

Metformin increases sensitivity of osteosarcoma stem cells to cisplatin by inhibiting expression of PKM2

DEPENG SHANG^{1*}, JU WU^{2*}, LIANYI GUO³, YANJU XU¹, LEZI LIU¹ and JIANMIN LU¹

Departments of ¹Orthopedics, ²General Surgery, Zhongshan Hospital Affiliated of Dalian University, Dalian, Liaoning 116001; ³Department of Gastroenterology, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning 121001, P.R. China

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Abstract. Multiple drug resistance is reported to be a major obstacle in treatment of osteosarcoma (OS). Research has demonstrated that small subsets of cells called cancer stem cells (CSCs) are responsible for multiple drug resistance. CSCs are potential targets for reversing chemoresistance. In the present study, we compared cisplatin sensitivity between OS stem cells and OS non-stem cells. We confirmed that OS stem cells showed significant cisplatin-resistance compared with the OS non-CSCs. Mechanically, we proved that over-expression of the pyruvate kinase isoenzyme M2 (PKM2) was responsible for the resistance to cisplatin in OS stem cells. As a potential strategy, we found that co-treatment with metformin significantly decreased the half maximal inhibitory concentration (IC₅₀) of cisplatin to HOS OS stem cells by downregulating the expression of PKM2. PKM2 downregulation resulted in, metformin inhibited glucose uptake, lactate production and ATP production in HOS CSCs. Therefore, metformin impaired the resistance of HOS CSCs to cisplatin and promoted cisplatin-induced apoptosis. In addition, antitumor effects of other chemotherapeutic drugs such as doxorubicin and 5-fluorouracil were proved to be enhanced by metformin on OS stem cells.

Introduction

Osteosarcoma (OS) is a malignant bone cancer commonly diagnosed in children and adolescents (1). At present, chemotherapy is still an effective treatment to kill OS cells and prolong the life of patients. However, repeated use of chemotherapeutic drugs induces inevitable multiple drug resistance

in cancer cells (2,3). Reliable strategies are urgently required to reverse the multiple drug resistance of OS.

Cancer stem cells (CSCs) are small cell subsets existing in cancer. Characteristics of CSCs include high self-renewal capacity and multilineage differentiation potential. They are important for tumor formation and development (4,5). CSCs are considered to be responsible for induction of acquired drug resistance. Continuous chemotherapy induces stemness in OS cells (6-8). CD133, a glycoprotein on cell surface, is identified as the molecular marker of cancer stem cells in many tumors including OS (9,10). CD133-positive OS cells are considered as effective targets for reducing chemoresistance (11).

Metformin is an anti-diabetic drug, which is the first-choice for patients with type-II diabetes (12). Of note, metformin therapy is shown to reduce cancer incidence in patient with diabetes. Metformin treatment reduces the risk of many tumors including breast cancer, lung cancer and colorectal cancers (13-16). However, the mechanism by which metformin inhibits malignancies still remains unclear. Some studies demonstrate that metformin can reduce the production of ATP in cancer cells, therefore inhibiting cell cycle and proliferation of cancer (17,18). In this study, we investigated the potential role of metformin in OS stem cells. We report that metformin treatment can inhibit glucose metabolism and increase sensitivity of OS stem cells to chemotherapy.

Materials and methods

Cell culture. Human osteosarcoma cell lines HOS, Saos-2 and MG-63 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). For separation of cancer stem cells from OS cell lines, HOS, Saos-2 and MG-63 cells were incubated with fluorescein isothiocyanate (FITC) conjugated CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at room temperature. Then, cells were washed with cold PBS twice followed by sorting the CD133-positive OS cells and CD133-negative OS cells on a FACS vantage (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). CD133-positive OS cells were considered as the OS stem cells, whereas the CD133-negative OS cells were identified as the OS non-stem cells (9). All of these cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen, Carlsbad, CA, USA).

Correspondence to: Dr Jianmin Lu, Department of Orthopedics, Zhongshan Hospital Affiliated of Dalian University, 6 Jiefang Street, Dalian, Liaoning 116001, P.R. China
E-mail: jnxuechengcao@yeah.net

*Contributed equally

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Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNAs were extracted from HOS, Saos-2 and MG-63 OS cells using Trizol reagent (Invitrogen). cDNA of these cells was synthesized using M-MLV Reverse Transcriptase (Invitrogen) by taking total RNAs as templates. For detecting the expression of PKM2, Real-time PCR was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) on ABI PRISM 7900 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). GAPDH gene was used as the internal reference to determine the relative expression of PKM2.

PKM2 plasmid and siRNA. To investigate the role of PKM2 in OS stem cells, we overexpressed or knocked down the PKM2 by transfecting with PKM2 plasmid or small interfering RNA (siRNA), respectively. For conducting PKM2 eukaryotic expression plasmid, open reading frame of PKM2 gene was amplified by PCR followed by linking the PCR products to pcDNA3.1 plasmid (Invitrogen). For knockdown of PKM2, siRNA targets to PKM2 was purchased from Genechem Co., Ltd. (Shanghai, China). For transfection, 2 μ g/ml PKM2 plasmid or 50 pmol/ml PKM2 siRNA were transfected into the OS stem cells by using Lipofectamine 2000 (Invitrogen).

Cell viability and IC_{50} . OS cells (1×10^4) were seeded in 96-well plates and cultured at 37°C. Cells were transfected with PKM2 vector or PKM2 siRNA. At 24 h after transfection, the cells were treated with 2 mM metformin and 8 μ M cisplatin for 48 h, cell viability was measured by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assays. The absorbance was measured at 450 nm by using an ELISA microplate reader (Sunrise microplate reader; Tecan, Mannedorf, Switzerland). Half maximal inhibitory concentration (IC_{50}) was calculated according to the results of CCK-8 assays.

