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CircHMGB2 Acts as a Molecular Decoy for IGF2BP1 to Promote Cisplatin Resistance via Grb10/Akt Signaling in Chondrosarcoma

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ABSTRACT

Chondrosarcoma is a common bone sarcoma in adults with limited therapeutic options due to frequent chemoresistance. Cisplatin (CDDP) is a key chemotherapeutic agent, but resistance often develops. This study investigated the role and mechanism of circular RNA HMGB2 (circHMGB2) in cisplatin resistance of chondrosarcoma. Clinically, circHMGB2 was upregulated in chondrosarcoma tumors and associated with cisplatin resistance and poor patient survival. Functionally, exogenous overexpression of circHMGB2 elevated cisplatin resistance in chondrosarcoma cells (SW1353, OUMS-27) in vitro, while its silencing re-sensitized resistant cells. Mechanistically, circHMGB2 did not function as a miRNA sponge but acted as a competitive molecular decoy for the RNA-binding protein IGF2BP1. We demonstrated that circHMGB2 directly binds IGF2BP1, competitively impairing IGF2BP1's binding to and stabilization of Grb10 mRNA, leading to its destabilization and subsequent downregulation of Grb10 protein. This suppression of Grb10, a known negative regulator of the PI3K/Akt pathway, resulted in constitutive Akt phosphorylation, driving resistance. Crucially, co-overexpression of IGF2BP1 rescued circHMGB2-induced Grb10 downregulation, Akt activation, and cisplatin resistance, validating the decoy mechanism. Furthermore, in a cisplatin-resistant xenograft model, silencing circHMGB2 synergized with cisplatin to significantly inhibit tumor growth, improve host survival, and upregulate intratumoral Grb10 while suppressing Akt phosphorylation. Our results delineate a novel circHMGB2/IGF2BP1/Grb10/Akt axis that drives cisplatin resistance in chondrosarcoma. Targeting circHMGB2 could therefore serve as a promising therapeutic strategy to overcome chemoresistance and enhance treatment efficacy.

Keywords: Chondrosarcoma, cisplatin, chemoresistance, circHMGB2, Grb10, Akt.

INTRODUCTION

Chondrosarcoma is an uncommon malignant mesenchymal tumor which accompanies rare differentiation of cartilaginous cells in the jaw[1,2]. After myeloma and osteosarcoma, chondrosarcoma is the third frequently occurred primary bone malignancy[2]. Even with adequate surgery, patients with chondrosarcoma have a poor prognosis[3]. Chondrosarcoma is radio- and chemo- therapy resistant due to the barriers from extracellular matrix and poor vascularity[3]. Currently, the molecular mechanisms underlying the chemoresistance of chondrosarcoma are partially understood. Therefore, this study aimed to identify the precis molecular mechanisms of the chemoresistant chondrosarcoma.

Cisplatin (CDDP) is a platinum-based anti-cancer agent against various tumors including breast, bladder, ovarian, testicular, colorectal, lung, head and neck cancers and chondrosarcoma[4-6]. However, a considerable fraction of chondrosarcoma patients eventually developed cisplatin resistance[7]. In addition, overdosed usage of cisplatin may result in the cytotoxicity of normal tissues[8]. Thus, investigating the mechanisms for overcoming chemoresistance could mitigate the main limitations of clinical application of cisplatin. Cisplatin-resistant chondrosarcoma cells were known to be associated with dysregulated oncogenic signaling pathways[9], indicating that selective targeting specific oncogenic pathway could be an effectively therapeutic strategy to sensitize chondrosarcoma cells to chemotherapeutic agents.

Circular RNAs (circRNAs) are a class of single-stranded and covalently closed RNA[10]. Previous reports indicated circRNAs were essential regulators for oncogenesis and progressions of tumors through modulating a vast number of oncogenic or tumor suppressive genes[11-13]. For instance, circROCK1 has been reported to suppress osteosarcoma proliferation and migration[12]. Moreover, circular RNA circ_001621 sponged miR-578 to promote osteosarcoma cells proliferation and migration[13]. CircRNA high-mobility group box 2 (circHMGB2) was known to be positively associated with lung cancer[14]. Currently, the biological roles and molecular targets of circHMGB2 in chemoresistant chondrosarcoma remain unclear.

Growth factor receptor binding protein-10 (Grb10) is an adaptor protein which has been reported to restrain the PI3K/AKT signaling pathway[15]. Currently, the precise roles and upstream regulators of Grb10 in chemoresistant chondrosarcoma remain elusive. This study explored the biological roles and molecular mechanisms of circHMGB2 in cisplatin sensitivity of chondrosarcoma cells. Our results revealed a circular RNA-mediated Grb10-Akt pathway which acted essential roles in cisplatin resistant chondrosarcoma, providing new molecular basis for development of therapeutic approaches against chemoresistant chondrosarcoma.

MATERIALS AND METHODS

Chondrosarcoma patient information and tissue collection

This study included a cohort of 30 chondrosarcoma patients diagnosed between 2015 and 2020 at our institution. The study protocol was approved by the Institutional Review Board of The First People's Hospital of Xuzhou, and written informed consent was obtained from all participants. During surgical resection, paired samples from the primary tumor and adjacent normal bone tissue were collected. Each sample was divided into two aliquots: one was snap-frozen in liquid nitrogen and stored at –80°C for subsequent molecular analysis, and the other was fixed in 10% neutral-buffered formalin and embedded in paraffin (FFPE) for histopathological evaluation. All tumor samples were independently reviewed by two pathologists to confirm a tumor cell content exceeding 80%.

Detailed clinicopathological data were retrospectively collected from medical records, including age, sex, tumor location, size, histological subtype and grade (according to the WHO classification), American Joint Committee on Cancer (AJCC) stage, treatment regimen, and response to cisplatin-based chemotherapy. Patients were classified as "cisplatin-sensitive" if they exhibited a partial or complete response to therapy, or "cisplatin-resistant" in cases of stable disease or progression. Overall survival (OS) was defined as the time from diagnosis to death from any cause or the last follow-up. The median follow-up duration was 36 months. To evaluate the independent prognostic value of circHMGB2, multivariate survival analysis was performed using the Cox proportional hazards regression model, adjusting for age, tumor size, histological grade, AJCC stage, and cisplatin response. Statistical significance was set at $p < 0.05$.

Chondrosarcoma cells and cell culture

Human OUMS-27 and SW1353 chondrosarcoma cells were obtained from ATCC (American Type Culture Collection). All cells were kept in DMEM/F12 medium supplied with FBS (10%) (Gibco, USA) with CO2 (5%) at 37˚C. Cisplatin-resistant cells were generated from SW1353 cells which were treated with increased amount of cisplatin for three months. The survival cells were collected and used for downstream analysis.

Reagents and antibodies

Antibodies are purchased from: total Akt (t-Akt) (mouse monoclonal; catalog no., 2966; Cell Signaling Technology, USA), p-Akt (S473; rabbit monoclonal; catalog no., 4060; Cell Signaling Technology, USA), Grb10 (rabbit polyclonal; catalog no., 3702; Cell Signaling Technology, USA) and β-actin (mouse monoclonal; catalog no., 3700; Cell Signaling Technology, USA.). Cisplatin was obtained from Sigma (Shanghai, China).

Transfections of miRNA, siRNA and plasmid DNA

CircHMGB2 siRNA, overexpression plasmid as well as their negative controls were provided by GenePharma (Shanghai, China). Akt and Grb10 overexpression plasmids and control plasmids were obtained from Addgene Inc. (Cambridge, USA). Chondrosarcoma cells were transfected with lipofectamine 3000 (Invitrogen, USA.) following protocol from the kit. Transfection was performed for 48 hours and cells were harvested for analysis.

RNA isolation and qRT-PCR

RNAs were isolated from chondrosarcoma cells by the Trizol reagent (ThermoFisher Scientific, Inc., USA).

For evaluating the level of circHMGB2 expression, the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Inc., USA) was applied for cDNA synthesis according to the protocols from the kit. Realtime PCR was conducted using the Real-time PCR Mixture assays kit (TaKaRa, China). RT-qPCR data were normalized to β-actin. Relative expression was calculated by the 2-ΔΔCt formular. Experiments were performed in triplicate.

Cell viability

Cell viability capacity was determined by MTT assay (ThermoFisher Scientific, Inc., USA) following standard protocol from the kit. Cells were plated in 96-well plate (3x103 per well) for twenty-four hours then incubated with MTT reagent (20 µl) at 37˚C for 4 hours. DMSO (100 µl) was added for 1 hour at 37˚C. The OD (optical density) value was measured at 490 nm wavelength. Cell viability was shown as the ratio of OD value from the treatment group to that from control group.

