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ORIGINAL PAPER

Hsc70 Interacts with β4GalT5 to Regulate the Growth of Gliomas

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Abstract
Heat shock cognate protein 70 (Hsc70) is a key mediator for the maintenance of intracellular proteins and regulates cellular activities. And it is elevated in various tumor tissues including glioma, which is closely related to the malignancy and poor prognosis of the tumors. However, the effects of Hsc70 on gliomas and its regulatory mechanism have not yet been elucidated. In the present study, we found that Hsc70 was overexpressed in glioma tissues and cultured glioma cells. Furthermore, Hsc70 expression exhibited positive correlation with the grades of gliomas. Knockdown of Hsc70 could effectively inhibit cell proliferation and increase cell apoptosis. Furthermore, we identified that β4GalT5 was a critical target for Hsc70-mediated anti-glioma effects. Blocking β4GalT5 activity could effectively reverse the anti-tumor effect of Hsc70. Taken together, these data indicate that Hsc70 regulates β4GalT5 levels, and possibly plays a role in cell proliferation and apoptosis of glioma.

Keywords Hsc70 · β4GalT5 · Proliferation · Apoptosis · Glioma

Introduction

Glioma is the most common malignant tumor in the central nervous system, accounting for more than 50% of the intracranial tumors in adults (Robertson et al. 2011). As the glioma grows rapidly, it is migrated to the surrounding area in the early stage and has an invasive growth. At present, microsurgery can only be performed by gross resection, but glioma cells growing into normal brain tissues cannot be completely resected, and thus become the source of recurrence and treatment resistance (Ferguson 2011).

Protein glycosylation can promote the occurrence and development of glioma by regulating various biological behaviors (D'Arrigo et al. 2017). Heat shock protein 70 (Hsc70) belongs to the HSP70 family. It is a molecular chaperon, which is synthesized rapidly after stimulation by various physical and chemical factors. Hsc70 is composed of three domains, including ATP domain, polypeptide domain, and carbon end domain (Ding et al. 2012). Reportedly, Hsc70 participates in protein synthesis, folding and assembly, transport and localization, and proteasome degradation, and participates in a variety of biological behaviors of the body (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002; Beckmann et al. 1990). Recent studies have shown that Hsc70 plays important roles in some types of tumors (Hantschel et al. 2000). Hsc70 is highly expressed in malignant tumors including esophageal cancer, lung adenocarcinoma, colon cancer, renal cell carcinoma, leukemia, lymphoma, and so on, and its upregulation is closely related to tumor grades and tumor metastasis (Wang et al. 2013; Tanaka et al. 2014; Ramp et al. 2007; Isomoto et al. 2003). The prognosis of patients with high expression of Hsc70 is often poor. Beaman et al. have found that the expression of Hsc70 is increased in gliomas, and its expression can also be used as a marker to judge the prognosis of gliomas (Beaman et al. 2014).

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Beta 4GalT5 (β4GalT5) is a single-chain type II transmembrane protein. β4GalT5 is mainly located in Golgi, and participates in the regulation of protein glycosylated modification through the formation of the catalytic protein Gal beta 1 to 4GlcNAc chain (Shirane et al. 1999). β4GalT5 regulates the glycosylation of proteins and participates in the development of the body, the occurrence of tumor and the recognition of the surface of the cells (Kumagai et al. 2010). Reportedly, β4GalT5 is overexpressed in glioma and involved in a variety of processes, such as the occurrence and development of glioma. Jiang et al. has found that the existence of β4GalT5 in glioma cells is critical for its survival. Deletion of β4GalT5 can cause apoptosis, and over expression of β4GalT5 can promote cell proliferation (Jiang et al. 2006). β4GalT5 also participates in the adhesion process of glioma cells (Chen et al. 2006). β4GalT5 can activate Notch1 signaling in glioma stem-like cells (Cui et al. 2017).

In this study, we investigated potential Hsc70-β4GalT5 interactions, and examined the role of Hsc70 in glioma proliferation and apoptosis through regulation of β4GalT5 signal pathway.

