenin protein level not only in the nucleus, but also in the cytoplasm and the whole cell after the treatment with ORI for 24h (Fig. 4A). To validate the role of β-catenin in the proliferation inhibitory effect of ORI in OS cells, we tested the effects of exogenous expression or knockdown of β-catenin on the proliferation inhibitory effect of ORI in OS cells. We found that exogenous expression of β-catenin attenuated the growth inhibitory function of ORI, while knockdown of β-catenin enhanced this function in 143B cells (Fig. 4B), suggesting that downregulation of β-catenin plays a critical role in the function of ORI in OS cells. To explore the mechanism by which ORI inhibits the β-catenin protein expression in 143B cells, we determined whether ORI can decrease the expression of β-catenin at mRNA level by RT-PCR analysis. However, the result showed that ORI did not affect the mRNA expression of β-catenin (Fig. 4C), suggesting that other cytokines involved in Wnt/β-catenin signaling may participate in ORI-induced downregulation of β-catenin.

The stability of β-catenin in cells is tightly regulated by the Axin/APC/GSK3β complex. Phosphorylation of β-catenin by GSK3β results in its degradation, which leads to the inactivation of Wnt/β-catenin signaling (27,28). Thus, we explored whether ORI can affect the expression of GSK-3β, the negative regulator of Wnt/β-catenin signaling. We found that its protein expression level was markedly increased in response to ORI (Fig. 4A). Previous studies have demonstrated that phosphorylation of GSK3β at Ser9 can lead to GSK3β inactivation (29,30), we therefore also examined the effect of ORI on the phosphorylation of GSK3β. As shown in Fig. 4A, the phosphorylation level of GSK3β was reduced under the action of ORI. In addition to GSK3β, we also found that the expression of another canonical Wnt inhibitor, Dickkopf-1 (Dkk-1), was significantly improved upon ORI treatment (Fig. 4A and C). Overexpression of Dkk-1 enhanced the proliferation inhibitory effect of ORI in 143B OS cells (Fig. 4B). The above results suggest that ORI may regulate Wnt/β-catenin signaling through enhancing the function of GSK3β and/or upregulation of Dkk-1 to exert its anticancer activity in OS cells.

ORI inhibits tumor growth in the xenograft model of human osteosarcoma. We further assessed the *in vivo* anti-osteosarcoma effects of ORI. Using a xenograft tumor model, we injected the 143B cells subcutaneously into the flanks of athymic nude mice. At one week after injection, animals were given different doses of ORI (50 or 100 mg/kg) or solvent as control by intragastric administration, once a day up to 4 weeks. The result showed that treatment with ORI resulted in significant suppression of tumor growth in nude mice dose-dependently, compared with the solvent control group (Fig. 5A and B). We subsequently conducted histologic evaluation of the tumor samples. Hematoxylin and eosin (H&E) staining showed that ORI treatment group exhibited more necrotic cells than those of solvent control group (Fig. 5C). Moreover, we also examined the expression pattern of β-catenin protein. Consistent with our *in vitro* result, β-catenin was dramatically decreased in ORI-treated groups (Fig. 5D). PCNA-positively stained cells were significantly decreased in the ORI treatment group, compared with control group (Fig. 5E). Collectively, these *in vivo* results