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Clonogenic assay

After transfection and cisplatin treatment, chondrosarcoma cells (5 x 102/well) were seeded into six-well plates and culture for 24 hours. Medium was refreshed with drug-free medium, and cells were cultured for an additional week. Cells were fixed with methanol and stained with a solution of 0.01% crystal violet. After washing with PBS, colonies were photographed under a bright-field microscope.

Apoptosis assay

Chondrosarcoma cells were seeded onto 96-well plates at a density of 1 × 103/well and cultured for 24 hours. Cells were then treated with different concentrations of cisplatin for 48 hours. Cell apoptosis rate was measured using the Annexin V-FITC Apoptosis Kit (Biovision, USA) according to the manufacturer's instructions. Cells were collected and washed with cold PBS. Binding buffer (500 µl) from the kit was added into tubes. Annexin-FITC and propidium iodide (PI) solutions (5 µl of each) were added into reaction, followed by incubation for 15 min in the dark. The FACS Calibur flowcytometry (Becton Dickinson, USA) was applied to analysis cell apoptosis/death rate. Experiments were performed in triplicate.

RNA Pull-down Assay

Biotinylated DNA probes (the antisense probe targeting the circHMGB2 backsplice junction and the antisense probe targeting the 3'UTR of human Grb10 mRNA) were designed and synthesized (GenePharma, Shanghai). Corresponding sense probes served as negative controls. For each reaction, 3 µg of biotinylated probe was incubated with 1 mg of whole-cell lysate (prepared in Pierce IP Lysis Buffer, ThermoFisher) and 20 µL of pre-washed Streptavidin Magnetic Beads (Pierce) at 4°C for 4 hours with rotation. Beads were then washed five times with lysis buffer. Bound proteins were eluted by boiling in 1× SDS loading buffer and analyzed by Western blot.

RNA Immunoprecipitation (RIP) and Competitive RIP Assay

The RIP assay was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's protocol. Briefly, SW1353 CDDP Res cells were lysed in RIP lysis buffer. For each immunoprecipitation, 100 µL of lysate was incubated with 5 µg of anti-IGF2BP1 antibody (Cell Signaling Technology, #8482) or normal mouse IgG (negative control) conjugated to magnetic beads overnight at 4°C. Beads were washed six times with RIP wash buffer. Co-precipitated RNA was then extracted using TRIzol LS reagent (Invitrogen). For the competitive RIP assay (Fig. 5C), cells were first transfected with circHMGB2 overexpression plasmid or control vector for 48 hours prior to lysis and RIP procedure. Purified RNA was reverse-transcribed and analyzed by qRT-PCR. The enrichment of specific RNAs was calculated as fold-change relative to the IgG control after normalization to the input sample.

Grb10 mRNA Stability Assay

SW1353 CDDP Res cells were transfected as indicated. Forty-eight hours post-transfection, transcription was halted by adding actinomycin D (Act D, Sigma) to the culture medium at a final concentration of 5 µg/mL. Total RNA was harvested at 0, 2, 4, 6, and 8 hours post-Act D treatment using TRIzol reagent. The remaining levels of Grb10 mRNA at each time point were quantified by qRT-PCR, normalized to the

internal control β-actin mRNA, and expressed as a percentage of the level at time 0. The mRNA half-life (t1/2) was calculated from the decay curve using one-phase exponential decay nonlinear regression in GraphPad Prism 9.0.

Western blot

Chondrosarcoma ells were lysed in RIPA buffer (Invitrogen, USA) on ice for 30 minutes. Protein samples (40 μg/lane) were loaded onto SDS electrophoresis then transferred onto nitrocellulose membranes. After blocking (5% BSA, 1 hour), membranes were incubated with primary antibodies (1:1000), including Grb10, p-Akt, t-Akt and β-actin at 4 oC for 16 hours. After washing by PBST for 5 times with 5 minutes each, membranes were incubated with secondary antibodies for 3 hours. Bands were visualized by a Super Signal Enhanced Chemiluminescence kit (Biorad, USA).

Animal xenograft study

Four- to six-week-old female BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under specific pathogen-free conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of The First People's Hospital of Xuzhou (Approval No. 20240321). For tumor inoculation, 5×106 SW1353 CDDP Res cells stably expressing either control shRNA (sh-NC) or circHMGB2-targeting shRNA (sh-circHMGB2) were suspended in 100 μL of PBS and subcutaneously injected into the right flank of each mouse. When tumors reached approximately 100 mm^3, mice were randomly assigned to four groups (n=10 per group) using a computer-generated randomization schedule: (1) sh-NC + saline; (2) sh-NC + cisplatin (3 mg/kg); (3) sh-circHMGB2 + saline; (4) sh-circHMGB2 + cisplatin (3 mg/kg). Cisplatin or saline was administered intraperitoneally once weekly for 5 weeks. Tumor volumes were measured twice weekly by an investigator blinded to the group allocation using digital calipers and calculated as length × $width^2$ × 0.5. Mice were euthanized when tumors exceeded 1500 mm^3 or at the study endpoint (day 35). Tumors were excised, weighed, and either snap-frozen in liquid nitrogen for molecular analysis.

Statistical analysis

All experiments were performed with at least three independent biological replicates, unless otherwise specified. The exact number of replicates (n) for each experiment is detailed in the corresponding figure legends. Data are presented as mean ± standard deviation (SD). For comparisons between two groups, unpaired two-tailed Student's t-test was used. For comparisons among multiple groups, one-way or two-way analysis of variance (ANOVA) was applied as appropriate, followed by Tukey's post-hoc test for multiple comparisons with adjustment for family-wise error rate. The half-maximal inhibitory concentration (IC50) values and their corresponding 95% confidence intervals (CIs) were calculated using nonlinear regression (log(inhibitor) vs. response -- variable slope) in GraphPad Prism 9.0.

For animal studies: Mice were randomly assigned to treatment groups using a computer-generated randomization schedule to ensure equal distribution of initial tumor volumes. Tumor volume measurements were performed by an investigator blinded to the group allocation. All animal experiments were performed in accordance with ARRIVE guidelines. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, USA).

RESULTS

CircHMGB2 is positively associated with cisplatin resistance of chondrosarcoma

Previous reports indicated that circHMGB2 played oncogenic roles in lung cancer[14], prompting us to investigate its potential involvement in chondrosarcoma chemoresistance. We first analyzed a well-characterized cohort of 30 chondrosarcoma patients. The clinicopathological features of this cohort are detailed in Table I. Briefly, the median age was 52 years, with a balanced sex distribution (60.0% male). Conventional chondrosarcoma was the predominant histological subtype (73.3%), and the majority of tumors were high-grade (Grade II/III, 80.0%) and presented at advanced stages (AJCC Stage II/III, 76.7%). Notably, high circHMGB2 expression was significantly associated with adverse clinical features, including larger tumor size, higher histological grade, and advanced AJCC stage (Table I).

To evaluate the independent prognostic value of circHMGB2, we performed multivariate Cox regression analysis adjusting for key covariates including age, tumor size, histological grade, AJCC stage, and cisplatin response. As summarized in Table II, high circHMGB2 expression emerged as a strong and independent predictor of poor overall

Table I. — Clinicopathological Characteristics of the Chondrosarcoma Cohort (n=30).

Characteristic	Category	Number of Patients (%) or Median (Range)
Age (years)		52 (28–75)
Sex	Male	18 (60.0%)
	Female	12 (40.0%)
Tumor Location	Femur	14 (46.7%)
	Pelvis	8 (26.7%)
	Humerus	4 (13.3%)
	Tibia	2 (6.7%)
	Other	2 (6.7%)
Tumor Size (cm)		8.5 (4.5–15.2)
Histological Subtype	Conventional	22 (73.3%)
	Dedifferentiated	5 (16.7%)
	Mesenchymal	3 (10.0%)
Histological Grade	Grade I	6 (20.0%)
	Grade II	16 (53.3%)
	Grade III	8 (26.7%)
AJCC Stage	I	7 (23.3%)
	II	15 (50.0%)
	III	8 (26.7%)
Treatment Modality	Surgery only	11 (36.7%)
	Surgery + Cisplatin-based therapy	19 (63.3%)
Response to Cisplatin	Sensitive	9 (30.0%)
	Resistant	21 (70.0%)
circHMGB2 Expression	Low	8 (26.7%)
	High	22 (73.3%)
Follow-up (months)		36 (12–60)

Table II. — Multivariate Cox Regression Analysis for Overall Survival.