Western blot analysis. Total proteins were extracted from OS stem cells and OS non-CSCs using radio immunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). The proteins in cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gels were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% non-fat dry milk for 2 h followed by incubating with primary antibodies against PKM2, pro-caspase-9, pro-caspase-3, cleaved caspase-9, cleaved caspase-3 and β -actin (Cell Signaling Technology, Inc.) overnight. Then, the proteins on PVDF were incubated with appropriate HRP-conjugated secondary antibodies followed by detecting the protein signals by using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Analysis of apoptosis. HOS CSCs were transfected with PKM2 vector followed by treating with 2 mM metformin and 8 μ M cisplatin. The cells were stained using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) and subsequently analyzed using flow cytometry (Becton-Dickinson, San Jose, CA, USA).

Proliferation assay. HOS CSCs were transfected with PKM2 vector followed by treating with 2 mM metformin and 8 μ M

cisplatin. Proliferation of HOS CSCs was measured by using 3 H-thymidine incorporation assay.

Glucose uptake measurement. HOS CSCs were transfected with PKM2 vector followed by treating with 2 mM metformin and 8 μ M cisplatin. Relative glucose uptake was measured using an Amplex Red Glucose/Glucose Oxidase assay kit (Molecular Probes, Camarillo, CA, USA) according to the manufacturer's instructions.

Detection of lactate production. HOS CSCs were transfected with PKM2 vector followed by treating with 2 mM metformin and 8 μ M cisplatin. Production of lactate in HOS CSCs was detected by using a Lactate assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol.

ATP measurement. HOS CSCs were transfected with PKM2 vector followed by treating with 2 mM metformin and 8 μ M cisplatin. After treatment, cells were harvested and washed with PBS twice. NP40 solution (1%) was used to split the cells. ATP in the cell lysates were measured by using ATP Colorimetric/Fluorometric Assay kit (BioVision).

Tumor growth in nude mice. Four-week-old female immunodeficient nude BALB/c mice were purchased from Shanghai Super-B&K Laboratory Animal Corp., Ltd. (Shanghai, China). For xenografts, mice were injected subcutaneously into the right armpit with 5×10^6 HOS CSCs. Cisplatin (5 mg/kg) and metformin (200 mg/kg) were administered by intraperitoneal injection twice a week. Tumor size was measured every five days. Tumor volume was calculated according to the following formula: volume (V) = $1/2 \times \text{length} \times \text{width}^2$. For detection of PKM2 expression in tumor tissues, collagenase type III (Worthington Biochemical Corp., Lakewood, NJ, USA) was used to digest the tissues. The animal care and experimental protocols were approved by the Animal Care Committee of The Affiliated Zhongshan Hospital of Dalian University and complied with the recommendations of the Chinese guidelines for the care and use of laboratory animals. The study was approved by ethics committee of The Affiliated Zhongshan Hospital of Dalian University.

Statistical analysis. Data are presented as mean \pm SD and analyzed by using SPSS 14.0. Two-tail Student's t-test and ANOVA were performed to determine the differences. A value of $P < 0.05$ was considered to indicate a statistically significant difference. All the experiments were independently repeated at least 3 times.

Results

Osteosarcoma stem cells are resistant to cisplatin treatment. To investigate the cisplatin sensitivity of OS stem cells and OS non-stem cells, we first separated them from the HOS, Saos-2 and MG-63 OS cell lines. As shown in Fig. 1A, the separation efficiency was evaluated by flow cytometry. CD133-positive OS cells were considered as the OS stem cells. Whereas, the CD133-negative OS cells were identified as the OS non-stem cells. Results of CCK-8 assays showed that the sensitivity of OS stem cells (HOS CSCs, Saos-2 CSCs and MG-63 CSCs)

