

# TGF- $\beta$ Suppressed Succinate Dehydrogenase to Promote Osteosarcoma Chemo-Resistance Through An HIF1 $\alpha$ Dependent Manner

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## Research Article

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# Abstract

**Background** Despite the widespread adoption of chemotherapy, drug resistance has been the major obstacle in tumor elimination of cancer patients. Our aim was to explore the role of TGF- $\beta$  in osteosarcoma chemo-resistance.

**Methods** We performed cytotoxicity analysis of methotrexate (MTX) and cisplatin (CIS) in TGF- $\beta$  treated osteosarcoma cells. Then a metabolite profile of the core energetic routes was analyzed by  $^1\text{H}$ -NMR in Saos-2 and MG-63 cell extracts. We detected the expression of succinate dehydrogenase (SDH), STAT1, and Hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) in TGF- $\beta$  treated osteosarcoma cells, and further tested the effects of these molecules on the cytotoxicity of chemotherapeutic agents. In vivo, we examined the tumor growth and survival time of Saos-2 bearing mice given the combination therapy of chemotherapeutic agents and a HIF1 $\alpha$  inhibitor.

**Results** Metabolic analysis revealed an enhanced succinate production of osteosarcoma cells after TGF- $\beta$  treatment. We further found the decrease in SDH expression and the increase in HIF1 $\alpha$  expression of TGF- $\beta$  treated osteosarcoma cells. Consistently, blockade of SDH [aggravated](#) the resistance of Saos-2/MG-63 cells to MTX and CIS. Also, a HIF1 $\alpha$  inhibitor significantly strengthened the anti-cancer efficacy of [chemotherapeutic](#) drugs in mice with osteosarcoma cancer.

**Conclusion** Our study demonstrated that TGF- $\beta$  attenuated the expression of SDH through reducing the transcription factor STAT1. The reduction of SDH then caused the up-regulation of HIF1 $\alpha$ , thereby rerouting the glucose metabolism and aggravating chemo-resistance in osteosarcoma cells. Linking tumor cell metabolism to the formation of [chemotherapy resistance](#), our study may guide the development of more effective treatments of osteosarcoma.

## Background

Osteosarcoma is the most prevalent malignant bone tumor with a high occurrence in children and adolescents. Surgery combined with chemotherapeutic agents is considered the major strategy for treatment. However, patients with osteosarcoma still undergo pulmonary metastasis and relapse, resulting in low survival rate [1, 2]. Over the past decades, resistance of tumor cells to drugs such as methotrexate (MTX), cisplatin (CIS) and doxorubicin has emerged as a major impediment to eradicating osteosarcoma [3]. Therefore, further research is warranted to better understand osteosarcoma chemo-resistance and formulate efficacious therapeutic strategies.

Drug resistance, a multifaceted process, is attributed to a combination of factors comprising apoptosis induction, autophagy induction, cancer stem cell regulation, DNA damage and repair, and epigenetic regulation [4]. Emerging evidence has supported the role of tumor metabolism in promoting drug resistance [5, 6]. Cancer cells rewire their metabolism to satisfy the high demand for both energy and biosynthesis. In this context, glycolysis is given a high-priority in cancer cells even under physiological oxygen conditions, which is named “Warburg effect”. Components of the glycolytic pathway such as

glucose transporters and hexokinase-2, the first rate-limiting enzyme in the glycolytic pathway, are intimately linked to chemo-resistance [7, 8]. These all raise awareness of intervening tumor metabolism to combat drug resistance.

It has been substantiated that transforming growth factor- $\beta$  (TGF- $\beta$ ) may function as a significant contributor to drug resistance in several types of tumor [9–13]. TGF- $\beta$ , a pleiotropic cytokine, plays a key role in regulating multiple biological processes, including cell proliferation, immune response and inflammation [14]. Reportedly, up-regulation of TGF- $\beta$  signaling has been found in erlotinib-resistant lung cancer cells [15]. Upon attaching to its receptors, TGF- $\beta$  spurs a series of events, among which epithelial-mesenchymal transition (EMT) imparts cancer cells with metastatic and invasive properties. Compelling reports have demonstrated that EMT confers chemo-resistance on cancer cells through increasing drug efflux pumps and anti-apoptotic effects [16]. TGF- $\beta$  has thus become a promising target in cancer therapy, and treatments targeting TGF- $\beta$  pathway such as neutralizing antibodies and soluble TGF- $\beta$  receptors have been evaluated in pre-clinical tests and even clinical trials [17].

In the current study, we observed a stronger TGF- $\beta$  expression in chemo-resistant osteosarcoma patients, and in vitro TGF- $\beta$  treatment obviously strengthened the multi-drug resistance of osteosarcoma cell lines. Mechanistically, we demonstrated that TGF- $\beta$  caused the decrease of STAT1 to inhibit the metabolic enzyme succinate dehydrogenase (SDH), giving rise to succinate accumulation in osteosarcoma cells. We further elucidated that the increased succinate promoted the expression of Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), and blockade of HIF1 $\alpha$  in turn augmented the antitumor effects of MTX and CIS. These findings revealed the molecular basis of TGF- $\beta$  implicated in the regulation of metabolic pathways and subsequent chemo-resistance of osteosarcoma.

## Methods

### Cell lines and reagents

Human osteosarcoma cell lines, Saos-2 and MG-63, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco). All cells were grown at 37°C in a 5% CO<sub>2</sub> incubator. MTX, CIS, succinate, and KC7F2 were from Sigma-Aldrich (ST, USA). Recombinant protein human and mouse TGF- $\beta$ 1 were bought from Pepro Tech (Rocky Hill, NJ).

### Patients and specimens

This study was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University. Written informed consent was attained from all patients, and all methods were performed according to the Declaration of Helsinki. A total of 20 osteosarcoma patients were recruited and provided informed consent between January 2017 and January 2020. These patients were divided into the chemo-sensitive and chemo-resistant groups according to the Response Evaluation Criteria in Solid Tumors

(RECIST). Then formalin-fixed, paraffin-embedded osteosarcoma specimens were collected for further detection.

For 3D matrix gel culture, tumor tissues were minced and digested with collagenase (Sigma-Aldrich, MA, USA) followed by filtration (BIOFIL). After centrifugation and removal of the red blood cells, osteosarcoma cells were seeded into 3D matrix gels in DMEM medium with 10% FBS.

### **Metabolic assessment of cells**

Metabolic assessment of Saos-2 and MG-63 cells ( $1 \times 10^7$  cells per sample) was performed by NMR as mentioned before [18,19]. Briefly, after using a methanol–chloroform–water extraction method, the upper aqueous phase was lyophilized and then redissolved in 550  $\mu$ l of phosphate buffer solution (60 mM  $K_2HPO_4/NaH_2PO_4$ , pH 7.4, 99.9%  $D_2O$ ) [20]. A Bruker 600-MHz spectrometer was used for the  $^1H$ -NMR experiments at 277 K temperature. Quantitative analysis of metabolites was performed using TopSpin (version 3.5) software. Metabolites were assigned according to published data. Metabolite concentrations were quantified per million cells and mean cell metabolite concentrations (fold change) were then calculated.

### **Metabolite quantification**

Quantitative analysis of succinate and fumarate was conducted using succinate (succinic acid) and fumarate colorimetric assay kit (BioVison, SF, USA), respectively, under the supplied instructions.