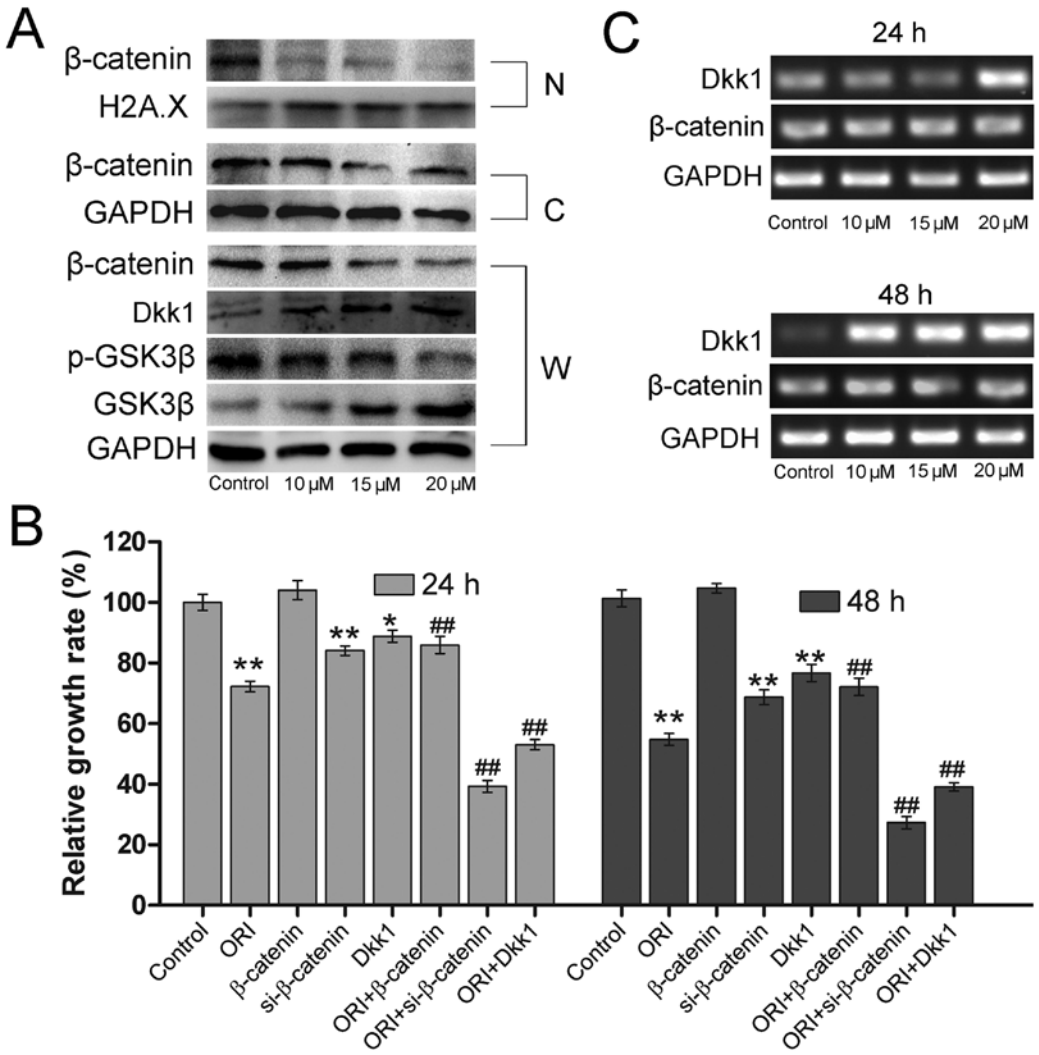

Figure 4. ORI targets the Wnt/β-catenin signaling for the proliferation inhibitory effect in human OS cells. (A) Western blot assay results show the effect of ORI on p-GSK3β (Ser 9), GSK3β, and β-catenin in the nucleus, cytoplasm and the whole cell (N, nucleus; C, cytoplasm; W, whole cell). GAPDH was used as loading control. The 143B cells were seeded in 6-well plates and treated with the indicated concentrations of ORI for 24 h and then harvested for western blot assay. (B) The effect of β-catenin and Dkk-1 on the proliferation inhibitory effects of ORI on OS cells. The 143B cells were seeded in 24-well plates and infected with Ad-BC, Ad-siBC or Ad-Dkk-1 in the presence or absence of 15 μM ORI. At 24 and 48 h after treatment, the cells were stained with crystal violet and growth rate was quantified. The assay was performed in triplicate. *p<0.05, compared with control; **p<0.01, compared with control; $^{\#\#}$p<0.01, compared with ORI. (C) The effect of ORI of mRNA expression of β-catenin and Dkk-1. 143B cells were treated with the indicated concentrations of ORI for 24 or 48 h, and then semiquantitative RT-PCR was performed to assess the expression of β-catenin and Dkk-1 in gene level. GAPDH was used as loading control.

further indicate that the inhibitory effect of ORI on OS cells may result from the inactivation of Wnt/β-catenin signaling.