Variable	Category	Hazard Ratio (HR)	95% Confidence Interval	*p*-value
circHMGB2 Expression	High vs. Low	3.42	1.56 – 7.48	0.002
Age	> 50 vs. ≤ 50 years	1.21	0.58 – 2.54	0.612
Tumor Size	> 5 cm vs. ≤ 5 cm	1.89	0.88 – 4.05	0.104
Histological Grade	Grade III vs. I/II	2.15	1.02 – 4.55	0.045
AJCC Stage	III vs. I/II	2.67	1.24 – 5.76	0.012
Cisplatin Response	Resistant vs. Sensitive	2.98	1.32 – 6.73	0.008
Note: Cox proportional hazards model was adjusted for all variables listed. HR > 1 indicates increased risk of death. Statistical significance was set at *p* < 0.05.				

survival (Hazard Ratio [HR] = 3.42, 95% Confidence Interval [CI]: 1.56–7.48, p = 0.002). Other factors retaining independent prognostic significance in this model were higher histological grade (HR=2.15, p=0.045), advanced AJCC stage (HR=2.67, p=0.012), and cisplatin-resistant disease (HR=2.98, p=0.008). This analysis solidifies the clinical relevance of circHMGB2 as a biomarker indicative of aggressive tumor behavior and therapy resistance.

From clinical chondrosarcoma specimen analysis, circHMGB2 was detected to be significantly upregulated in chondrosarcoma tumors compared with adjacent normal bone tissues (Fig. 1A). Moreover, Kaplan-Meier analysis illustrated that higher

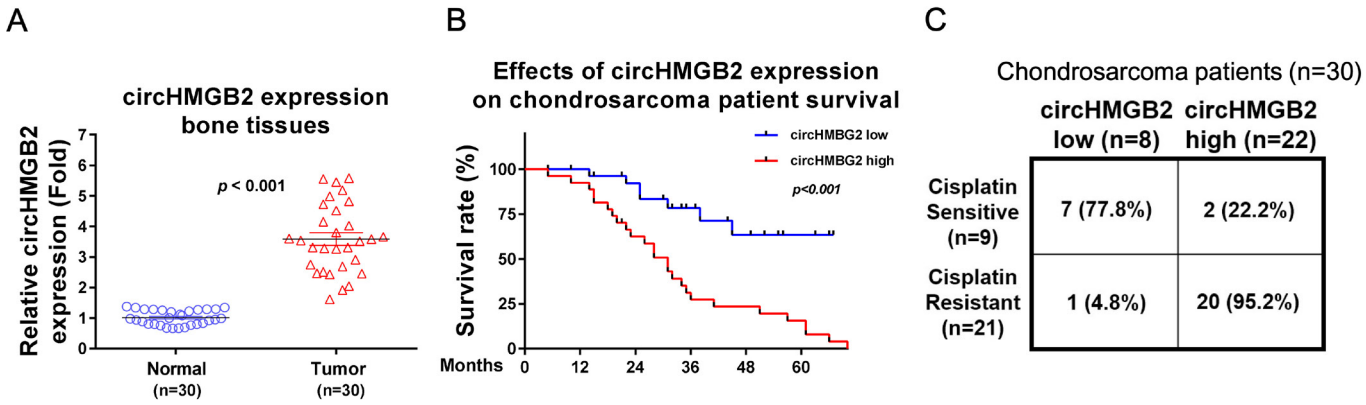

Fig. 1 — CircHMGB2 is positively associated with chondrosarcoma and cisplatin resistance.
*(A) Expressions of circHMGB2 were detected from chondrosarcoma specimens (n=30) and adjacent normal bone tissues (n=30). Data are presented as mean ± SD (n=30). ***p<0.001 by unpaired t-test. (B) Effects of circHMGB2 expression levels on chondrosarcoma patient survival (n=30 per group). Log-rank test was used for comparison. (C) Correlation between expression level of circHMGB2 and cisplatin resistance in chondrosarcoma patients (n=30). Fisher's exact test was used for statistical analysis.*

circHMGB2 expression was associated with lower survival rate of chondrosarcoma patients (p<0.001) (Fig. 1B), suggesting that circHMGB2 functioned as a chondrosarcoma-favorite molecule. Due to the characteristics of chondrosarcoma that it was frequently insensitive to chemo and radiotherapy[3], we then evaluated the roles of circHMGB2 in cisplatin resistant chondrosarcoma. Out of the thirty chondrosarcoma patients, 8 of them displayed low circHMGB2 expression and 22 patients showed high circHMGB2 expression. Moreover, among thirty patients, 9 of them were cisplatin sensitive and 21 patients were characterized as cisplatin resistant (Fig. 1C). Notably, 7 of the 9 (77.8%) cisplatin sensitive chondrosarcoma patients were associated with low circHMGB2 expression, while only 2 (22.2%) of them showed high circHMGB2 expression (Fig. 1C). On the other way, among the 21 cisplatin resistant chondrosarcoma patients, the majority (20 cases, 95.2%) exhibited high circHMGB2 expression and only 1 (4.8%) of them displayed low circHMGB2 expression (Fig. 1C). Summarily, this clinical analysis uncovered that circHMGB2 was positively associated with cisplatin resistance of chondrosarcoma.

CircHMGB2 promotes cisplatin resistance of chondrosarcoma cells in vitro

To investigate the underlying cellular mechanisms of the circHMGB2-accelerated cisplatin resistance, a CDDP resistant chondrosarcoma cell line, SW1353 CDDP Res was generated by gradual treating cells with elevated concentrations of CDDP for three months. The CDDP resistant cells were pooled for analysis. Figure 2A showed that SW1353 CDDP Res cells could apparently tolerate higher concentrations of cisplatin. Parental cells exhibited a half maximal inhibitory concentration (IC50) of 60.3 μM (95% CI: 55.8-65.2 μM). The IC50 of CDDP Res cells was 250.52 μM (95% CI: 235.6-266.7 μM), representing an approximately 4-fold increase in resistance compared to parental cells. Expectedly, circHMGB2 was remarkedly upregulated in CDDP resistant cells (Fig. 2B), suggesting circHMGB2 plays essential roles in chemoresistant chondrosarcoma. To verify the above results, circHMGB2 was overexpressed in OUMS-27 and SW1353 cells. Overexpression of circHMGB2 significantly promoted the survival capacity of chondrosarcoma cells under cisplatin treatments (Fig. 2C and 2D), indicating that inhibition of circHMGB2 could potentially serve as a therapeutic approach to enhance the cisplatin efficacy in chondrosarcoma cells.

Akt is hyper-phosphorylated and Grb10 is downregulated in cisplatin-resistant chondrosarcoma cells

We then evaluated the molecular mechanisms for the circHMGB2-promoted cisplatin resistance. Previous results reveal that Akt pathway, which acted as oncogene to facilitate cancer cell chemoresistance, was negatively regulated by Grb10[15]. The effects of cisplatin treatments on the phosphorylation of Akt and the protein expressions of Grb10 were subsequently assessed. As shown in figure 3A, 3B, cisplatin treatment at low concentrations could slightly (p<0.05) induce Akt phosphorylation and suppress GRB10. But at high concentrations which are above the IC50 level, cisplatin treatment significantly diminished the Akt phosphorylation and induced Grb10 expression, suggesting phosphorylation of Akt contributes to the survival of chondrosarcoma cells and Grb10 promotes cancer cell death under cisplatin treatments. As the