### **Cytotoxicity analysis**

Cytotoxicity analysis was performed using the FITC-Annexin V/ PE-PI apoptosis detection kit (BD, NJ, USA) under the manufacturer's instructions. Briefly, after treatment with 75 mM MTX or 40  $\mu$ M CIS for 48 hours, osteosarcoma cells were stained with FITC-Annexin V and PE-PI staining solution. Apoptosis was detected on a C6 flow cytometer (BD, NJ, USA). Each experiment was repeated independently in triplicate.

### **SiRNA silence**

Transfection of siRNAs was performed with Lipofectamine 8000 (Beyotime, Beijing, China) according to the suppliers' protocol. The relevant siRNA sequence was as follows: SDHD-si#1:5'-GCTCACAATAAGGAAGAAATA-3'; SDHD-si#2:5'-GCCGAGCTCTGTTGCT TCGAA-3'; STAT1-si#1:5'-CTGGAAGATTTACAAGATGAA-3'; STAT1-si#2:5'-CCCTGAAGTATCTGTATCCAA-3'.

### **Real-time PCR**

Total RNA was extracted with TRIzol (Invitrogen, CA, USA) and transcribed into cDNA by using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). PCR was performed on ABI stepone plus (Applied Biosystems, MA, USA). The primer sequences are shown as follows: STAT1, 5' - CAGCTTGACTCAAAATTCCTGGA-3' (sense) and 5' -TGAAGATTACGCTTGC TTTTCCT-3' (antisense).

SDHD, 5'-CATCTCTCCACTGGACTAGCG-3' (sense) and 5'-TCCATCGCAGAGCAAGGATTC-3' (antisense). GAPDH, 5'-GGAGCGA GATCCCTCCAAAT-3' (sense) and 5'-GGCTGTTGTCATACTTCT CATGG-3' (antisense). Results were confirmed by at least three independent experiments.

### **Western blot analysis**

Cells were collected and lysed in NP40 solution. Then the protein samples were run on an SDS-PAGE gel and transferred to nitrocellulose. Nitrocellulose membranes were blocked in 5% bovine serum albumin (BSA) and probed with primary antibodies against:  $\beta$ -actin (Cell Signaling, Cat No. 3700; 1:1,000); SDHD (Abcam, ab189945; 1:500); Phospho-Jak1 (Cell Signaling, Cat No. 74129; 1:1,000); Jak1 (Cell Signaling, Cat No. 50996; 1:1,000); Phospho-Jak2 (Cell Signaling, Cat No. 3776; 1:1,000); Jak2 (Cell Signaling, Cat No. 3230; 1:1,000); Phospho-Stat1 (Cell Signaling, Cat No. 9167; 1:1,000); Stat1 (Cell Signaling, Cat No. 9172; 1:1,000); Phospho-Stat2 (Cell Signaling, Cat No. 88410; 1:1,000); Stat2 (Cell Signaling, Cat No. 72604; 1:1,000); Phospho-Stat3 (Cell Signaling, Cat No. 9145; 1:1,000); Stat3 (Cell Signaling, Cat No. 9139; 1:1,000); HIF1 $\alpha$  (Abcam, ab179483; 1:1,000). Incubation with secondary antibodies conjugated to horseradish peroxidase was performed for one hour at room temperature. The proteins detected were visualized by enhanced chemiluminescence (Thermo fisher, MA, USA).

### **Immunohistochemical and immunofluorescence staining**

Tumor tissues from patients were fixed in 37% formalin and embedded in paraffin. After retrieval of antigens, sections were stained with primary antibodies against: TGF- $\beta$  (Abcam, ab215715; 1:500), SDHD (Abcam, ab189945; 1:200), and phospho-Stat1 (Cell Signaling, Cat No. 9167; 1:500) at 4°C overnight. Immunohistochemical staining was performed using the DAB Horseradish Peroxidase Color Development Kit (Beyotime, Shanghai, China) according to the supplied protocol. In brief, tissue slides were stained with HRP-conjugated secondary antibodies for 1 hour at room temperature and counterstained with hematoxylin (Solarbio, Beijing, China). For immunofluorescent staining, tissue slides were incubated with secondary antibody followed by incubation with DAPI (Solarbio, Beijing, China). The intensity of immunostaining was analyzed by Image J 9.0 software.

### **Animal experiments**

NSG mice (4-6 weeks old) were purchased from HFK Bioscience Company (Beijing, China) and maintained under pathogen-free conditions. For tumor growth analysis,  $2 \times 10^6$  Saos-2 or MG-63 cells were subcutaneously injected into NSG mice. Then these mice were randomized into different groups 10 days later after inoculation and treated with or without TGF- $\beta$  (20  $\mu$ g/kg), MTX (5 mg/kg), CIS (1 mg/kg), KC7F2 (10 mg/kg) twice a week for 14 days. The mice of control groups received an equal volume of saline. Tumor growth was examined every other day and the survival of mice was recorded. Tumor volume was calculated using the formula: tumor volume=length $\times$ width<sup>2</sup>/2.

For tumorigenesis analysis, NSG mice received injections of  $2 \times 10^5$  Saos-2 or MG-63 cells subcutaneously. 10 days later after inoculation, these mice were treated with or without TGF- $\beta$  (20  $\mu$ g/kg), KC7F2 (10

mg/kg) twice a week for 14 days. The tumorigenesis was calculated 20 days after injection. The above experimental procedures were approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were conducted in accordance with the Public Health Service Policy and complied with the ARRIVE guidelines for the humane use and care of animals.

## **Statistical analysis**

All experiments were independently performed in triplicate. Results are presented as mean  $\pm$  SEM, and analyzed by Student's t-test or one-way ANOVA. The survival rates were determined by Kaplan-Meier survival analysis. P value < 0.05 was considered statistically significant. The analysis was performed using Graphpad 8.0 software.

# **Results**

## **TGF- $\beta$ promoted osteosarcoma chemo-resistance**

Compelling reports have implicated that tumor tissues with increasing TGF- $\beta$  expression revealed enhanced migratory features and poor prognosis. Our study further explored the role of TGF- $\beta$  in osteosarcoma progression, including drug resistance and tumorigenicity. To do this, we isolated tumor tissues from chemo-resistant/sensitive osteosarcoma patients and examined the expression of TGF- $\beta$  in tumor tissues. Notably, those tumor tissues isolated from chemo-resistant patients exhibited elevated TGF- $\beta$  expression compared to the chemo-sensitive group (Fig. 1A), suggesting the potential role of TGF- $\beta$  in chemo-resistance development of osteosarcoma. Given the limited samples size, we further performed cytotoxicity analysis in TGF- $\beta$  treated osteosarcoma cell lines Saos-2 and MG-63. As a result, TGF- $\beta$  treatment significantly strengthened the resistance of Saos-2/MG-63 to chemotherapeutic MTX (Fig. 1B) and CIS (Fig. 1C). Similar results were observed in TGF- $\beta$  treated Saos-2/MG-63 bearing mice (Fig. 1D). Those results suggested that TGF- $\beta$  could facilitate the chemo-resistance in osteosarcoma cells. Subsequently, we further assessed the influence of TGF- $\beta$  in tumor growth. Though no significant difference was observed in tumor volumes of Saos-2/MG-63 bearing mice (Fig. 1E), Saos-2 and MG-63 cells treated with TGF- $\beta$  revealed strengthened capability of tumorigenesis (Fig. 1F), reminding us of the potential relationship between TGF- $\beta$  and stem associated transcription factors in osteosarcoma. Together, those results suggested TGF- $\beta$  could promote osteosarcoma chemo-resistance, resulting in poor outcome in patients.