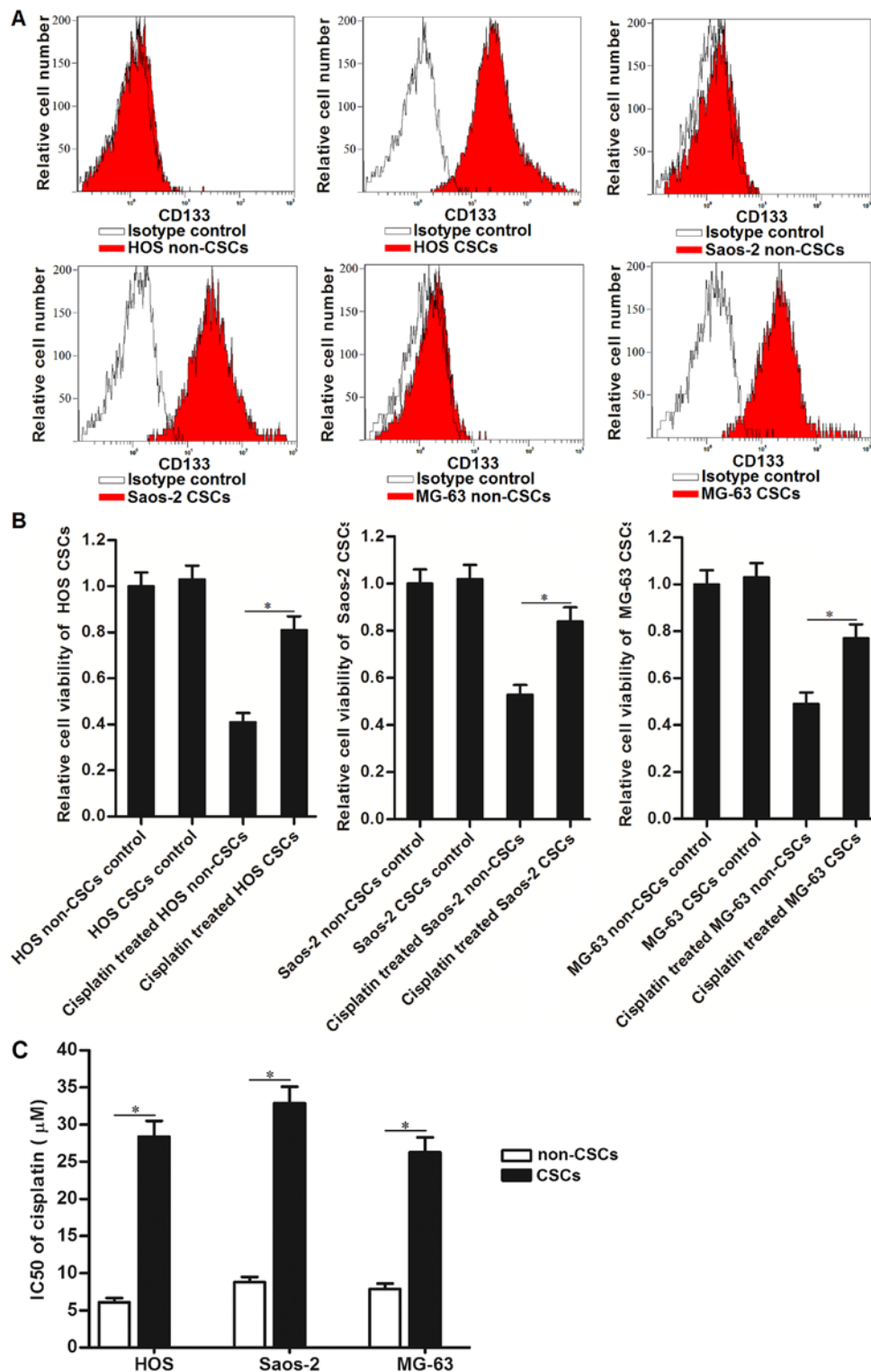


Figure 1. Osteosarcoma stem cells are resistant to cisplatin treatment. (A) HOS, Saos-2 and MG-63 osteosarcoma (OS) cells were incubated with CD133 antibodies followed by sorting the cancer stem cells (CSCs) and non-CSCs on flow cytometry. (B) HOS, Saos-2 and MG-63 CSCs and non-CSCs were treated with 8 μ M cisplatin for 48 h. Relative cell viability was measured by CCK-8 assays. *P<0.05. (C) IC₅₀ of cisplatin to OS stem cells and OS non-stem cells was determined according to the results of CCK-8 assays. *P<0.05.

to cisplatin was significantly lower than the OS non-stem cells (HOS non-CSCs, Saos-2 non-CSCs and MG-63 non-CSCs) (Fig. 1B). IC₅₀ of cisplatin to OS CSCs (28.4 μ M to HOS CSCs, 32.9 μ M to Saos-2 CSCs and 26.3 μ M to MG-63 CSCs) was significantly higher than their corresponding OS non-CSCs (6.1 μ M to HOS non-CSCs, 8.8 μ M to Saos-2 non-CSCs and

7.9 μ M to MG-63 non-CSCs) (Fig. 1C). Taken together, we demonstrated that osteosarcoma stem cells showed resistance to cisplatin treatment.

Cisplatin-resistance in OS stem cells is dependent on the overexpression of PKM2. PKM2 plays an important role in