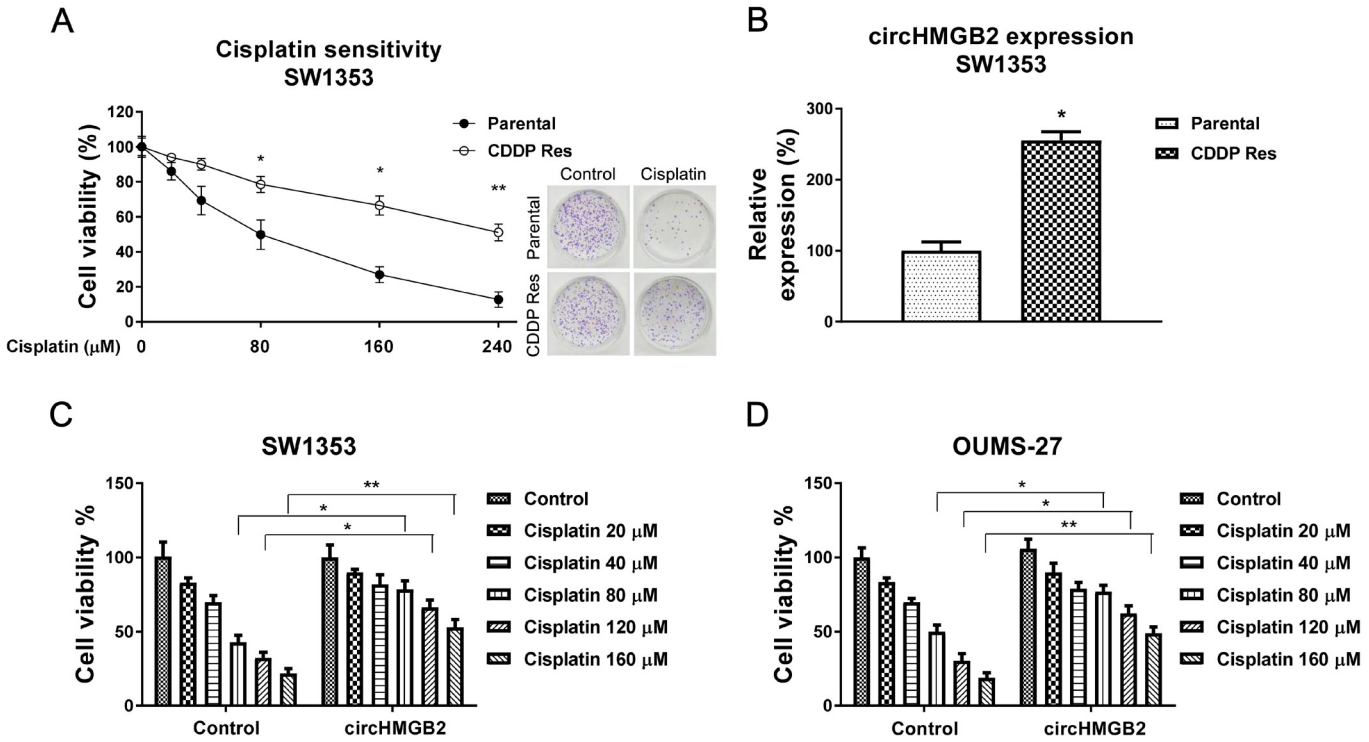

Fig. 2 — CircHMGB2 promotes cisplatin resistance of chondrosarcoma cells.
*(A) Validation of the CDDP resistance from SW1353 CDDP resistant cells. IC50 values: Parental cells: 60.3 μM (95% CI: 55.8-65.2 μM); CDDP Res cells: 250.52 μM (95% CI: 235.6-266.7 μM). (B) Expressions of circHMGB2 in SW1353 parental and cisplatin resistant cells (n=3 independent experiments). (C) SW1353 and (D) OUMS-27 cells were transfected with control plasmid or circHMGB2. Cells were treated with 20, 40, 80, 120 and 160 μM cisplatin for 48 h. Cell survival capacities were determined by cell viability assay (n=3 independent experiments). Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. *, p<0.05; **, p<0.01.*

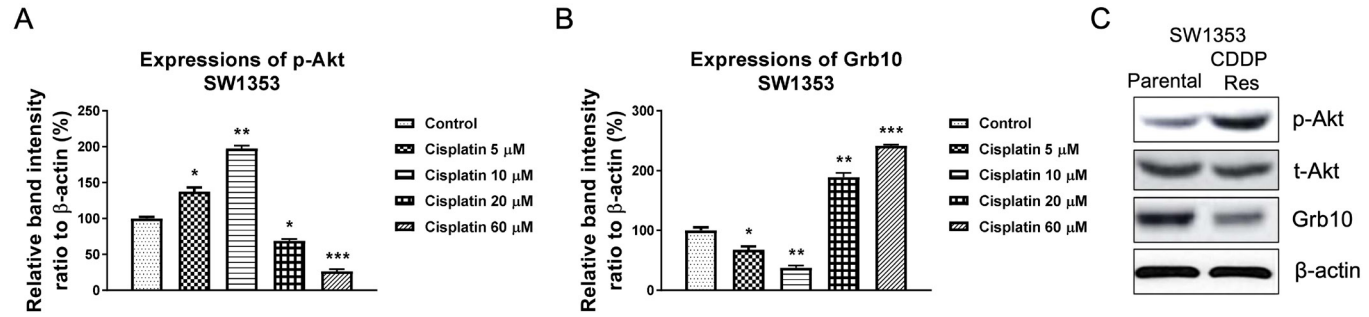

Fig. 3 — Akt is activated in CDDP resistant chondrosarcoma cells.
*(A) SW1353 cells were treated with cisplatin at various concentrations, p-Akt and (B) Grb10 were measured by Western blot and results were shown as the relative intensity of protein bands. (C) SW1353 parental and CDDP resistant cells were examined by western blot. Expressions of total Akt, phosphorylated-Akt and Grb10 were shown. β-actin was a loading control. *, p<0.05; **, p<0.01, ***, p<0.001.*

above results have revealed a positive association between circHMGB2 and cisplatin resistance, we evaluated whether the circHMGB2-promoted cisplatin resistance was correlated with Grb10 suppression and Akt pathway activation. The Akt phosphorylation and Grb10 protein levels were examined in SW1353 parental and CDDP resistant cells. Results in figure 3C reveals that the phosphorylation of Akt was increased but Grb10 expression was downregulated in cisplatin-resistant cells. These results imply a tight association between the circHMGB2 promoted cisplatin resistance and the Grb10-Akt pathway in chondrosarcoma cells.

CircHMGB2 promotes Akt phosphorylation and blocks Grb10 expression chondrosarcoma cells

We investigated whether circHMGB2 regulated Akt phosphorylation and Grb10 expression. CircHMB2 was overexpressed in SW1353 cells (Fig. 4A). Western blot results demonstrated that overexpression of circHMGB2 significantly promoted Akt phosphorylation and

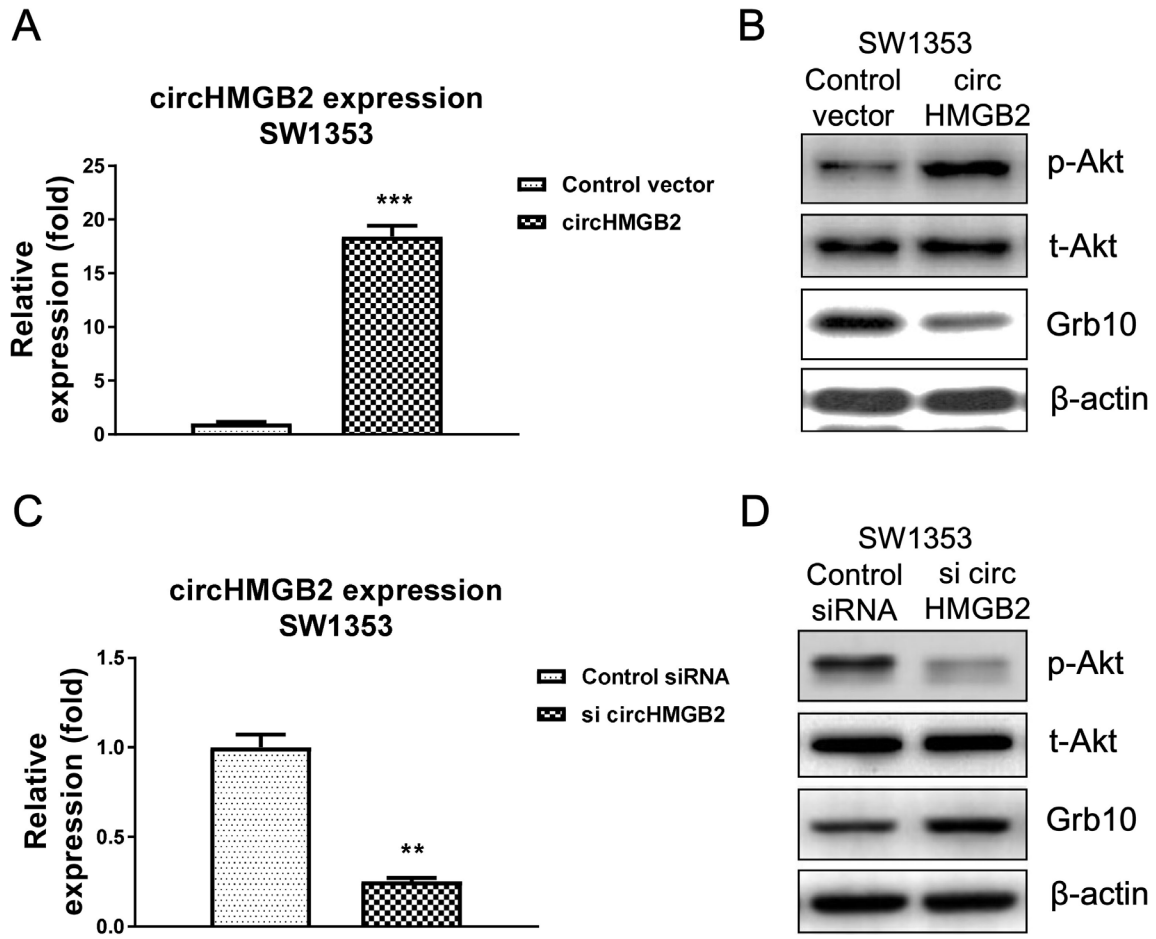

Fig. 4 — CircHMGB2 upregulates Akt phosphorylation in chondrosarcoma cells.
*(A) Control plasmid or circHMGB2 overexpression plasmid was transfected into SW1353 cells, circHMGB2 expression was detected. (B) Expressions of total Akt, phosphorylated-Akt and Grb10 were examined in the above cells. (C) Control siRNA or circHMGB2 overexpression plasmid was transfected into SW1353 cells, circHMGB2 expression was detected. (B) Expressions of total Akt, phosphorylated-Akt and Grb10 were examined in the above cells. **, $p<0.01$; ***, $p<0.001$.*