Materials and Methods

Tumor Tissue Samples

Glioma samples used in this study were obtained from discarded tumor tissue at the time of surgery. Specimens, including 16 normal brain tissues, 21 cases of grade I–II glioma tissues as low-grade glioma tissues, and 30 cases of grade III–IV glioma tissues as high glioma tissues. Normal brain tissue specimens were obtained from patients with severe craniocerebral trauma who needed internal decompression during emergency surgery. The collection and use of the patient samples were reviewed and approved by the Institutional Ethics Committee of Yancheng City No. 1 People's Hospital, The Second People's Hospital of Huai'An, Zoucheng Peoples' Hospital, and Jiangning Hospital affiliated with Nanjing Medical University, and written informed consent from all patients was appropriately obtained.

Cell Culture and Treatments

U251 and MO59J cells were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. At the time the cells were 70% confluent, cells were transfected with 100 pmol of Hsc70-targeting siRNA (Hsc70 siRNA, Santa Cruz Biotechnology) or scrambled RNA (Hsc70 siRNAnc, Santa Cruz Biotechnology) using FuGENE HD (Promega). At 48 h after transfection, cells were harvested and lysed for western blotting (WB).

Antibodies, Western Blotting, and Co-immunoprecipitation

Anti-Hsc70 (ab2788, Abcam), anti-β4GalT5 (ab110398, Abcam), anti-HA (ab18181, Abcam), anti-GST (ab19256, Abcam), and anti-His (ab18184, Abcam) were used for WB or immunoprecipitation (IP) analyses. For WB, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with a corresponding gel concentration using the discontinuous Laemmli buffer system (Bio-Rad Laboratories, Richmond, CA). The electrophoresed proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis with antibodies. The reaction was detected by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). For IP, lysates were incubated with the indicated antibody overnight at 4 °C. Immune complexes recovered with protein-A Sepharose (Sigma) were washed three times with washing buffer and analyzed by WB.

GST Pull-down Assay

GST-Hsc70 expression plasmid was generated by cloning GST-Hsc70 into pGEX4T-1 vector. Hsc70-His and β4GalT5-His expression plasmids were generated by cloning Hsc70-His or β4GalT5-His into pET23a vectors, respectively. Recombinant proteins were produced from *Escherichia coli* transformed with above-mentioned plasmids. Recombinant GST or GST-Hsc70 protein was incubated with glutathione-agarose beads (GE Healthcare), and then incubated with recombinant Hsc70-His or recombinant β4GalT5-His protein in lysis buffer for 2 h at 4 °C in a rotator. After washing three times, the precipitated proteins on the glutathione beads were analyzed by WB.

MTT Assay for Cell Proliferation

MTT assay was used to determine relative cell growth levels as follows. Cells were plated at 5×10^3 cells per well in 96-well plates with six replicate wells at the indicated concentrations. After incubation for 1, 2, or 3 days, 50 μL of MTT dilution (5 mg/mL, KeyGEN, China) was added into each well at each day of consecutive 3 days after transfection and the cells were incubated at 37 °C for additional 4 h. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay plate reader. Cell growth inhibition rates formula is $(A_C - A_T)/A_C \times 100\%$ (A_C absorbance value of the blank control group, A_T absorbance value of the experimental group).

Flow Cytometry for Cell Apoptosis

Cell apoptosis was analyzed using an Annexin V/FITC Apoptosis Detection Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were harvested after the treatment, washed with cold PBS, and subjected to Annexin V/FITC and propidium iodide staining in binding buffer at room temperature for 15 min in the dark. Apoptotic cells were analyzed by FACSCalibur (BD Biosciences, San Jose, CA).

Statistics Analysis

Data were analyzed with SPSS 19.0. Statistical evaluation for data analysis was determined by *t* test or ANOVA. Differences with $P < 0.05$ were considered statistically significant.

Results

Hsc70 and β4GalT5 Expression is Increased in Glioma Tissues and Cell Lines

We adopted qRT-PCR to analyze the expression of Hsc70 and β4GalT5 in normal brain tissues and glioma tissues. Results showed that both Hsc70 and β4GalT5 were obviously overexpressed in glioma tissues than that in normal brain tissues, and the highest expression was observed in high-grade gliomas (Fig. 1a). Their expression was further identified in normal astrocytes and U251 and MO59J cell lines, and similar results were observed (Fig. 1b). Correlation analysis showed that their expression was significantly correlative in glioma tissues ($P < 0.05$, Fig. 1c).