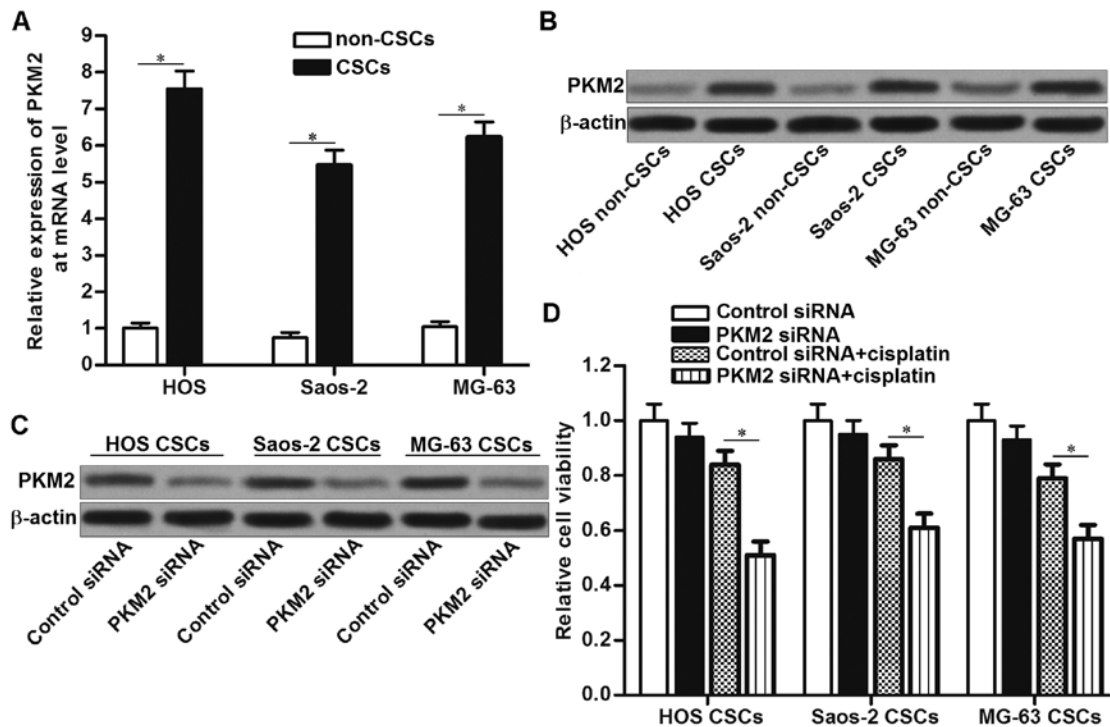


Figure 2. Role of PKM2 in cisplatin-resistance in osteosarcoma (OS) stem cells. (A) mRNA expression of PKM2 in HOS, Saos-2 and MG-63 cancer stem cells (CSCs) and their corresponding non-CSCs was detected by qRT-PCR analysis. * $P < 0.05$. (B) Protein expression of PKM2 in HOS, Saos-2 and MG-63 CSCs and their corresponding non-CSCs was detected by western blot analysis. (C) Expression of PKM2 in HOS, Saos-2 and MG-63 CSCs was knocked down by PKM2 specific siRNA. Western blot analysis was performed to detect the transfection efficiency of PKM2 siRNA. (D) PKM2 siRNA increased the sensitivity of HOS, Saos-2 and MG-63 CSCs to cisplatin ($8 \mu\text{M}$) treatment. Cell viability was measured by CCK-8 assays. * $P < 0.05$.

tumor growth, development and chemoresistance (19,20). In our study, we found that the expression of PKM2 was significantly overexpressed in OS stem cells compared with that in their corresponding OS non-stem cells at mRNA (Fig. 2A) and protein levels (Fig. 2B). To investigate the role of the overexpressed PKM2, we transfected the HOS, Saos-2 and MG-63 CSCs with PKM2 siRNA. Effects of PKM2 siRNA on OS stem cells are shown in Fig. 2C. Noteworthy, we observed that knockdown of PKM2 by the specific siRNA restored the sensitivity of OS stem cells to cisplatin treatment (Fig. 2D). These results emphasized the key role of PKM2 in cisplatin-resistance in OS stem cells.

Metformin resensitizes HOS CSCs to cisplatin by down-regulating the expression of PKM2 in vitro. To investigate the potential role of metformin in the sensitivity of OS stem cells to cisplatin, we treated the HOS CSCs and non-CSCs with $8 \mu\text{M}$ cisplatin and 2 mM metformin for evaluating their synergistic effects. We found that metformin markedly enhanced the cytotoxicity of cisplatin to HOS CSCs, whereas the effect of metformin was slight on enhancing the cytotoxicity of cisplatin on HOS non-CSCs (Fig. 3A). Combination with metformin decreased the IC_{50} of cisplatin to HOS CSCs by 74.9%. In contrast, the reduction of cisplatin IC_{50} to HOS non-CSCs was 31.2% (Fig. 3B). These data demonstrated that metformin was able to enhance the antitumor effect of cisplatin, and HOS CSCs were more sensitive to metformin-promoted cell death than their corresponding HOS non-CSCs. Mechanically, results of western blot analysis showed that metformin treatment significantly downregulated the expres-

sion of PKM2 in HOS CSCs (Fig. 3C). We therefore inferred that synergistic effect of metformin on cisplatin-induced cell death was dependent on the inhibition of PKM2. To prove this inference, we transfected the HOS CSCs with PKM2 expression vector. We found that enforced expression of PKM2 'rescued' the HOS CSCs from the combination treatment with cisplatin and metformin (Fig. 3D). These results indicated that metformin had the ability to resensitize OS stem cells to cisplatin by targeting PKM2.

Metformin resensitizes OS CSCs to cisplatin in vivo. To investigate whether metformin enhanced the antitumor effect of cisplatin on OS CSCs *in vivo*, we established the *in vivo* model of OS by using HOS CSCs. We observed that single administration with cisplatin slightly reduced the tumor sizes in mice. Of note, although metformin single treatment did not reduce the tumor volume obviously, it strongly enhanced the antitumor effect of cisplatin on HOS CSC model (Fig. 4A and B). After analyzing the expression of PKM2 in tumor tissues formatted by HOS CSCs, we found that metformin significantly decreased the expression of PKM2 in HOS CSC *in vivo* model (Fig. 4C). These results suggested that metformin have to the ability to sensitize the OS CSCs to cisplatin by inhibiting the expression of PKM2 *in vivo*.

Metformin weakens the glucose metabolism. As PKM2 is a key regulator of glucose metabolism in tumor cells (21), we next studied the effect of metformin on glucose metabolism in HOS CSCs. As shown in Fig. 5A and B, cisplatin treatment did not obviously influence the glucose metabolism in HOS