suppressed Grb10 expression (Fig. 4B). In addition, silencing of circHMGB2 (Fig. 4C) effectively suppressed Akt phosphorylation and upregulated Grb10 (Fig. 4D). These data suggest the circHMGB2-regulated activation of Akt pathway and suppression of Grb10 could contribute to cisplatin resistance in chondrosarcoma.

CircHMGB2 functions as a molecular decoy for IGF2BP1 to destabilize Grb10 mRNA

To elucidate the molecular mechanism underlying circHMGB2-mediated Grb10 downregulation, we aimed to identify the key RNA-binding protein(s) involved and establish its relationship with both circHMGB2 and its target. Parallel RNA pull-down assays were performed using biotinylated probes designed against circHMGB2 or the 3' untranslated region (3'UTR) of Grb10 mRNA. In lysates from cisplatin-resistant SW1353 CDDP Res cells, the circHMGB2-specific probe, but not its sense control, specifically enriched the RNA-binding protein IGF2BP1 (Fig. 5A). In a complementary experiment, the Grb10 mRNA 3'UTR probe also specifically pulled down IGF2BP1 (Fig. 5A). These in vitro binding assays establish that IGF2BP1 is a common direct binding partner for both circHMGB2 and Grb10 mRNA.

We next confirmed that these interactions occur endogenously within the cellular context. RNA immunopcipitation (RIP) assay was performed using an antibody against IGF2BP1 in SW1353 CDDP Res cells. As shown in Fig. 5B, the anti-IGF2BP1 antibody, but not the control IgG, specifically co-precipitated significant amounts of both endogenous circHMGB2 and Grb10 mRNA, as quantified by qRT-PCR. This result demonstrates that IGF2BP1 simultaneously associates with both the regulatory circRNA and its target mRNA in vivo, solidifying its role as the critical molecular node linking circHMGB2 to post-transcriptional regulation of Grb10.

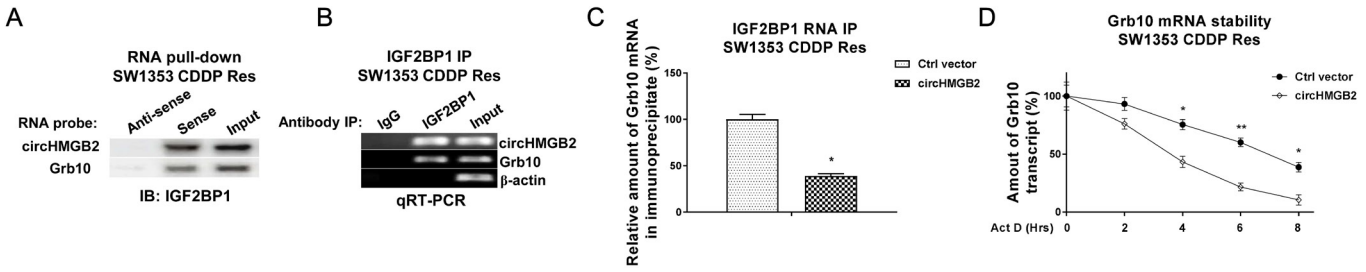

Fig. 5 — CircHMGB2 sequesters IGF2BP1 to destabilize Grb10 mRNA.
*(A) Biotinylated antisense or sense control probes against circHMGB2 (left panel) or the 3'UTR of Grb10 mRNA (right panel) were incubated with SW1353 CDDP Res cell lysates. Captured RNA-protein complexes were analyzed by immunoblotting (IB) for IGF2BP1. (B) Lysates from SW1353 CDDP Res cells were subjected to RIP using an anti-IGF2BP1 or control IgG antibody. The enrichment of endogenous circHMGB2 and Grb10 mRNA in the precipitates was quantified by qRT-PCR (n=3 independent experiments). Data are presented as fold enrichment relative to the IgG control (mean ± SD). ***p < 0.001 (Student's t-test). (C) SW1353 CDDP Res cells were transfected with a control vector or a circHMGB2 overexpression plasmid (circHMGB2 OE). IGF2BP1 was immunoprecipitated, and the relative amount of co-precipitated Grb10 mRNA was quantified by qRT-PCR (n=3). Data are presented as mean ± SD. **p < 0.01 (Student's t-test). (D) SW1353 CDDP Res cells transfected as in (C) were treated with the transcriptional inhibitor actinomycin D (Act D, 5 μg/mL). (Left panel) Grb10 mRNA levels at the indicated time points were measured by qRT-PCR and normalized to the 0-hour time point (n=3). (Right panel) The calculated mRNA half-life (1/2) is shown. Data are presented as mean ± SD. **p < 0.01 for comparison of half-lives (Student's t-test).*

These findings led us to hypothesize that elevated circHMGB2 may act as a competitive molecular decoy, sequestering IGF2BP1 and thereby reducing its binding to and stabilization of Grb10 mRNA. To test this directly, we performed a competitive RIP assay. In SW1353 CDDP Res cells, overexpression of circHMGB2 resulted in a significant decrease in the amount of Grb10 mRNA co-precipitated with IGF2BP1, compared to control cells (Fig. 5C). This result provides direct evidence that circHMGB2 competes with Grb10 mRNA for IGF2BP1 binding. The logical consequence of reduced IGF2BP1 binding is decreased mRNA stability. As predicted, actinomycin D chase experiments showed that overexpression of circHMGB2 significantly accelerated the decay of Grb10 mRNA, markedly reducing its half-life (Fig. 5D). Collectively, the data in Figure 5 delineate a coherent post-transcriptional regulatory axis: circHMGB2 directly binds to IGF2BP1, competitively displacing Grb10 mRNA, which leads to the destabilization of Grb10 mRNA and ultimately the downregulation of Grb10 protein.

Blocking circHMGB2 re-sensitizes cisplatin resistant chondrosarcoma cells through modulating the Grb10/Akt pathway

To test whether inhibiting circHMGB2 could enhance the cytotoxicity of cisplatin to chondrosarcoma cells, endogenous circHMGB2 was knocked down in SW1353 parental and cisplatin-resistant cells. Blocking circHMGB2 effectively enhanced the cisplatin sensitivity of SW1353 cells (Fig. 6A, 5B), resulting in a synergistic inhibitory effect on chondrosarcoma cells. Knockdown of circHMGB2 significantly decreased the CDDP IC50 from 245.8 μM (95% CI: 230.5-262.3 μM) in control cells to 102.4 μM (95% CI: 92.7-113.1 μM) (Fig. 6A). Consistently, inhibition of circHMGB2 re-sensitized SW1353 cisplatin-resistant cells to cisplatin (Fig. 6C, 6D).

We subsequently validated whether circHMGB2 promoted cisplatin resistance of chondrosarcoma cells through regulating the Akt-Grb10 pathway. Overexpression of Akt (Fig. 7A) or knockdown of Grb10 (Fig. 7B) significantly sensitized SW1353 CDDP resistant cells to CDDP. Subsequently, SW1353 CDDP resistant cells were co-transfected with si circHMGB2 plus control vector or plus Akt overexpression plasmid. Western blot results demonstrate that rescue of Akt in circHMGB2-silencing cells effectively recovered total and phosphorylation Akt (Fig. 7C). Additionally, recovery of Akt pathway successfully restored the CDDP resistance (Fig. 7D). Furthermore, knockdown the low-circHMGB2 upregulated Grb10 significantly recovered Akt phosphorylation (Fig. 7E) and cisplatin resistance (Fig. 7F) of SW1353 CDDP resistant cells. Taken together, these rescue experiments unveiled essential roles of circHMGB2-Grb10-Akt axis in cisplatin resistant chondrosarcoma.