Hsc70 Regulates β4GalT5 Protein Levels

To examine whether Hsc70 is associated with β4GalT5, IP analysis was performed. As shown in Fig. 2a, the result showed that exogenous β4GalT5 was associated with exogenous Hsc70 in 293T cells. Further, GST pull-down experiment showed that Hsc70 directly interacted with β4GalT5 (Fig. 2b). Exogenous IP was also performed in U251 cells, and the results showed that Hsc70 interacted with β4GalT5 in glioma cells (Fig. 2c). These results suggest that Hsc70 physically interacts with β4GalT5.

Hsc70 Impacts Cell Proliferation and Apoptosis of Glioma Cells via β4GalT5

To explore the effects of Hsc70 on cell proliferation and apoptosis of glioma cells, U251 and MO59J cells were transfected with Hsc70 siRNA to knockdown the expression of Hsc70 (Fig. 3a). As expected, MTT assay showed that the growth of U251 and MO59J cells was significantly inhibited after Hsc70 knockdown (Fig. 3b). Furthermore, flow cytometry demonstrated that Hsc70 knockdown induced significant apoptosis of U251 and MO59J cells (Fig. 3c). In view of the interaction of Hsc70 and β4GalT5, we hypothesized that Hsc70 probably regulates cell proliferation of glioma cells via β4GalT5. Western blot showed that the expression of β4GalT5 was reduced after knockdown of Hsc70 (Fig. 3d). And recent studies have confirmed that Ras/MAPK and PI3K/AKT signaling pathways are involved in β4GalT5 transcription activation. As shown in Fig. 3e, Hsc70 reduction decreased the levels of p-ERK1, p-JNK1, and p-AKT, which were the downstream signaling proteins of β4GalT5.

Overexpression of β4GalT5 Reverses the Anti-tumor Effects of Hsc70 siRNA

We further examined whether β4GalT5 overexpression counteracted the anti-tumor effects of Hsc70 siRNA in U251 and MO59J cells. As shown in Fig. 4a, β4GalT5 overexpression effectively reversed the decrease of β4GalT5 induced by Hsc70 siRNA. And as shown in Fig. 4b, c, overexpression of β4GalT5 rescued the growth arrest and apoptosis induced by Hsc70 siRNA. Therefore, Hsc70 impacted cell proliferation and apoptosis of glioma cells in a β4GalT5-dependent manner.

Discussion

The purpose of this study was to identify the Hsc70 interactome and to reveal its critical function for glioma cell survival. Early studies have demonstrated that Hsc70 is highly overexpressed in gliomas. However, as far as we know, Hsc70 function and interactome mechanism in gliomas are still unknown. Here, we first revealed that Hsc70 functioned as a positive growth regulator in gliomas and β4GalT5 was required for its anti-glioma effects.

HSP70 is one of the most important members of the heat shock protein family, including HSP60, HSP70, HSP90, HSP100, and ubiquitin. Among them, HSP70 family is the most important and highest expression after stress. Hsc70 belongs to HSP70 family, which is a molecular chaperone. Hsc70 can promote its maturation by regulating subcellular localization of cell surface nucleolin (NCL) in tumor cells. Hsc70 is overexpressed in many cancer cells and be reliant for the survival of malignant cells. For example, Liu et al. have reported inhibiting Hsc70 expression is a potential use