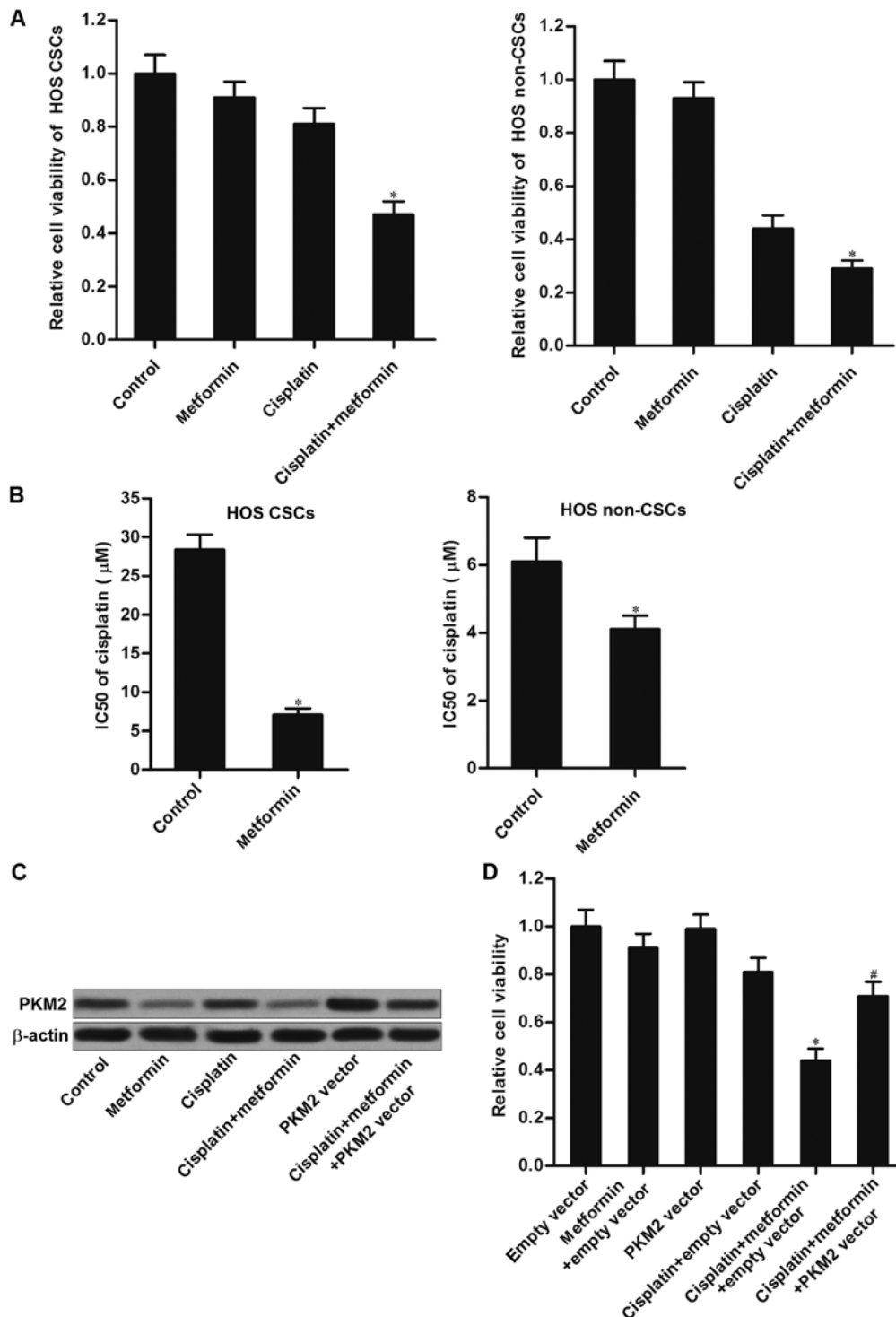


Figure 3. Metformin resensitizes HOS cancer stem cells (CSCs) to cisplatin by downregulating the expression of PKM2. (A) Cell viability of HOS CSCs was measured by CCK-8 assays after they were treated with cisplatin (8 μ M) and metformin (2 mM). * $P < 0.05$ vs. cisplatin group. (B) IC₅₀ of cisplatin to HOS CSCs was determined according to the results of CCK-8 assays. * $P < 0.05$ vs. control group. (C) Western blot analysis was performed to evaluate the effect of metformin (2 mM), cisplatin (8 μ M) and PKM2 vector on changing the expression of PKM2. (D) PKM2 vector abolished the synergistic effect of metformin (2 mM) on cisplatin (8 μ M)-induced cell death. * $P < 0.05$ vs. cisplatin + empty vector group. # $P < 0.05$ vs. cisplatin + metformin + empty vector group.

CSCs. However, metformin treatment significantly decreased the consumption of glucose as well as increased the production of lactate in HOS CSCs. In addition, transfection with PKM2 vector restored the glucose uptake and lactate production in HOS CSCs treated with metformin. These results demonstrated that metformin had the ability to weaken the glucose metabolism by inhibiting the expression of PKM2. Furthermore, as the

results of glucose metabolism reduction, metformin decreased the production of ATP in HOS CSCs (Fig. 5C).

Combination with metformin and cisplatin induce proliferation inhibition and apoptosis in HOS CSCs. In tumor cells, glucose metabolism and intracellular ATP regulate cell proliferation and chemoresistance (22,23). In our study, we found

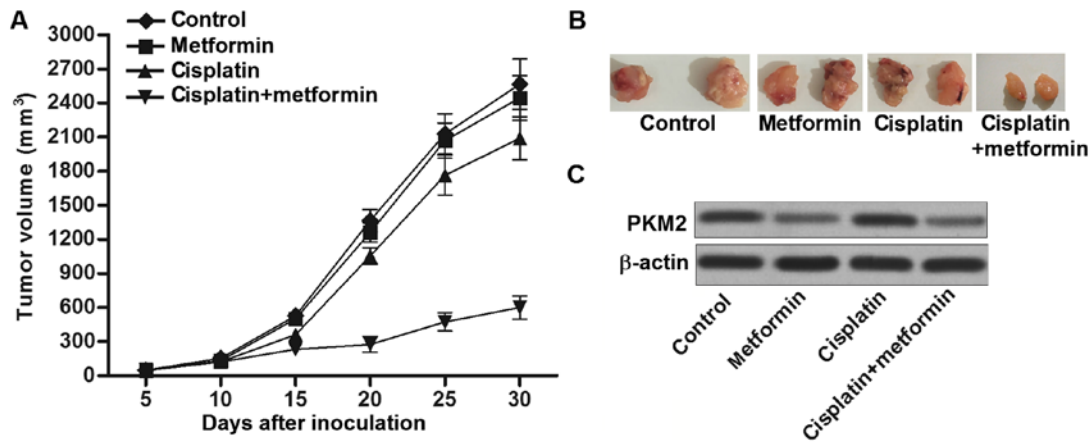


Figure 4. Metformin resensitizes HOS cancer stem cells (CSCs) to cisplatin *in vivo*. (A) Antitumor effect of cisplatin and metformin on HOS CSCs *in vivo* model. (B) Effect of cisplatin and metformin on changing the expression of PKM2 in tumor tissues formatted by HOS CSCs.