To definitively establish that circHMGB2 exerts its oncogenic function by sequestering IGF2BP1, we performed a critical rescue experiment. SW1353 CDDP Res cells were divided into three groups: transfected with a control vector, a circHMGB2 overexpression plasmid (circHMGB2 OE), or both circHMGB2 and IGF2BP1 overexpression plasmids (circHMGB2 OE + IGF2BP1 OE). Western blot analysis confirmed successful overexpression of IGF2BP1 protein

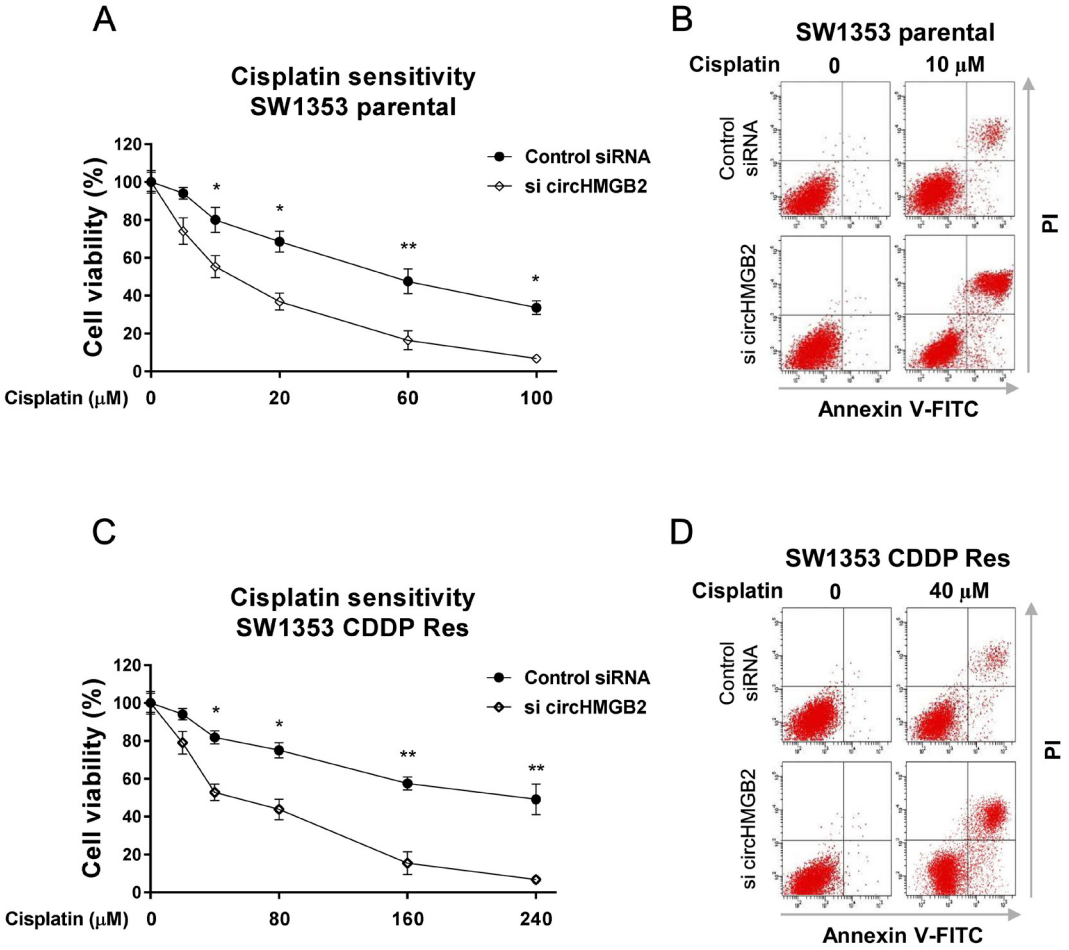

Fig. 6 — Silencing circHMGB2 sensitizes chondrosarcoma cells to cisplatin.
*(A) Control siRNA or circHMGB2 siRNA was transfected into SW1353 parental cells, followed by treatment with cisplatin at multiple concentrations. Cell response to cisplatin treatments were evaluated by cell viability assay (n=3 independent experiments). IC50 values: Control: 58.2 μM (95% CI: 52.4-64.7 μM); si-circHMGB2: 25.4 μM (95% CI: 22.1-29.2 μM). (B) Apoptosis assay under indicated conditions (n=3). (C) Control siRNA or circHMGB2 siRNA was transfected into SW1353 CDDP resistant cells, followed by treatment with cisplatin at multiple concentrations. Cell response to cisplatin treatments were evaluated by cell viability assay (n=3). IC50 values: Control: 248.6 μM (95% CI: 233.2-265.1 μM); si-circHMGB2: 102.4 μM (95% CI: 92.7-113.1 μM). (D) Apoptosis assay under indicated conditions (n=3). Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test for viability assays, and by unpaired t-test for apoptosis assays. *, p<0.05; **, p<0.01.*

specifically in the co-transfected group (Fig. 7G). As expected, circHMGB2 overexpression alone resulted in the characteristic molecular phenotype: a significant downregulation of Grb10 protein and a concomitant increase in Akt phosphorylation (p-Akt) compared to the control. Strikingly, co-overexpression of IGF2BP1 completely reversed these effects, restoring Grb10 protein levels to baseline and suppressing the circHMGB2-induced hyperphosphorylation of Akt (Fig. 7H). This demonstrates that the molecular consequences of circHMGB2 are dependent on and can be rescued by increasing the pool of available IGF2BP1.

We next assessed the functional outcome of this molecular rescue on chemoresistance. A cisplatin dose-response assay revealed that circHMGB2 overexpression significantly enhanced cell survival, shifting the viability curve to the right. Importantly, co-expression of IGF2BP1 substantially re-sensitized the cells to cisplatin, shifting the survival curve back towards the left, similar to the control group (Fig. 7I). Quantitative analysis confirmed a significant reduction in the cisplatin IC_{50} value in the rescue group compared to the circHMGB2 overexpression alone group. Together, the data in Fig. 7G-I provide direct functional validation of the proposed "molecular decoy" mechanism. They conclusively show that the cisplatin resistance driven by circHMGB2 is executed through the functional impairment of IGF2BP1, and that restoring IGF2BP1 activity is sufficient to counteract both the molecular signaling alterations and the chemoresistant phenotype.