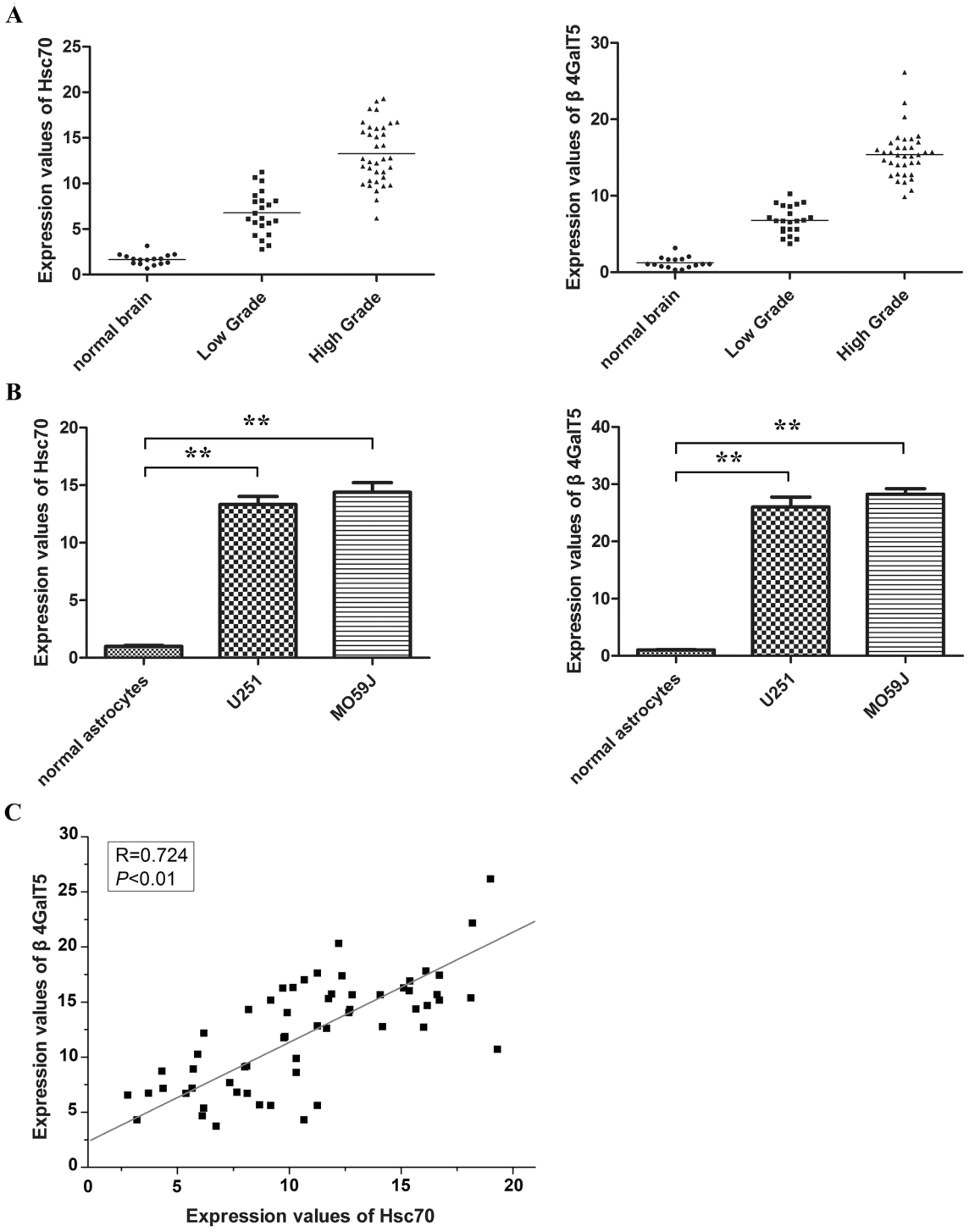


Fig. 1 Hsc70 and β4GalT5 expression in glioma tissues and normal tissues. **a** Levels of Hsc70 and β4GalT5 expression in human glioma tissues and normal brain tissues were measured by qRT-PCR. **b** Levels of Hsc70 and β4GalT5 expression in normal astrocytes and U251 and MO59J cell lines were measured by qRT-PCR. **c** Correlation between Hsc70 and β4GalT5 expression in glioma tissues