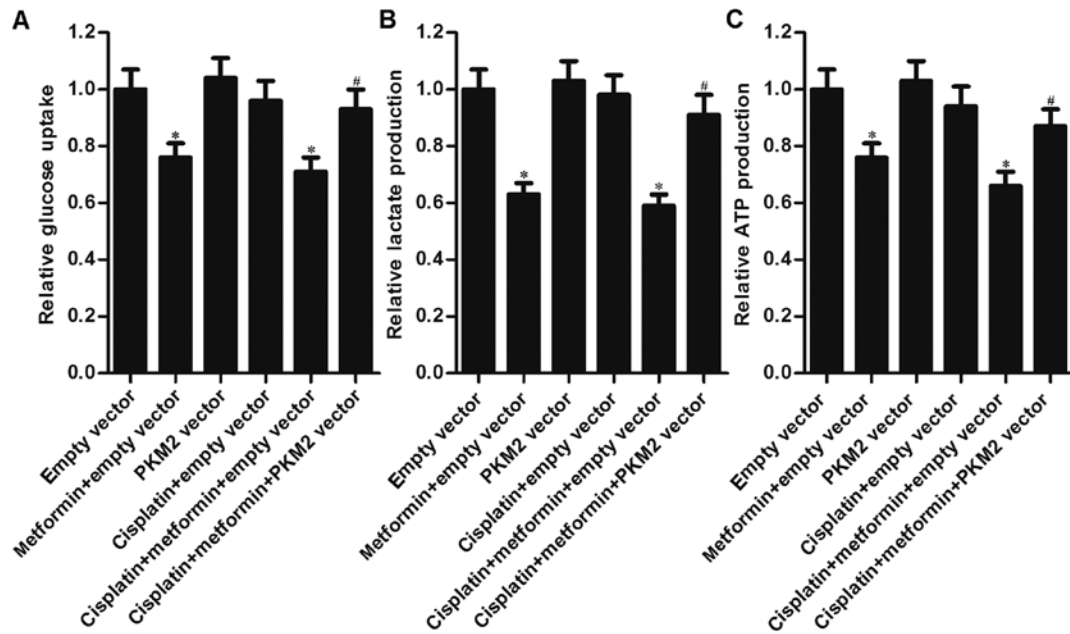


Figure 5. Metformin weakens the glucose metabolism in HOS cancer stem cells (CSCs). (A) Effect of metformin (2 mM) and cisplatin (8 μ M) on the consumption of glucose in HOS CSCs. * $P < 0.05$ vs. empty vector group. # $P < 0.05$ vs. cisplatin + metformin + empty vector group. (B) Effect of metformin (2 mM) and cisplatin (8 μ M) on the production of lactate in HOS CSCs. * $P < 0.05$ vs. empty vector group. # $P < 0.05$ vs. cisplatin + metformin + empty vector group. (C) Effect of metformin (2 mM) and cisplatin (8 μ M) on the production of ATP in HOS CSCs. * $P < 0.05$ vs. empty vector group. # $P < 0.05$ vs. cisplatin + metformin + empty vector group.

that combination with metformin and cisplatin induced drastic proliferation inhibition in HOS CSCs. However, enforced expression of PKM2 restored the proliferation of HOS CSCs which were co-treated with metformin and cisplatin (Fig. 6A). Furthermore, we observed that metformin treatment reduced the resistance of HOS CSCs to cisplatin-induced apoptosis. On the other hand, transfection with PKM2 vector impaired the effect of cisplatin plus metformin (Fig. 6B and C). Taken together, we demonstrated that metformin promoted cisplatin-induced proliferation inhibition and apoptosis in HOS CSCs by downregulating the expression of PKM2.

Effect of metformin on multidrug resistance in OS stem cells. Multidrug resistance is a major obstacle for chemotherapy of OS. We therefore treated OS stem cells (HOS CSCs, Saos-2 CSCs and MG-63 CSCs) with three kinds of chemotherapeutic

drugs (cisplatin, doxorubicin and 5-fluorouracil). As shown in Fig. 7A, co-treatment with metformin significantly increased the sensitivity of HOS CSCs, Saos-2 CSCs and MG-63 CSCs to cisplatin, doxorubicin and 5-fluorouracil. Metformin treatment obviously decreased the IC_{50} of cisplatin, doxorubicin and 5-fluorouracil to HOS, Saos-2 and MG-63 OS stem cells (Fig. 7B). We demonstrated that metformin can increase the sensitivity of OS stem cells to chemotherapy.