## **TGF- $\beta$ suppressed succinat dehydrogenase to promote chemo-resistance**

Alteration of energy metabolism is a biological fingerprint of tumor cells, and enhanced lactate production (caused by glycolysis) correlated with drug resistance in several tumor types. Here, a metabolite profile of the core energetic routes was analyzed by  $^1\text{H-NMR}$  in Saos-2/MG-63 cell extracts. After TGF- $\beta$  treatment, Saos-2/MG-63 cells revealed obviously higher consumption of glucose and increasing production of lactate (Fig. 2A), suggesting that tumor cells might enhance glycolysis by

preferential conversion of pyruvate to lactate instead of oxidation. Meanwhile, enhanced succinate production and an increased succinate/fumarate ratio were observed in Saos-2/MG-63 cells treated with TGF- $\beta$ , as shown in Fig. 2A. These results prompted us to speculate that TGF- $\beta$  might affect the SDH metabolism, resulting in the accumulation of succinate and suppression of oxidation in the tricarboxylic acid cycle. In that case, the expression of SDHD, a subunit of SDH, was examined. Intriguingly, suppression of SDHD expression was observed in mRNA (Fig. 2B) and protein (Fig. 2C) levels of TGF- $\beta$  treated Saos-2/MG-63 cells. To further confirm the role of SDH in osteosarcoma progression, siRNA interference was conducted to suppress the expression of SDHD (Fig. 2D). Blockade of SDHD obviously strengthened the resistance of Saos-2/MG-63 cells to MTX and CIS (Fig. 2E and F). Meanwhile, addition of succinate to suppress the oxidation also promoted MTX/CIS resistance of osteosarcoma cells (Fig. 2G and H). Consistently, suppression of SDHD was observed in chemo-resistant tumor tissues from patients (Fig. 2I). Together, those results suggested that TGF- $\beta$  could suppress SDH to promote chemo-resistance in osteosarcoma.

### **TGF- $\beta$ suppressed STAT1 to decrease SDH expression**

Current studies provided evidence to suggest that TGF- $\beta$  could regulate JAK/STAT associated signaling pathways to promote cancer progression. To further clarify the mechanism in SDH associated osteosarcoma progression, the expression of JAK1, JAK2, STAT1, STAT2, and STAT3 was examined by western blotting. Notably, reduced expression of phosphorylated JAK2 and STAT1 were observed in TGF- $\beta$  treated Saos-2 cells (Fig. 3A and B). Meanwhile, reduced nucleus entry of STAT1 in TGF- $\beta$  treated Saos-2 and MG-63 cells were found in our immunofluorescent staining (Fig. 3C), suggesting TGF- $\beta$  suppressed STAT1 activation in osteosarcoma cells. Next, siRNA interference was performed to suppress the STAT1 expression in Saos-2 and MG-63 cells (Fig. 3D). Consequently, silence of STAT1 down-regulated the expression of SDHD in Saos-2 and MG-63 cells (Fig. 3E). And enhanced succinate/ fumarate ratio were found in STAT1 silenced Saos-2 and MG-63 cells (Fig. 3F). These results implicated that TGF- $\beta$  suppressed STAT1 to down-regulate SDH activity. Subsequently, we observed similar chemo-resistance to MTX (Fig. 3G) and CIS (Fig. 3H) in STAT1 silenced Saos-2 and MG-63 cells, suggesting that STAT1 signals were involved in the TGF- $\beta$  associated chemo-resistance. Consistently, STAT1 level was reduced in chemo-resistant tumor tissues from osteosarcoma patients (Fig. 3I). Those results suggested that TGF- $\beta$  suppressed STAT1 signals to down-regulate SDH metabolism.

### **Metabolic succinate facilitated osteosarcoma chemo-resistance through an HIF1 $\alpha$ dependent manner**

Compelling findings implicated that succinate served as a crucial oncometabolite by suppressing PHDs, which target HIF1 $\alpha$  for proteasomal degradation or glycolysis. Hence, we sought to investigate whether TGF- $\beta$  could mediate the HIF1 $\alpha$  up-regulation by controlling SDH metabolism. TGF- $\beta$  or succinate treatment promoted the expression of HIF1 $\alpha$  expression in Saos-2/MG-63 cells (Fig. 4A). And silence of STAT1 or SDHD also contributed to the elevated expression of HIF1 $\alpha$  (Fig. 4B), indicating that TGF- $\beta$  could up-regulate HIF1 $\alpha$  through an SDH dependent manner. Next, we used HIF1 $\alpha$  inhibitor KC7F2 to treat Saos-2/MG-63 cells for cytotoxicity analysis and no obvious cytotoxicity was observed in bulk Saos-

2/MG-63 cells (Fig. 4C). However, blockade of HIF1 $\alpha$  efficiently reversed the drugs resistance caused by TGF- $\beta$  (Fig. 4D and E), indicating that TGF- $\beta$  promoted chemo-resistance through HIF1 $\alpha$ . More importantly, our previous results implicated that TGF- $\beta$  strengthened the capability of tumorigenesis in osteosarcoma cells (Fig. 1F), and HIF1 $\alpha$  has been proved to be associated with tumor stemness regulation. Herein, we further suppressed HIF1 $\alpha$ , then treated Saos-2/MG-63 cells with TGF- $\beta$ . Intriguingly, blockade of HIF1 $\alpha$  efficiently weakened the tumorigenesis of Saos-2 and MG-63 cells in vivo (Fig. 4F), demonstrating that TGF- $\beta$  could further promote osteosarcoma stemness through HIF1 $\alpha$ . Together, those results suggested that TGF- $\beta$  suppressed SDH activity to up-regulate HIF1 $\alpha$ , resulting in chemo-resistance in osteosarcoma.

### **Blockade of HIF1 $\alpha$ improved outcome of chemotherapy in osteosarcoma**

Given the crucial role of HIF1 $\alpha$  in tumorigenesis and chemo-resistance, it might be feasible to target HIF1 $\alpha$  to improve the outcome of chemotherapy in osteosarcoma. To assess our hypothesis, Saos-2 cells were subcutaneous injected into the NOD-SCID mice, following with HIF1 $\alpha$  inhibitor KC7F2, and MTX/CIS treatment. Intriguingly, addition of KC7F2 significantly strengthened the anticancer effects of MTX (Fig. 5A) and CIS (Fig. 5B), though no TGF- $\beta$  was used for tumor-bearing mice. We conjectured that osteosarcoma cells could produce TGF- $\beta$  through an autocrine manner, thereby resulting in the activation of HIF1 $\alpha$  pathway. Consistently, KC7F2 treatment also significantly prolonged the survival time of Saos-2 bearing mice (Fig. 5C and D), indicating that suppression of HIF1 $\alpha$  could efficiently improve anticancer effects of chemotherapy. To further evaluate the anticancer effects of KC7F2, we isolated tumor cells from chemo-resistant patients and seeded those tumor cells into matrix gels. Tumor cells isolated from patient #2 succeed to generate spherical colonies and revealed proliferative phenotypes in two months (Fig. 5E). Here, we treated those colonies with MTX, CIS and KC7F2 in matrix gels. As anticipated, slight cytotoxicity was observed in MTX or CIS treatment group, which might be due to the tumor cells isolated from chemo-resistant tumor tissue. However, addition of KC7F2 significantly strengthened the cytotoxicity of MTX and CIS (Fig. 5F and G), indicating the potential anticancer effects of HIF1 $\alpha$  inhibitors in clinic. Together, those results suggested that blockade of HIF1 $\alpha$  could improve the outcome of chemotherapy, which described a novel strategy in clinical osteosarcoma treatment.