Discussion

ORI is a diterpenoid compound extracted from the Chinese traditional medicine herb *Rabdosia rubescens* (3). It has been reported that ORI can inhibit proliferation and induce apoptosis in various cancer cells (4). However, the effect of ORI on the proliferation of OS cells remains unclear, as well as the exact mechanism underlaying this function. In the present study, our results demonstrated that ORI can inhibit the proliferation and induce apoptosis in 143B OS cells. Mechanistically, we found that the anti-proliferation activity of ORI in 143B OS cells may be mediated by downregulating Wnt/β-catenin signaling transduction through upregulating the expression of Dkk-1 and/or promoting the activity of GSK3β.

ORI has been shown to be able to target several crucial genes and signaling pathways that are responsible for regulating apoptotic cell death and the cell cycle (31), a few studies have attempted to clarify the possible molecular mechanism by which ORI exerts its anticancer activities. For example, ORI can inactivate Akt and ERK, and activate p38 MAPK and JNK signaling pathways in OS cells, resulting in the suppression of proliferation and induction of apoptosis (8). However, another study showed a converse observation that ERK served as a tumor suppressor and linked mitochondrial-related apoptotic pathway to MAPK-mediated pathways in ORI-treated human melanoma A375-S2 cells (32). Furthermore, it was also evidenced that ORI could induce cell cycle arrest and apoptosis through activating ERK-p53 apoptotic pathway and inhibiting PTK-Ras-Raf-JNK survival pathway in murine fibrosarcoma L929 cells (33). These findings suggest that the regulatory effect of ORI on MAPK signaling is cell type-specific. In