Discussion

Cisplatin is a cytotoxic drug that is used for treatment of OS (24,25). Mechanism by which cisplatin kills tumor cells is dependent on the formation of cross-linking DNA by interacting with it. When the DNA repair mechanism in tumor fails to neutralize the DNA damage induced by cisplatin, apoptosis

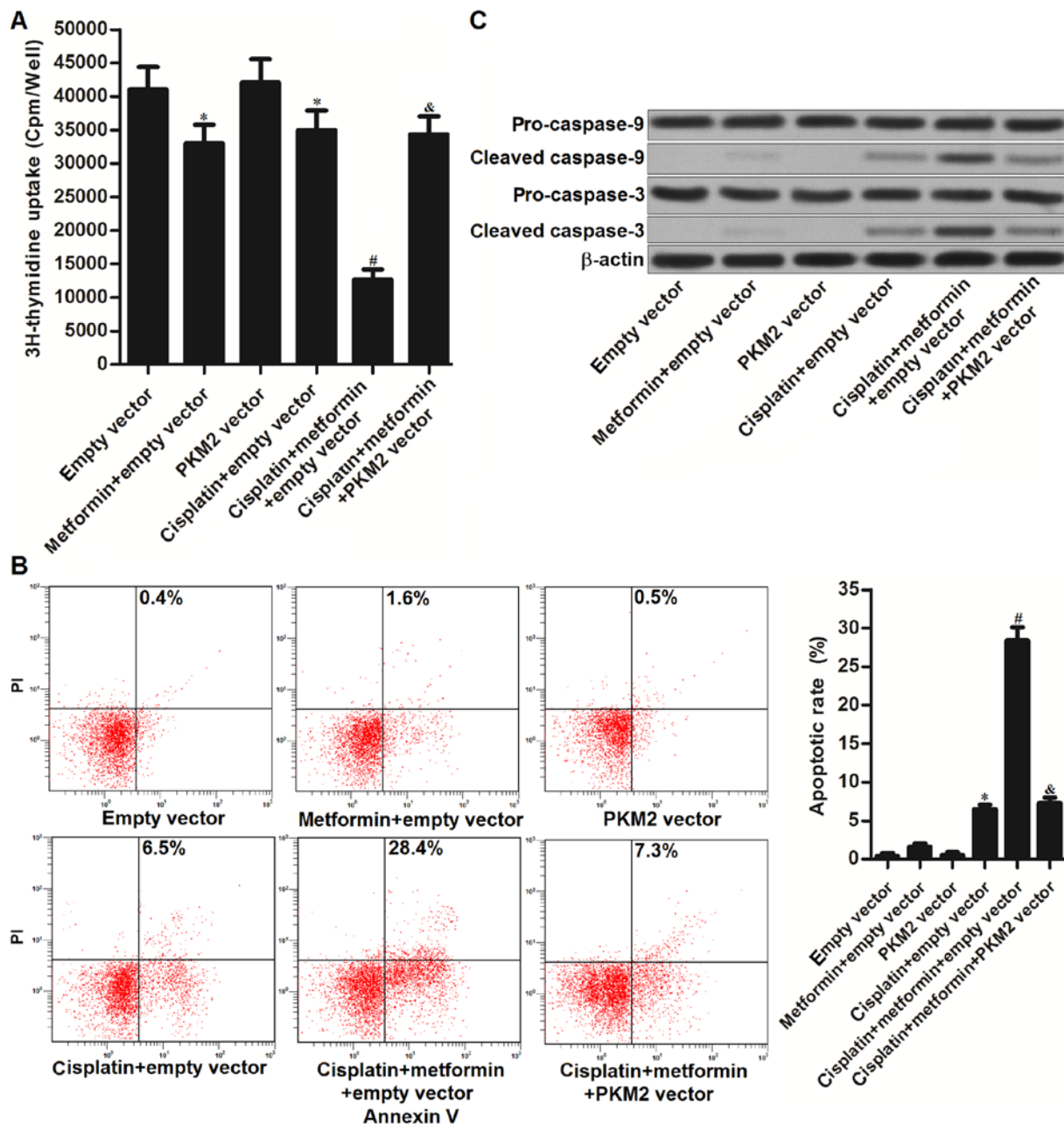


Figure 6. Metformin promoted cisplatin-induced proliferation inhibition and apoptosis in HOS. (A) ^3H -thymidine incorporation assays were performed to detect the proliferation of HOS cancer stem cells (CSCs) treated with metformin (2 mM), cisplatin (8 μM) and PKM2 vector. * $P < 0.05$ vs. empty vector group. # $P < 0.05$ vs. cisplatin + empty vector group. & $P < 0.05$ vs. cisplatin + metformin + empty vector group. (B) Flow cytometry analysis was performed to measure apoptosis induced by metformin (2 mM), cisplatin (8 μM) and PKM2 vector. (C) Western blot analysis was performed to detect the cleavage of caspase-9 and caspase-3 in HOS CSCs treated with metformin (2 mM), cisplatin (8 μM) and PKM2 vector.

occurs (26,27). However, chemotherapy resistance often occurs in OS due to the repeated use of cisplatin (28). Recent studies demonstrate that one important factor in tumor recurrence is the existence of cancer stem cells (CSCs) which are resistant to chemotherapy (29,30). Similarly in the present study, HOS, Saos-2 and MG-63 CSCs exhibited significant resistance to cisplatin compared with their corresponding non-CSCs. We proved that OS stem cells showed high IC_{50} of cisplatin. In view of the high proliferation and oncogenicity of stem cells, increasing sensitivity of OS stem cells to chemotherapy may represent an effective strategy against treatment failure.