## **Discussion**

TGF- $\beta$  is a pleiotropic cytokine which has a dual action in tumor development. Activation of TGF- $\beta$  pathway results in a variety of gene responses, which modulate cell-cycle arrest and apoptosis in early-stage cancer, as well as metastasis and angiogenesis in advanced cancer [21]. A growing body of research has indicated that TGF- $\beta$  is also linked to chemo-resistance and the union application of drugs and TGF- $\beta$  inhibitors achieve remarkable effects in several tumor types [9, 22]. However, our current understanding of TGF- $\beta$  induced drug resistance is incomplete. Given its role in gene expression and cell differentiation, cellular metabolism is emerging as a key player in tumor initiation and progression [23]. Here, our study provided evidence of the contribution of TGF- $\beta$  to glucose metabolism of tumor cells, thus exacerbating drug resistance in osteosarcoma.



Variation in energetic routes is a hallmark of tumor cells, which is characterized by enhancement of glucose uptake, glycolysis hyperactivation, reduction of oxidative phosphorylation, and lactate accumulation [24]. The altered cellular metabolism not only brings an abundance of energy, but also gives rise to metabolic intermediates that exert important effects in supporting tumor proliferation, metastasis, and even chemo-resistance [25]. In a study of breast cancer, elevated expression of lactate dehydrogenase which converted pyruvate into lactate was proved to mediate trastuzumab resistance [26]. Shi et al. reported that knockdown of pyruvate kinase M2 (PKM2), the final rate-limiting enzyme of the glycolytic pathway, caused an accumulation of docetaxel in lung cancer cells and synergistically strengthened the efficiency of chemotherapy in mice [27]. Apart from enzymes in control of glycolysis, glucose transporters also contributed to drug resistance [28]. Here, our study provided a quantitative analysis of metabolite levels and observed increasing glucose consumption as well as lactate generation in TGF- $\beta$  treated osteosarcoma cells. Importantly, we demonstrated that the TGF- $\beta$  down-regulated SDH expression in osteosarcoma cells, thus leading to the collection of succinate and conversion to glycolysis pathway. Previous research has revealed that TGF- $\beta$  could control SDH expression through transcriptional and posttranslational regulation of STAT1 [29, 30]. Consistently, we further indicated that TGF- $\beta$  attenuated STAT1 phosphorylation so as to suppress SDH in osteosarcoma cells. Decrease of SDH expression in osteosarcoma cells brings about an accumulation of succinate. Succinate has been regarded as a crucial oncometabolite which functions through enhancing angiogenesis and suppressing histone and DNA demethylases. [31, 32]. Our results indicated that succinate treatment caused a rise in HIF1 $\alpha$  in osteosarcoma cells. Hypoxia is probably the most pervasive condition within the tumor tissue. Tumor cells have to struggle with the low oxygen tension by activating several pathways, among which HIF1 $\alpha$  signaling plays a vital role in cancer progression. There is increasing evidence that HIF1 $\alpha$  mediates tumor metastasis, angiogenesis and development of resistance to various therapeutic modalities [33]. Sowa et al. reported that HIF-1 facilitated drug resistance of lung adenocarcinoma in part due to the induction of carbonic anhydrase IX (CAIX) [34]. In another study of bladder cancer, cisplatin-resistant cells exhibited higher levels of HIF1 $\alpha$ , which was correlated with increased expression of MDR1 encoding multidrug efflux pump P-glycoprotein (P-gp) [35]. Here, we demonstrated that HIF1 $\alpha$  up-regulation resulting from elevated succinate exerted regulative effects on the glycolytic state and chemotherapy resistance of osteosarcoma cells.

## Conclusions

Our study indicated that TGF- $\beta$  exhibited an inhibitory effect on SDH expression in osteosarcoma cells, therefore having a central function in tumor metabolism rerouting and subsequent drug resistance. Chemotherapeutic agents in combination with a HIF-1 $\alpha$  inhibitor significantly abrogated TGF- $\beta$  mediated chemo-resistance and enhanced the curative effects, which revealed a potentially promising method for combating osteosarcoma.

## Abbreviations

TGF- $\beta$ : transforming growth factor- $\beta$ ; SDH: succinate dehydrogenase; HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$ ; MTX: methotrexate; CIS: cisplatin; EMT: epithelial-mesenchymal transition; CAIX: carbonic anhydrase IX; P-gp: P-glycoprotein; PKM2: pyruvate kinase M2.

## Declarations

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University. Written informed consent was attained from all individual participants, and all methods were performed according to the Declaration of Helsinki. All animal experiments were approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Consent for publication

Not applicable.

### Availability of data and materials

The anonymized data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

DW conceived the project and wrote the manuscript. YX, YL, FX, YD, ZL, YY and DW performed the experiments. YX and YL performed data analysis. All authors read and approved the final manuscript.

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Not applicable.

## References

1. Lilienthal I, Herold N. *Targeting Molecular Mechanisms Underlying Treatment Efficacy and Resistance in Osteosarcoma: A Review of Current and Future Strategies. Int J Mol Sci.*

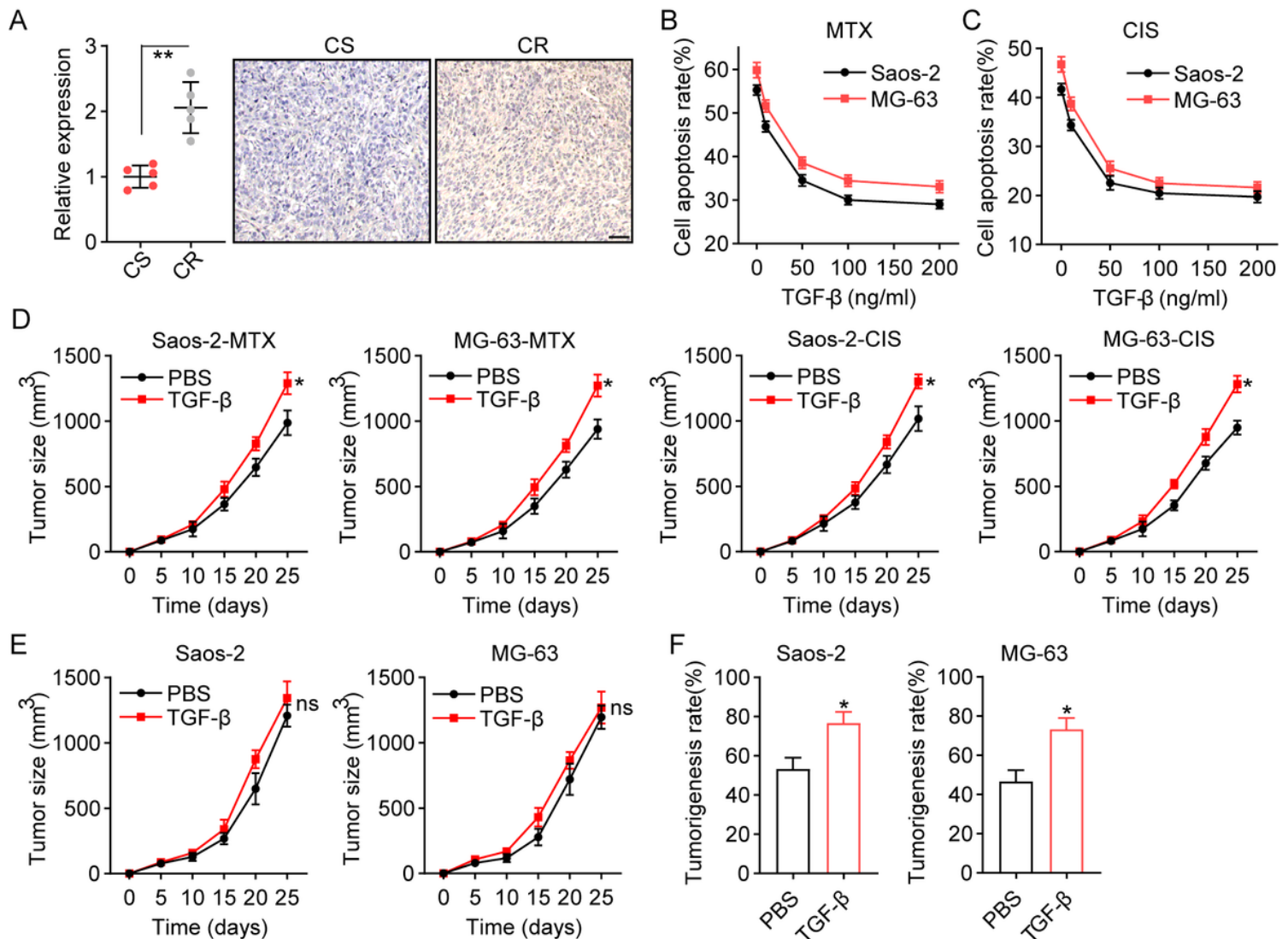
2020;21(18):6885.