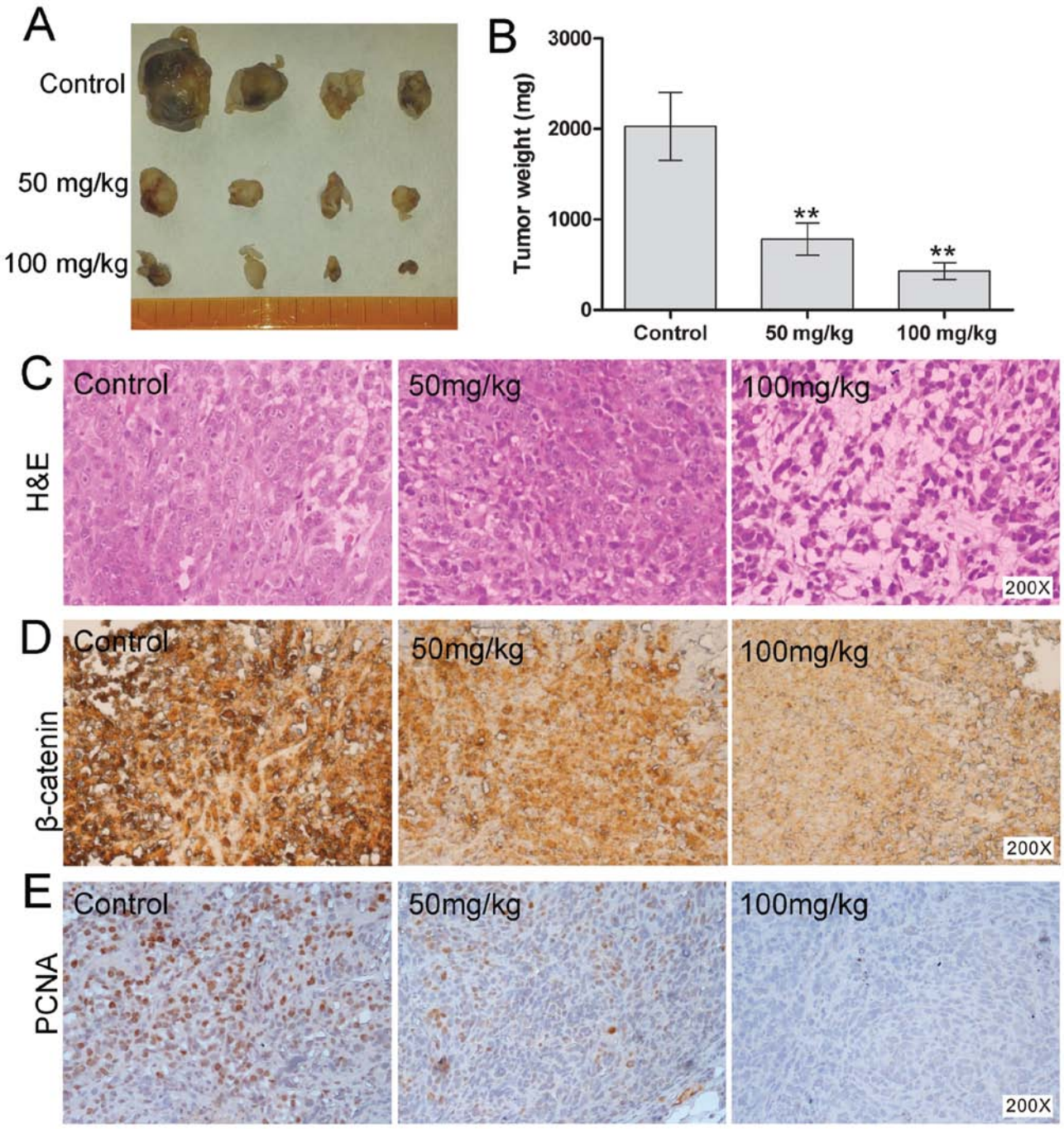

Figure 5. The effect of ORI on xenograft OS tumor growth. (A and B) The inhibition of xenograft tumor growth of human OS cells by ORI. After the last treatment, tumor samples were retrieved from the OS tumor animal model and tumor weight was measured. $^{**}p<0.01$, compared with control. (C-E) Histologic and immunohistochemical staining of ORI treated OS tumors. Tumor samples derived from 143B xenografts were retrieved, fixed and paraffin embedded. Sections were used for hematoxylin and eosin (H&E) staining. Deparaffinized slides were stained with antibody against β-catenin and PCNA. Representative results are shown.

addition to MAPK signaling, ORI was reported to be able to inhibit proliferation and induce caspase-dependent apoptosis via downregulation of PI3K/Akt pathway in cervical carcinoma HeLa cell line (34). Treatment of prostate cancer cells with ORI also caused the upregulation of P21, autophagy and apotosis (35). Although these studies have provided important insights into the molecular mechanism through which ORI exhibits anticancer activity, it is still conceivable that other signaling pathways may also participate in the anticancer activity of ORI.

It has been well demonstrated that Wnt/β-catenin signaling pathway plays a critical role in various cancers. In the canonical Wnt/β-catenin signaling, Wnt ligands bind to the dual receptor complex comprised of frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). This leads to inactivation of the β-catenin destruction complex, Axin/APC/GSK-3β, thus relieving the critical mediator β-catenin from its constitutive proteosomal degradation. β-catenin subsequently accumulates in the cytoplasm and translocates into the nucleus, where it associates with transcription factors

to regulate the downstream target genes (36,37). Deregulation of Wnt signaling has been implicated in the development and pathogenesis of a wide range of cancers (38), including OS. A previous study has evidenced that increased cytoplasmic and/or nuclear accumulation of β-catenin protein is a common occurrence in human OS that is implicated in the pathogenesis of OS (39). Additionally, it was also reported that the OS often expresses the Wnt co-receptor LRP5 which significantly correlates with metastaic disease in human OS (14). Moreover, the Wnt inhibitory factor 1 (WIF1), which encodes an endogenous secreted Wnt pathway antagonist, was reported to be epigenetically silenced in human OS; targeted deletion of mouse WIF1 could accelerate osteosarcomagenesis *in vivo* (18). Conversely, overexpression of WIF1 and dominant negative mutation of LRP5 effectively decreased tumorigenicity and metastasis of OS *in vivo* (40,41). Based on these observations, the compounds from Chinese herbal medicine targeting Wnt/β-catenin signaling can be considered as attractive candidates for OS treatment.