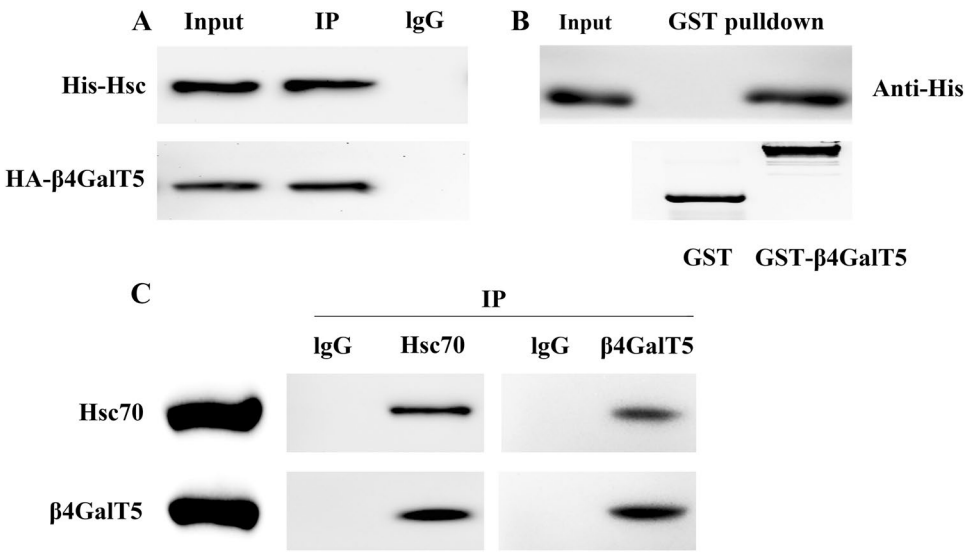


Fig. 2 Hsc70 regulates β4GalT5 protein. **a** Interaction between β4GalT5 and Hsc70 was identified by exogenous IP analysis in 293T cells. **b** Interaction between Hsc70 and β4GalT5 was identified by GST Pull-down assay in 293T cells. **c** Interaction between β4GalT5 and Hsc70 in U251 glioma cells was detected by IP analysis

for the treatment of prostate cancer (Liu et al. 2015). Tanaka et al. have showed that Hsc70 is highly expressed in colon cancer cells and impact the malignant biological behavior (Tanaka et al. 2014). In gliomas, its high expression was confirmed by Beaman et al., which showed its expression levels in glioblastoma tissue were between 1.8- and 8.8-fold higher than in lower-grade glioma and normal brain tissue, respectively (Beaman et al. 2014). Zeise et al. have showed that Hsc70 determines the process of S phase entry and/or progression in C6 glioma cells (Vila-Carriles et al. 2007). Vila-Carriles et al. have reported that decreasing Hsc70 expression promotes the reversion of a high-grade glioma cell to a more normal astrocytic phenotype (Vila-Carriles et al. 2007). However, till now it is still largely unknown how Hsc70 contributes to glioma cell survival.

To explore its function of Hsc70 on glioma cell survival, Hsc70 siRNA was applied to knockdown the expression of Hsc70 in gliomas and the results showed that the proliferation of glioma cells was significantly blocked and cellular apoptosis was increased, indicating that Hsc70 functions as an oncogene in gliomas. Reportedly, Hsc70 contributes to cancer cell survival by preventing Rab1A degradation in colon cancers (Tanaka et al. 2014). Here we found that β4GalT5 was the targeted regulatory protein in gliomas. β4GalT5 is a member of 1,4-galactosyltransferase (GalT) family. β4GalT5 can effectively galactosylate the GlcNAcβ1 6Man arm of the highly branched *N*-glycans that are characteristic of glioma (Jiang et al. 2006). β4GalT5 is overexpressed in gliomas and involved in a variety of processes, such as the occurrence and development of gliomas. Overexpression of β4GalT5 can promote the malignant transformation of glioma cell biological behavior (Shirane et al. 1999). Our present study showed that the expression of Hsc70 was positively correlated with the expression of β4GalT5, both of which were high expressed in gliomas. To confirm the interactome between Hsc70 and β4GalT5, GST pull-down experiment and exogenous IP were applied and the result was consistent with what we envisaged. Thus, we speculated that in the glioma cells, Hsc70 binds to β4GalT5 and form complexes. Hsc70 regulates the glycosylation and maturation of substrate integrin β1 by regulating the protein folding of β4GalT5, thereby promoting the proliferation of glioma cells. Our data showed that knockdown of Hsc70 resulted in decreased β4GalT5 and upregulation of β4GalT5 reversed the anti-tumor effects of Hsc70 siRNA. These results demonstrated that the anti-tumor effects of Hsc70 were dependent on β4GalT5. Reportedly, β4GalT5 promoter can dominate active Ras, ERK1, JNK1, and constitutively active AKT, demonstrating that β4GalT5 regulates the pro-survival role of AKT or Ras/MAPK signaling pathways in gliomas (Jiang et al. 2006). Thus, the downstream effectors of p-ERK1, p-JNK1, and p-AKT were also detected in the further studies. And as we expected, knockdown of Hsc70 decreased the expression of these effectors, while upregulation of β4GalT5 restored the expression of these effectors.

In conclusion, these findings indicate that Hsc70 directly interacts with β4GalT5 and performs a positive growth regulator in gliomas. And β4GalT5 is an important protein required for the anti-glioma effects of Hsc70.