2. Harrison DJ, Geller DS, Gill JD, Lewis VO, Gorlick R. *Current and future therapeutic approaches for osteosarcoma. Expert Rev Anticancer Ther.* 2018;18(1):39–50.
3. Isakoff MS, Bielack SS, Meltzer P, Gorlick R. *Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. J Clin Oncol.* 2015;33(27):3029–3035.
4. Wu Q, Yang Z, Nie Y, Shi Y, Fan D. *Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches. Cancer Lett.* 2014;347(2):159–166.
5. Pan C, Wang X, Shi K, et al. *MiR-122 Reverses the Doxorubicin-Resistance in Hepatocellular Carcinoma Cells through Regulating the Tumor Metabolism. PLoS One.* 2016;11(5):e0152090. doi:10.1371/journal.pone.0152090
6. Grasso C, Jansen G, Giovannetti E. *Drug resistance in pancreatic cancer: Impact of altered energy metabolism. Crit Rev Oncol Hematol.* 2017;114:139–152.
7. Varghese E, Samuel SM, Lišková A, Samec M, Kubatka P, Büsselberg D. *Targeting Glucose Metabolism to Overcome Resistance to Anticancer Chemotherapy in Breast Cancer. Cancers (Basel).* 2020;12(8):2252.
8. Min JW, Kim KI, Kim HA, et al. *INPP4B-mediated tumor resistance is associated with modulation of glucose metabolism via hexokinase 2 regulation in laryngeal cancer cells. Biochem Biophys Res Commun.* 2013;440(1):137–142.
9. Oshimori N, Oristian D, Fuchs E. *TGF- $\beta$  promotes heterogeneity and drug resistance in squamous cell carcinoma. Cell.* 2015;160(5):963–976.
10. Cai J, Fang L, Huang Y, et al. *Simultaneous overactivation of Wnt/ $\beta$ -catenin and TGF $\beta$  signalling by miR-128-3p confers chemoresistance-associated metastasis in NSCLC [published correction appears in Nat Commun. 2018 Mar 30;9:16196]. Nat Commun.* 2017;8:15870.
11. Katsuno Y, Meyer DS, Zhang Z, et al. *Chronic TGF- $\beta$  exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. Sci Signal.* 2019;12(570):eaau8544.
12. Bhagyaraj E, Ahuja N, Kumar S, et al. *TGF- $\beta$  induced chemoresistance in liver cancer is modulated by xenobiotic nuclear receptor PXR. Cell Cycle.* 2019;18(24):3589–3602.
13. Tripathi V, Shin JH, Stuelten CH, Zhang YE. *TGF- $\beta$ -induced alternative splicing of TAK1 promotes EMT and drug resistance. Oncogene.* 2019;38(17):3185–3200.
14. Akhurst RJ, Hata A. *Targeting the TGF $\beta$  signalling pathway in disease. Nat Rev Drug Discov.* 2012;11(10):790–811.
15. Yao Z, Fenoglio S, Gao DC, et al. *TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. Proc Natl Acad Sci U S A.* 2010;107(35):15535–15540.
16. Du B, Shim JS. *Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. Molecules.* 2016;21(7):965.

17. Colak S, Ten Dijke P. *Targeting TGF- $\beta$  Signaling in Cancer*. *Trends Cancer*. 2017;3(1):56–71.
18. Duarte IF, Lamego I, Marques J, Marques MP, Blaise BJ, Gil AM. *Nuclear magnetic resonance (NMR) study of the effect of cisplatin on the metabolic profile of MG-63 osteosarcoma cells*. *J Proteome Res*. 2010;9(11):5877–5886.
19. Lin C, Dong J, Wei Z, et al. *<sup>1</sup>H NMR-Based Metabolic Profiles Delineate the Anticancer Effect of Vitamin C and Oxaliplatin on Hepatocellular Carcinoma Cells*. *J Proteome Res*. 2020;19(2):781–793.
20. Le Belle JE, Harris NG, Williams SR, Bhakoo KK. *A comparison of cell and tissue extraction techniques using high-resolution <sup>1</sup>H-NMR spectroscopy [published correction appears in NMR Biomed. 2016 Apr;29(4):527]. NMR Biomed*. 2002;15(1):37–44.
21. Seoane J, Gomis RR. *TGF- $\beta$  Family Signaling in Tumor Suppression and Cancer Progression*. *Cold Spring Harb Perspect Biol*. 2017;9(12):a022277.
22. Brunen D, Willems SM, Kellner U, Midgley R, Simon I, Bernards R. *TGF- $\beta$ : an emerging player in drug resistance*. *Cell Cycle*. 2013;12(18):2960–2968.
23. DeBerardinis RJ, Chandel NS. *Fundamentals of cancer metabolism*. *Sci Adv*. 2016;2(5):e1600200.
24. Pavlova NN, Thompson CB. *The Emerging Hallmarks of Cancer Metabolism*. *Cell Metab*. 2016;23(1):27–47.
25. Bhattacharya B, Mohd Omar MF, Soong R. *The Warburg effect and drug resistance*. *Br J Pharmacol*. 2016;173(6):970–979.
26. Zhao Y, Liu H, Liu Z, et al. *Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism*. *Cancer Res*. 2011;71(13):4585–4597.
27. Shi HS, Li D, Zhang J, et al. *Silencing of pkm2 increases the efficacy of docetaxel in human lung cancer xenografts in mice [published correction appears in Cancer Sci. 2010 Jun;101(6):1575]. Cancer Sci*. 2010;101(6):1447–1453.
28. Le Calvé B, Rynkowski M, Le Mercier M, et al. *Long-term in vitro treatment of human glioblastoma cells with temozolomide increases resistance in vivo through up-regulation of GLUT transporter and aldo-keto reductase enzyme AKR1C expression*. *Neoplasia*. 2010;12(9):727–739.
29. Zhou X, Zöller T, Kriegelstein K, Spittau B. *TGF $\beta$ 1 inhibits IFN $\gamma$ -mediated microglia activation and protects mDA neurons from IFN $\gamma$ -driven neurotoxicity*. *J Neurochem*. 2015;134(1):125–134.
30. Reardon C, McKay DM. *TGF-beta suppresses IFN-gamma-STAT1-dependent gene transcription by enhancing STAT1-PIAS1 interactions in epithelia but not monocytes/macrophages*. *J Immunol*. 2007;178(7):4284–4295.
31. Mu X, Zhao T, Xu C, et al. *Oncometabolite succinate promotes angiogenesis by upregulating VEGF expression through GPR91-mediated STAT3 and ERK activation*. *Oncotarget*. 2017;8(8):13174–13185.
32. Xiao M, Yang H, Xu W, et al. *Inhibition of  $\alpha$ -KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors*

33. Fallah J, Rini BI. *HIF Inhibitors: Status of Current Clinical Development.* *Curr Oncol Rep.* 2019;21(1):6.
34. Sowa T, Menju T, Chen-Yoshikawa TF, et al. *Hypoxia-inducible factor 1 promotes chemoresistance of lung cancer by inducing carbonic anhydrase IX expression.* *Cancer Med.* 2017;6(1):288–297.
35. Sun Y, Guan Z, Liang L, et al. *HIF-1 $\alpha$ /MDR1 pathway confers chemoresistance to cisplatin in bladder cancer.* *Oncol Rep.* 2016;35(3):1549–1556.