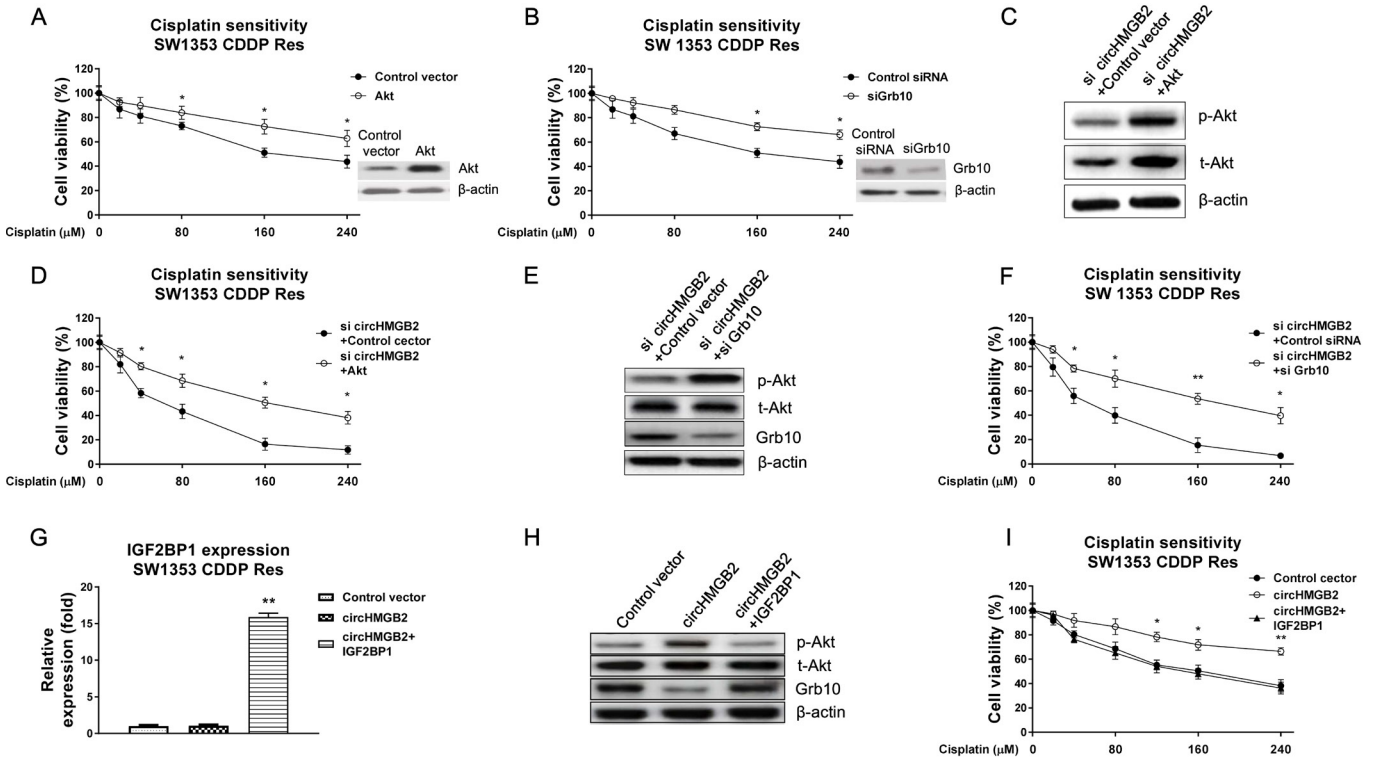

Fig. 7 — CircHMGB2 promotes cisplatin resistance of chondrosarcoma cells through the Grb10-Akt pathway.
*(A) Control vector or Akt overexpression vector was transfected into SW1353 CDDP resistant cells. Cell survival rate under CDDP treatment was determined by MTT assay. (B) Control siRNA or Grb10 siRNA was transfected into SW1353 CDDP resistant cells. Cell survival rate under CDDP treatment was determined by MTT assay. (C) SW1353 CDDP resistant cells were transfected with si circHMGB2 plus control plasmid or plus Akt overexpression plasmid. Expressions of Akt phosphorylation and total Akt were detected by Western blot. (D) The above transfected cells were treated with cisplatin at the indicated concentrations, cell responses to cisplatin were evaluated by cell viability assay. (E) SW1353 CDDP resistant cells were transfected with si circHMGB2 plus control plasmid or plus Grb10 siRNA. Expressions of Akt phosphorylation, total Akt and Grb10 were detected by Western blot. (F) The above transfected cells were treated with cisplatin at the indicated concentrations, cell responses to cisplatin were evaluated by cell viability assay. (G) SW1353 CDDP Res cells were transfected with a control vector, a circHMGB2 overexpression plasmid (circHMGB2 OE), or both circHMGB2 OE and an IGF2BP1 overexpression plasmid (circHMGB2 OE + IGF2BP1 OE). IGF2BP1 protein levels were analyzed by Western blot and quantified using Image J software. (H) Protein lysates from the transfected cells in (G) were analyzed by Western blot for the expression of Grb10, phosphorylated Akt (p-Akt, Ser473), and total Akt. Representative blots are shown. (I) IGF2BP1 overexpression re-sensitizes cells to cisplatin. The viability of the transfected cells from (G) was assessed by MTT assay after 48-hour treatment with the indicated concentrations of cisplatin. Dose-response curves are presented (mean ± SD, n=3 independent experiments). The calculated half-maximal inhibitory concentration (IC_{50}) for cisplatin is shown for each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant (one-way ANOVA with Tukey's post-hoc test for IC_{50} comparison).*

CircHMGB2 knockdown synergizes with cisplatin to improve survival and inhibit tumor growth in vivo via the Grb10/Akt axis

To evaluate the therapeutic potential of targeting the circHMGB2/IGF2BP1 axis in a physiological setting, we established a cisplatin-resistant chondrosarcoma xenograft model. Mice bearing SW1353 CDDP Res-derived tumors were randomized into four treatment groups: (1) control shRNA (sh-NC) + saline; (2) sh-NC + cisplatin (CDDP); (3) circHMGB2-targeting shRNA (sh-circHMGB2) + saline; (4) sh-circHMGB2 + cisplatin. Survival analysis revealed a striking therapeutic benefit. While cisplatin monotherapy offered a limited survival advantage over saline control, knockdown of circHMGB2 alone markedly extended host survival. Importantly, the combination of circHMGB2 knockdown and cisplatin treatment yielded the most significant survival benefit, with the majority of mice surviving to the study endpoint (Fig. 8A). This potent synergistic effect was mirrored in tumor control. At the experimental endpoint, tumors from the combination group (sh-circHMGB2 + CDDP) were significantly smaller than those from all other groups, demonstrating powerful growth inhibition (Fig. 8B).

We next examined whether the molecular axis defined in our cellular studies remained operative in vivo. Analysis of excised tumor tissues confirmed that circHMGB2 knockdown effectively upregulated Grb10 protein levels (Fig. 8C). Concordantly, the activation status of the downstream effector Akt was significantly attenuated, as shown by reduced phosphorylation (p-Akt) in tumors from the sh-circHMGB2 groups (Fig. 8D).