Pyruvate kinase isoenzyme M2 (PKM2) in cancer cells catalyzes the final step reaction of glycolysis by converting

phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP. Therefore PKM2 is a key regulator of glucose metabolism in tumor cells (31). Recent studies indicate that overexpression of PKM2 is associated with chemoresistance. PKM2 inhibition can restore sensitivity of colon cancer cells, liver cancer cells and multiple myeloma cells to chemotherapy (20,32,33). In our study, we observed significant overexpression of PKM2 in OS stem cells rather than the non-CSCs. As PKM2 siRNA enhanced the cisplatin-induced cell death of HOS, Saos-2 and MG-63 CSCs, we proved that knockdown of PKM2 can restore the sensitivity of OS stem cells to cisplatin treatment. Metformin has been reported to show biological effects of reducing the occurrence of some cancers, and the mecha-

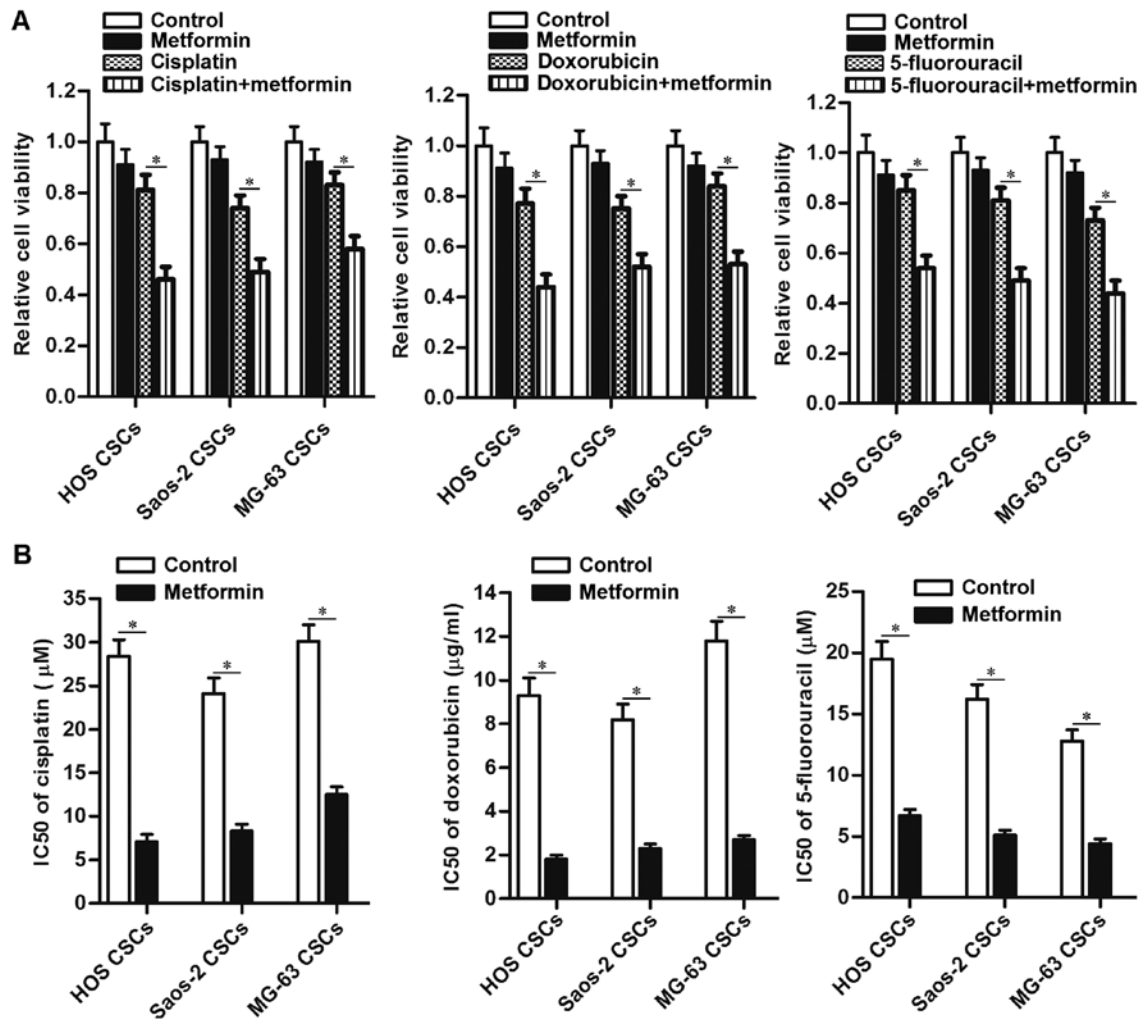


Figure 7. Effect of metformin on multidrug resistance in OS stem cells. (A) HOS, Saos-2 and MG-63 CSCs were treated with metformin (2 mM), cisplatin (8 μ M), doxorubicin (2 μ g/ml) and 5-fluorouracil (5 μ M) for 48 h. CCK-8 assays were performed to detect the sensitivity of OS stem cells to these chemotherapeutic drugs. * $P < 0.05$. (B) Comparison of IC₅₀ for cisplatin, doxorubicin and 5-fluorouracil to OS stem cells between control group and metformin group. * $P < 0.05$.

nism is associated with the inhibition of PKM2 induced by metformin (34). Consistent with this, our results proved that metformin can decrease the expression of PKM2 in OS stem cells. Sensitization of metformin to cisplatin-induced cell death is dependent on the inhibition of PKM2.

PKM2 regulates glucose metabolism and ATP production in tumor cells (31). Previous research has demonstrated that intracellular ATP levels are the switch to chemoresistance. Bioprocesses of drug efflux, drug inactivation and DNA damage repair require the consumption of ATP. Therefore low-levels of ATP reduce the resistance of drug-induced apoptosis (23,35).

Herein, we demonstrated that metformin treatment reduced the production of ATP in OS stem cells by inhibiting the PKM2 expression. Subsequently, metformin sensitized the OS stem cells to cisplatin-induced apoptosis and activation of caspase-9 and caspase-3. Furthermore, resistance of OS stem cells to some other chemotherapeutic agents such as doxorubicin and 5-fluorouracil was also inhibited by metformin co-treatment. It is proved that metformin/PKM2 axis is associated with chemosensitivity in OS stem cells.

In conclusion, we provide strong evidence that metformin mediates chemosensitivity in OS stem cells by targeting PKM2.

These findings suggest that combination with metformin and chemotherapeutic agents is a promising strategy for treatment of OS.

Acknowledgements

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