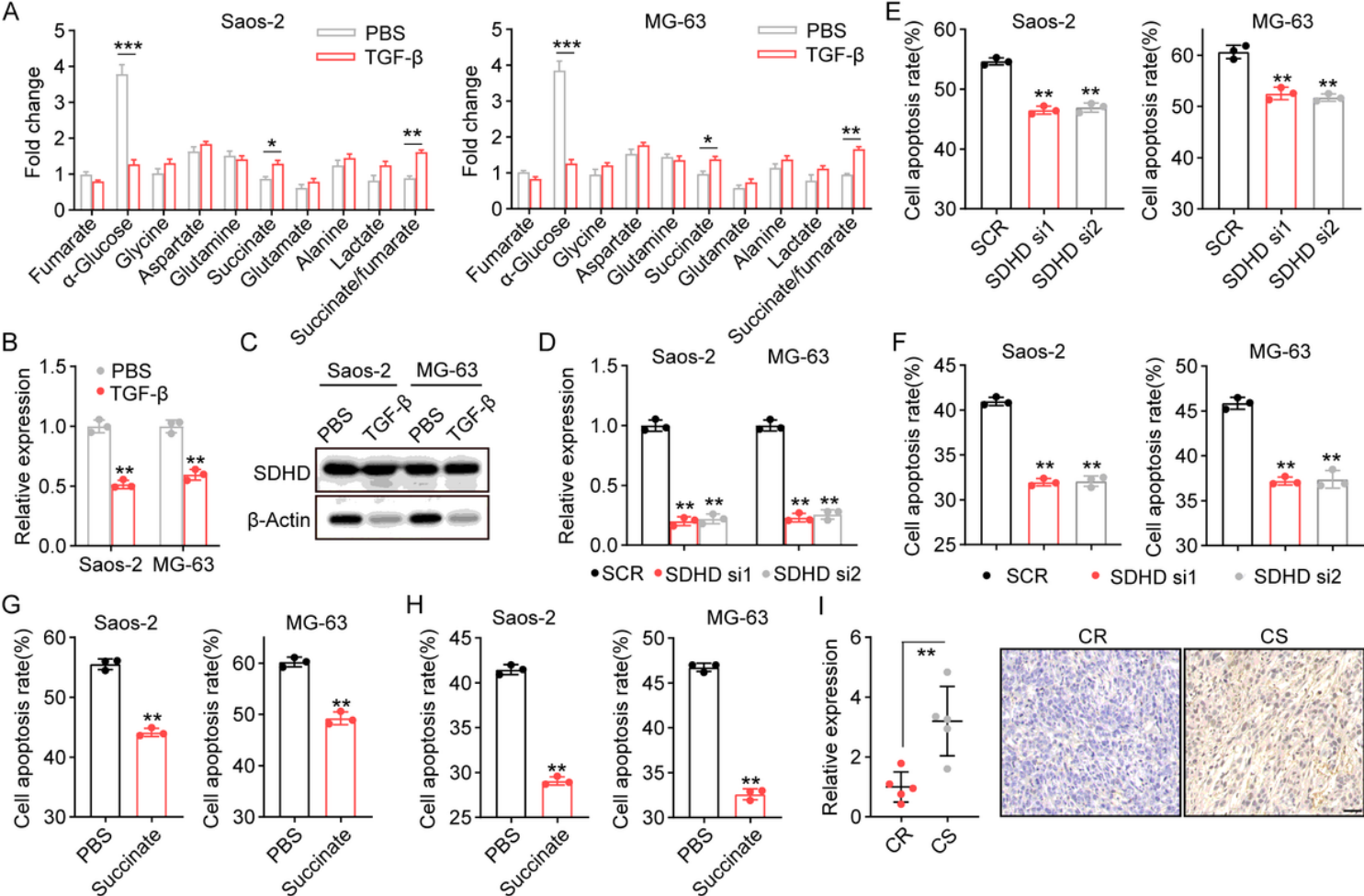
## Figures



**Figure 1**

TGF- $\beta$  promoted osteosarcoma chemo-resistance. A, Immunohistochemistry of TGF- $\beta$  in tumor tissues from chemoresistant (CR) and chemosensitive (CS) patients. The scale bar is 100  $\mu$ m. B, Saos-2 and MG-63 cells were treated with MTX (75 mM) plus different concentrations of TGF- $\beta$  for 48h. Cell apoptosis

was determined by flow cytometry. C, The same as B, except that Saos-2 and MG-63 cells were treated with CIS (40  $\mu$ M) plus different concentrations of TGF- $\beta$ . D, The NSG mice with Saos-2 and MG-63 osteosarcoma were treated with MTX (5 mg/kg)/CIS (1 mg/kg) plus TGF- $\beta$  (20  $\mu$ g/kg). The tumor growth was measured. E, The NSG mice with Saos-2 and MG-63 osteosarcoma were treated with TGF- $\beta$  (20  $\mu$ g/kg). The tumor growth was measured. F, Tumorigenicity of Saos-2 and MG-63 cells after treatment with TGF- $\beta$  (20  $\mu$ g/kg). MTX, methotrexate; CIS, cisplatin.