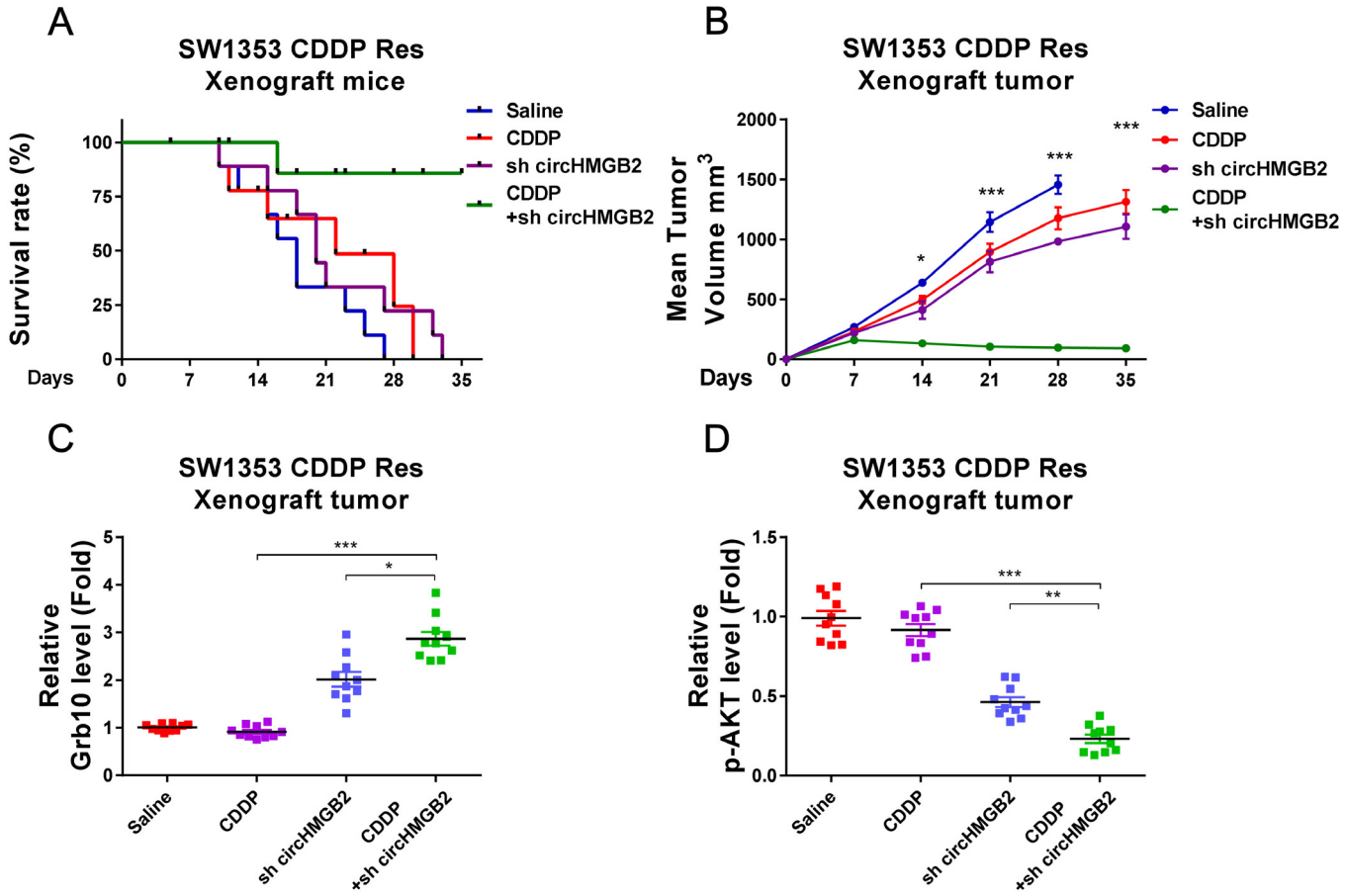

Fig. 8 — In vivo therapeutic efficacy of circHMGB2 knockdown and its synergistic effect with cisplatin.
*(A) Kaplan-Meier survival curves of nude mice bearing SW1353 CDDP Res xenograft tumors under the indicated treatments (n=10 mice per group). The combination of circHMGB2 knockdown and cisplatin (sh-circHMGB2 + CDDP) significantly extended survival compared to all other groups (Log-rank test, $p < 0.001$). (B) Representative images (left panel) and quantitative analysis (right panel) of excised tumors from each treatment group at the study endpoint. Tumor volume is presented as mean ± SD (n=10 tumors per group). ***, $p < 0.001$ (One-way ANOVA with Tukey's post-hoc test). (C, D) Molecular analysis of the in vivo mechanism. (C) Grb10 and (D) Phospho-Akt (Ser473) protein levels in tumor lysates were analyzed by Western blot (representative blots shown) and quantified by densitometry (graphs). Data are normalized to β-actin and presented as mean fold change relative to the 'sh-NC + Saline' group ± SD (n=4 independent tumor samples per group). *, $p < 0.05$; **, $p < 0.01$ (One-way ANOVA with Tukey's post-hoc test).*

These in vivo results provide compelling translational evidence that silencing circHMGB2 is an effective strategy to overcome cisplatin resistance. They demonstrate that circHMGB2 blockade not only exerts a single-agent anti-tumor effect but also profoundly synergizes with chemotherapy, resulting in superior survival outcomes. Furthermore, the consistent upregulation of Grb10 and suppression of Akt phosphorylation in treated tumors confirm that the circHMGB2-IGF2BP1-Grb10-Akt signaling axis is a critical and therapeutically targetable driver of chemoresistance in vivo.

DISCUSSION

Chondrosarcoma is a rare type of cancer that develops from cartilaginous cells in the jaw and is considered the third most common primary bone malignancy after myeloma and osteosarcoma[1,2]. Despite undergoing surgery, patients with chondrosarcoma have a bleak prognosis as the cancer is resistant to radiation and chemotherapy, mainly due to the obstacles posed by the extracellular matrix and limited blood supply[3]. The molecular mechanisms responsible for the chemoresistance of chondrosarcoma are not fully understood at this time. Recent studies revealed that circHMGB2 was positively associated with lung cancer and contributed to immunosuppression of lung cancer cells through the miR-181a-5p/CARM1 axis[14], indicating that circHMGB2 functions as an oncogenic circular RNA in chondrosarcoma. In this study, results demonstrated that circHMGB2 was significantly upregulated in chondrosarcoma, and negatively correlated with survival rate of chondrosarcoma patients. Furthermore, in an established cisplatin resistant chondrosarcoma cell line, we uncovered circHMGB2 was upregulated. These results consistently imply that circHMGB1 is a

potentially therapeutic target for anti-chemoresistant chondrosarcoma treatment.

Cisplatin acts as an anticancer chemotherapeutic agent through the formation of DNA adducts, leading to activation of cell cycle arrest and apoptosis signaling pathways to induce cancer cell death[4-6]. However, certain cancer cells develop cisplatin resistance, which limits the clinical application of cisplatin for cancer therapy[7]. Currently, the precise molecular mechanisms of CDDP resistance remain unclear. The PI3-K/Akt/mTOR signaling pathway is a central part of the proliferation, growth, survival and metabolism of cells[16]. In addition, activation of the Akt/mTOR pathway is important for the development of cisplatin resistance[17]. This study indicated that the Akt pathway was activated in CDDP resistant chondrosarcoma cells due to inhibition of Grb10 by circHMGB2.

To understand the mechanisms of the acquired cisplatin resistance, cisplatin-resistant chondrosarcoma cells were established. Results indicated that Akt phosphorylation and circHMGB2 were elevated in SW1353 CDDP resistant cells. Grb10 was significantly downregulated by circHMGB2. In addition, Grb10 negatively regulated Akt phosphorylation, suggesting an indirect regulation of Akt activity by circHMGB2. Notably, the present study also showed that inhibition of Akt by blocking circHMGB2 increased cisplatin sensitivity. Restoration of Akt activity via overexpression of Akt rendered chondrosarcoma cells resistant to cisplatin, presenting an important role of the circHMGB2-Grb10-Akt axis in the acquired cisplatin resistance of chondrosarcoma cells.

Our study elucidates a novel post-transcriptional regulatory circuit in chondrosarcoma chemoresistance. We identified that circHMGB2 does not function through the canonical miRNA sponge mechanism but rather acts as a competitive molecular decoy for the RNA-binding protein IGF2BP1. This is supported by direct evidence: circHMGB2 directly binds IGF2BP1 in vitro, and both molecules concurrently bind IGF2BP1 within cells. Most crucially, elevated circHMGB2 competitively reduces the binding of IGF2BP1 to Grb10 mRNA, leading to significant destabilization of the transcript (Fig. 5D). This "decoy" mechanism represents a distinct mode of action for oncogenic circRNAs in chemoresistance[11].

The role of IGF2BP1 as the central node in this axis is particularly intriguing. Typically, IGF2BP1 is considered an oncogenic factor that stabilizes numerous target mRNAs to promote cancer progression[18,19]. Our findings reveal a paradoxical, context-dependent function: in chondrosarcoma, its activity is hijacked by circHMGB2. Sequestration of IGF2BP1 away from its physiological target (Grb10 mRNA) ultimately executes a pro-survival, chemoresistant program via Akt activation. This highlights the complexity of RBP regulation in cancer, where the functional outcome is determined by the competitive landscape of its RNA ligands.

The definitive proof for this model comes from our rescue experiments. Co-overexpression of IGF2BP1 completely reversed the Grb10 downregulation, Akt hyperphosphorylation, and cisplatin resistance induced by circHMGB2. These data not only establish causality but also pinpoint the circHMGB2-IGF2BP1 interaction as a theoretically actionable vulnerability. Furthermore, the consistency between this molecular mechanism and our in vivo findings—where silencing circHMGB2 sensitized tumors to cisplatin—strengthens the translational relevance of this axis.

Most importantly, our in vivo xenograft study provides direct preclinical evidence supporting the therapeutic potential of targeting circHMGB2. We demonstrated that knockdown of circHMGB2 not only exerted a significant single-agent anti-tumor effect but also potently synergized with cisplatin, leading to markedly improved host survival and superior tumor growth control. The efficacy of this combination therapy was underscored by the consistent molecular changes observed in the treated tumors: upregulation of the tumor suppressor Grb10 and concomitant downregulation of activated, phosphorylated Akt. These data confirm that the circHMGB2/IGF2BP1/Grb10/Akt axis is not merely a cell culture phenomenon but is operational and therapeutically relevant in a living organism. The robust synergy between circHMGB2 silencing and cisplatin suggests that a combination strategy could be particularly effective in overcoming intrinsic and acquired chemoresistance in chondrosarcoma patients.

Therefore, our work extends the understanding of circRNA-mediated resistance beyond transcriptional regulation or miRNA sponging. We describe a precise mechanism wherein a circRNA post-transcriptionally modulates a key signaling adapter (Grb10) by competing for a shared RBP (IGF2BP1). This circHMGB2/IGF2BP1/Grb10/Akt axis provides a novel molecular basis for cisplatin resistance in chondrosarcoma. Therapeutic strategies aimed at disrupting the circHMGB2-IGF2BP1 interaction (e.g., using antisense oligonucleotides) or restoring Grb10 function could potentially re-sensitize resistant

tumors, offering a promising direction for overcoming chemoresistance in this recalcitrant malignancy.

Collectively, results from this study demonstrated that inhibition of circHMGB2 suppresses the Akt signaling pathway by upregulating Grb10, leading to cisplatin sensitization of chondrosarcoma cells. The circHMGB2-modulated Grb10/Akt pathway could be a therapeutic target for improvement of chemotherapy efficacy.

Ethical Compliance: Research experiments conducted in this article with animals or humans were approved by the Ethical Committee (#20240321) and responsible authorities of our research organization(s) following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

Consent for publication: Written informed consent for publication was obtained from all participants.

Conflicts of Interest: There are no conflicts to declare.

Availability of supporting data: The Supporting datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: Y.ZH. and L.C. designed the experiments. Y.ZH., H.S. and SH.Y.D. carried out the experiments and analyzed the data. All authors participated in the manuscript preparation.

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