Up to now, athough ORI can mediate various signaling pathways to exert its anticancer activities in OS, the effect of ORI on Wnt/β-catenin signaling still remains to be elucidated. Given that ORI could activate GSK3β activity and suppress the expression of c-Myc (8,19), we speculated that the anticancer effect of ORI on OS cells may result from targeting Wnt/β-catenin signaling. In this study, our data showed that ORI can reduce the protein level of β-catenin in both nucleus and cytoplasm in a concentration-dependent manner. Overexpression of β-catenin can attenuate the function of ORI, while knockdown of β-catenin can enhance the growth inhibitory effect of ORI. Using a xenograft tumor model of human OS, we demonstrated that ORI can also inhibit cancer cell proliferation *in vivo*. The histologic examination result has revealed a decreased staining intensity of β-catenin in ORI treatment group. Therefore, these results suggest that the inhibitory effect of ORI on OS is at least resulted from reducing nuclear translocation of β-catenin protein. However, the exact mechanism through which ORI downregulates β-catenin is still unclear. Herein, we examined the β-catenin mRNA expression level in 143B cells and found that ORI has no effect on the mRNA expression of β-catenin, suggesting that ORI may target some other components of Wnt/β-catenin signaling pathway to promote the degradation of β-catenin.

The stability of β-catenin in cells is tightly regulated by the Axin/APC/GSK3β complex. The phosphorylation of β-catenin by GSK3β results in ubiquitin-mediated degradation of β-catenin leading to the inactivation of Wnt/β-catenin signaling. Therefore, GSK3β has been identified as a tumor suppressor that is frequently inactivated in various tumors (42). However, studies have provided evidence that the exact role of GSK3β in tumorigenesis of human OS is controversial (43-46). In the present study, we explored the effect of ORI on GSK-3β and found that the total GSK-3β protein level was significantly elevated in response to the treatment with ORI. Moreover, the phosphorylation of GSK3β at Serine 9 was diminished. This result is supported by another study in which ORI decreased the phosphoralation level of GSK-3β in U2OS, SaOS-2 and MG63 OS cell lines (8). Our data indicate that downregulation of β-catenin protein in OS cells may result from the degradation initialized by GSK-3β, at least. In addition to GSK-3β, other natural Wnt antagonists have been identified, including the Dickkopf (Dkk) family consisting of four secretory proteins (Dkk-1, Dkk-2, Dkk-3, and Dkk-4) (47,48). Dkk-3 has been shown to have a negative impact on the progression of OS (49,50). To further explore the mechanism underlying the regulatory role of ORI in Wnt/β-catenin signaling, we examined whether Dkks could be influenced by ORI. Though we found no changes in the mRNA expression pattern of Dkk2, Dkk3 and Dkk4 (data are not shown), ORI dramatically upregulated the expression of Dkk-1. Moreover, overexpression of Dkk-1 potentiated the anti-proliferative effect of ORI in OS cells. It suggests that upregulation of Dkk-1 by ORI may also relate to the anticancer activities of ORI by inactivation of Wnt/β-catenin signaling in OS cells.

Taken together, our data suggest that ORI can be used as an effective chemotherapy agent for human OS. The anticancer effect of ORI in OS cells may result from inactivating Wnt/β-catenin signaling transduction through increasing GSK-3β activity and/or upregulation of Dkk-1. Future studies should be directed to the identification of more ORI target proteins, which is essential to elucidate the molecular mechanism underlying ORI anticancer activity. On the other hand, more strict and robust pre-clinical examinations should also be performed to ensure the safety of ORI before developing ORI to an antitumor drug.

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