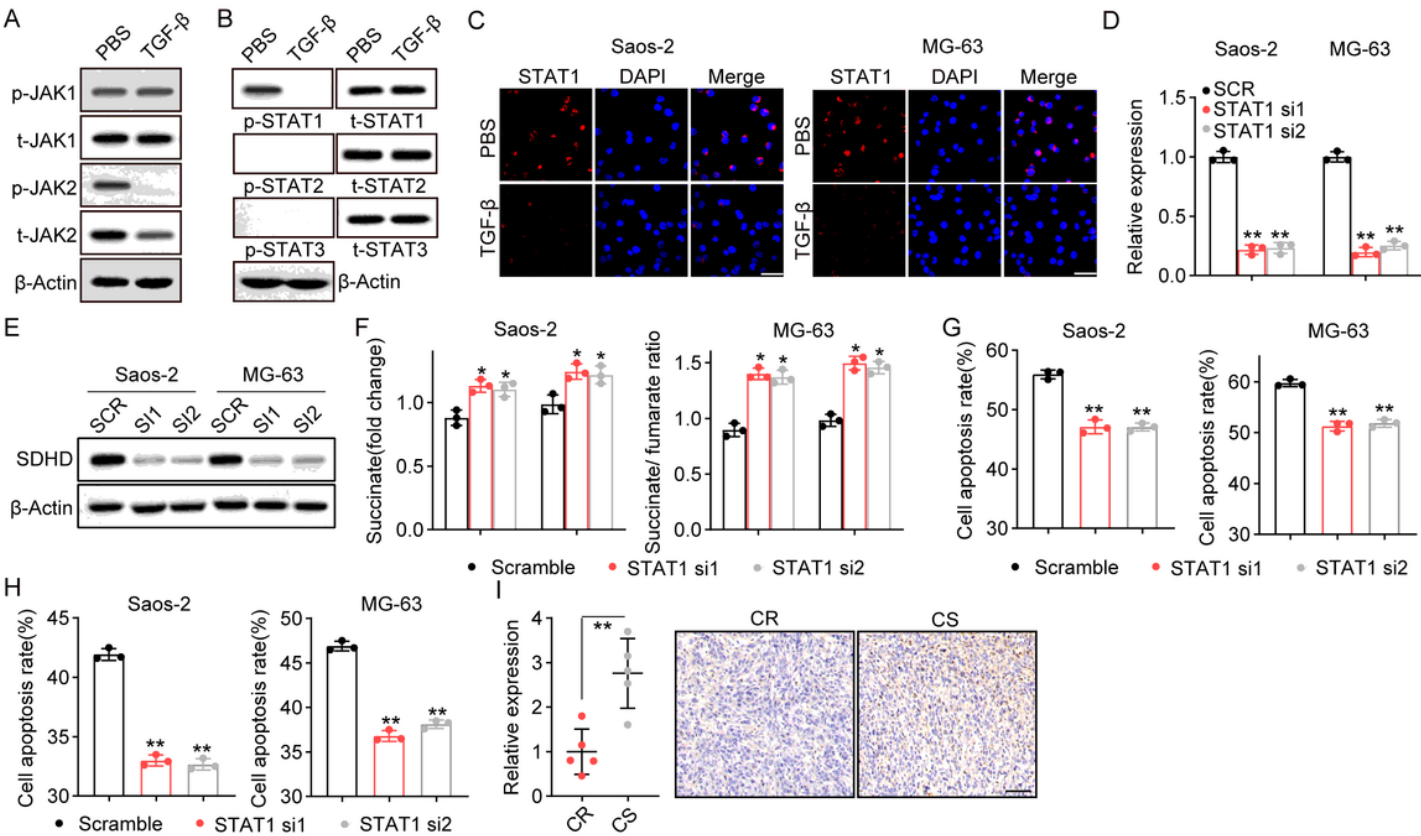


**Figure 2**

TGF- $\beta$  suppressed succinate dehydrogenase to promote chemo-resistance. A, Metabolite quantification by <sup>1</sup>H-NMR. Results show the metabolite ratio in Saos-2/MG-63 cells after treatment with TGF- $\beta$  (50 ng/ml). B, C, The expression of SDHD in Saos-2 and MG-63 cells after treatment with TGF- $\beta$  (50 ng/ml) was determined by quantitative real-time PCR (B) and western blot (C). D, The knockdown of SDHD in Saos-2 and MG-63 cells was analyzed by quantitative real-time PCR. E, F, Saos-2 and MG-63 cells were transfected with scramble siRNA (SCR), SDHD siRNA1 or SDHD siRNA2, and treated with 75 mM MTX (E) or 40  $\mu$ M CIS (F) for 48h. Scramble-Saos-2/MG-63 cells were used as control. Cell apoptosis was determined by flow cytometry. G, Saos-2 and MG-63 cells were treated with MTX (75 mM) plus succinate (20 mM) for 48h. Cell apoptosis was determined by flow cytometry. H, The same as G, except that Saos-2 and MG-63 cells were treated with CIS (40  $\mu$ M) plus succinate for 48h. I, Immunohistochemistry of SDHD

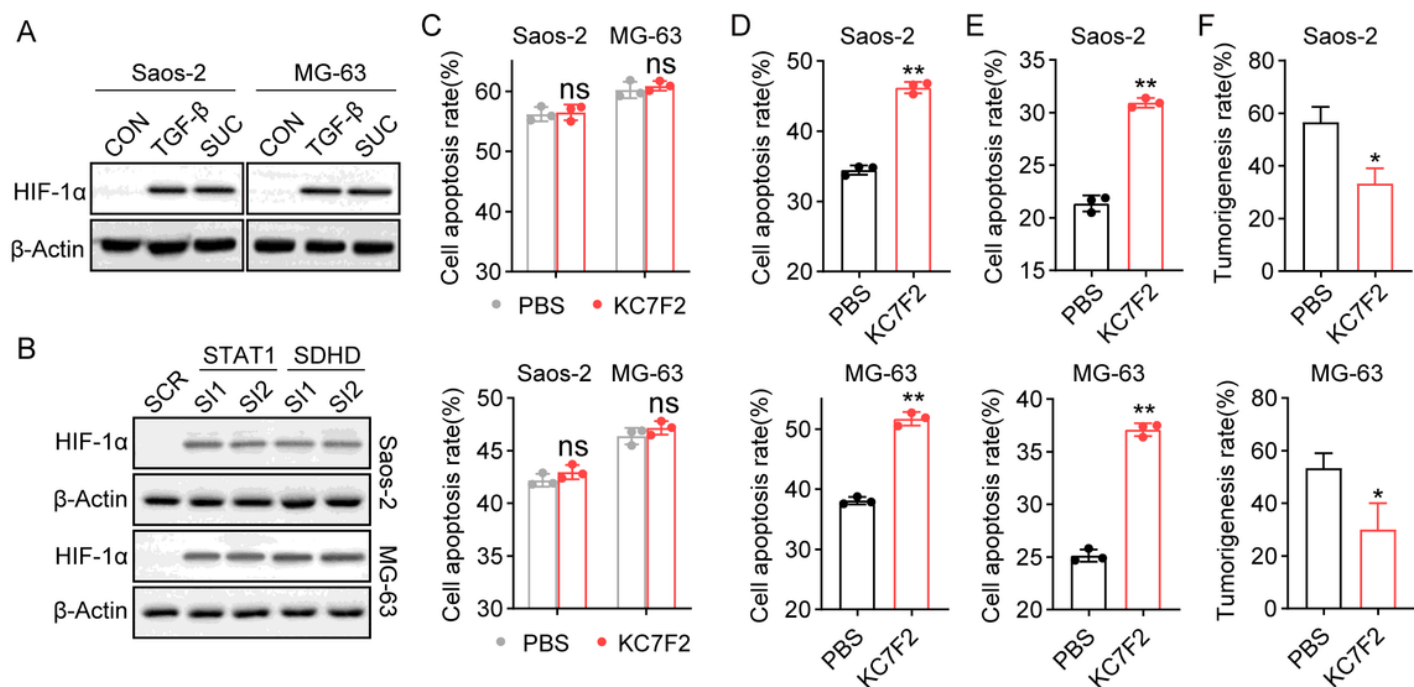


in tumor tissues from chemoresistant (CR) and chemosensitive (CS) patients. The scale bar is 50  $\mu$ m. MTX, methotrexate; CIS, cisplatin.



**Figure 3**

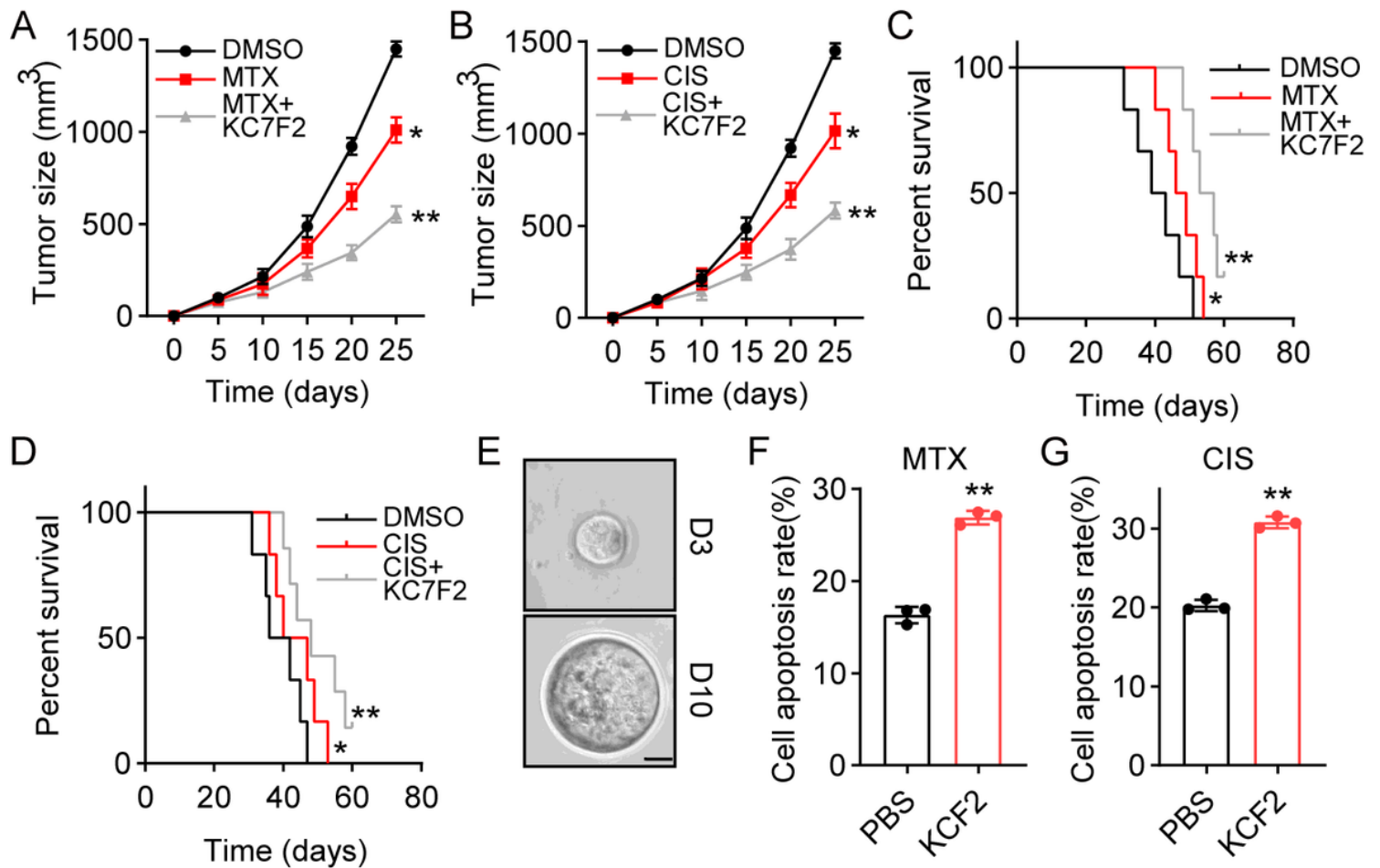
TGF- $\beta$  suppressed STAT1 to decrease SDH expression. A, Saos-2 cells were treated with TGF- $\beta$  (50 ng/ml) for 48h and then the expression of phosphorylated JAK1, JAK1, phosphorylated JAK2 and JAK2 was determined by western blot. B, Saos-2 cells were treated with TGF- $\beta$  (50 ng/ml) for 48h and then the expression of phosphorylated STAT1, STAT1, phosphorylated STAT2, STAT2, phosphorylated STAT3 and STAT3 was determined by western blot. C, Immunofluorescence staining of phosphorylated STAT1 in Saos-2 and MG-63 cells with or without TGF- $\beta$  (50 ng/ml) treatment. The scale bar is 50  $\mu$ m. D, The knockdown of STAT1 in Saos-2 and MG-63 cells was analyzed by quantitative real-time PCR. E, Saos-2 or MG-63 cells were transfected with scramble siRNA (SCR), STAT1 siRNA1 or STAT1 siRNA2, and the expression of SDHD was determined by western blot. F, Metabolite quantification in scramble- or shSTAT1-Saos-2/MG-63 cells. G, H, Scramble- or shSTAT1-Saos-2/MG-63 cells were treated with 75 mM MTX (G) or 40  $\mu$ M CIS (H) for 48h. Cell apoptosis was determined by flow cytometry. I, Immunohistochemistry of phosphorylated STAT1 in tumor tissues from chemoresistant (CR) and chemosensitive (CS) patients. The scale bar is 100  $\mu$ m. MTX, methotrexate; CIS, cisplatin.



**Figure 4**

Metabolic succinate facilitated osteosarcoma chemo-resistance through an HIF1α dependent manner. A, The expression of HIF1α in Saos-2 and MG-63 cells after treatment with TGF-β (50 ng/ml) or succinate (20 mM) was determined by western blot. B, The expression of HIF1α in shSTAT1- or shSDHD-Saos-2/MG-63 cells was determined by western blot. C, Saos-2 and MG-63 cells were treated with MTX (75 mM)/CIS (40 μM) plus KC7F2 (20 μM). Cell apoptosis was determined by flow cytometry. D, Saos-2 and MG-63 cells were treated with MTX (75 mM) in the presence of TGF-β (50 ng/ml) plus KC7F2 (20 μM). Cell apoptosis was determined by flow cytometry. E, The same as D, except that Saos-2 and MG-63 cells were treated with CIS (40 μM) in the presence of TGF-β (50 ng/ml) plus KC7F2 (20 μM). F, Tumorigenicity of Saos-2 and MG-63 cells after treatment with TGF-β (20 μg/kg) plus KC7F2 (10 mg/kg). SUC, succinate; MTX, methotrexate; CIS, cisplatin.





**Figure 5**

Blockade of HIF1 $\alpha$  improved outcome of chemotherapy in osteosarcoma. A, The tumor growth of the Saos-2 tumor was detected after treatment with DMSO, MTX (5 mg/kg), KC7F2 (10 mg/kg) and MTX combined with KC7F2. B, The tumor growth of the Saos-2 tumor was detected after treatment with DMSO, CIS (1 mg/kg), KC7F2 (10 mg/kg) and CIS combined with KC7F2. C, The survival time of the Saos-2 tumor was detected after treatment with DMSO, MTX (5 mg/kg), KC7F2 (10 mg/kg) and MTX combined with KC7F2. D, The survival time of the Saos-2 tumor was detected after treatment with DMSO, CIS (1 mg/kg), KC7F2 (10 mg/kg) and CIS combined with KC7F2. E, Osteosarcoma cells from chemo-resistant patients were cultured in 3D matrix gel. Colony size was indicated after 3 (D3) and 10 days (D10). The scale bar is 10  $\mu$ m. F, Osteosarcoma cells from chemo-resistant patients were cultured in 3D matrix gel and treated with MTX (75 mM) plus KC7F2 (20  $\mu$ M). Cell apoptosis was determined by flow cytometry. G, The same as F, except that osteosarcoma cells were treated with CIS (40  $\mu$ M) plus KC7F2 (20  $\mu$ M). MTX, methotrexate; CIS, cisplatin.