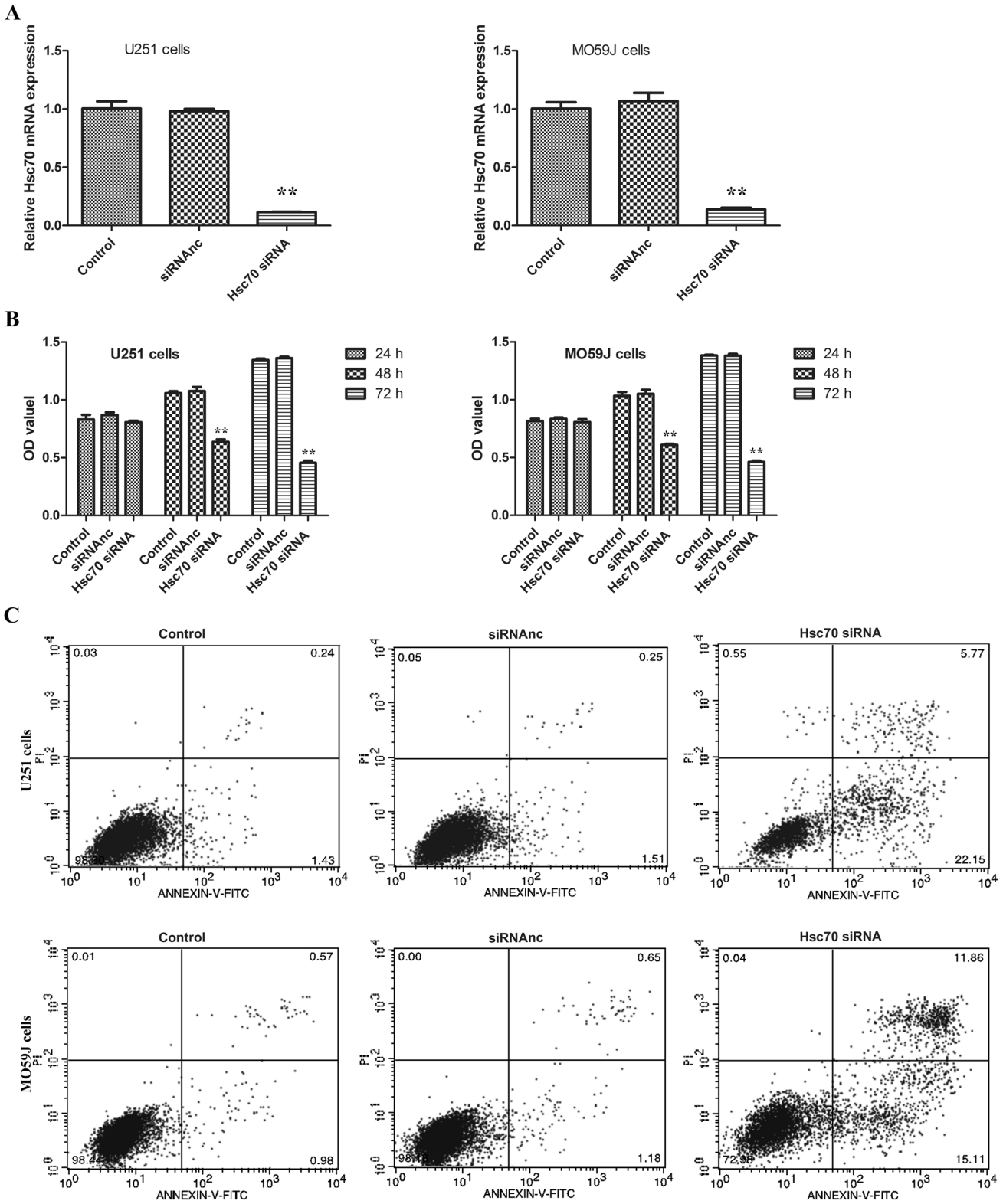


Fig. 3 Effects of reduction in Hsc70 expression on U251 and MO59J cell proliferation and apoptosis. **a** Expression levels of Hsc70 in U251 and MO59J cells were measured by qRT-PCR at 48 h after Hsc70 siRNA transfection. **b** Cell viability of U251 and MO59J cells transfected with Hsc70 siRNA was measured by MTT assay. **c** Cell apoptosis of U251 and MO59J cells transfected with Hsc70 siRNA was measured by Annexin V/PI staining. **d** The expression of β4GalT5 transfected with Hsc70 siRNA was measured by western blot. **e** The expression of p-ERK1, p-JNK1, and p-AKT transfected with Hsc70 siRNA was measured by western blot

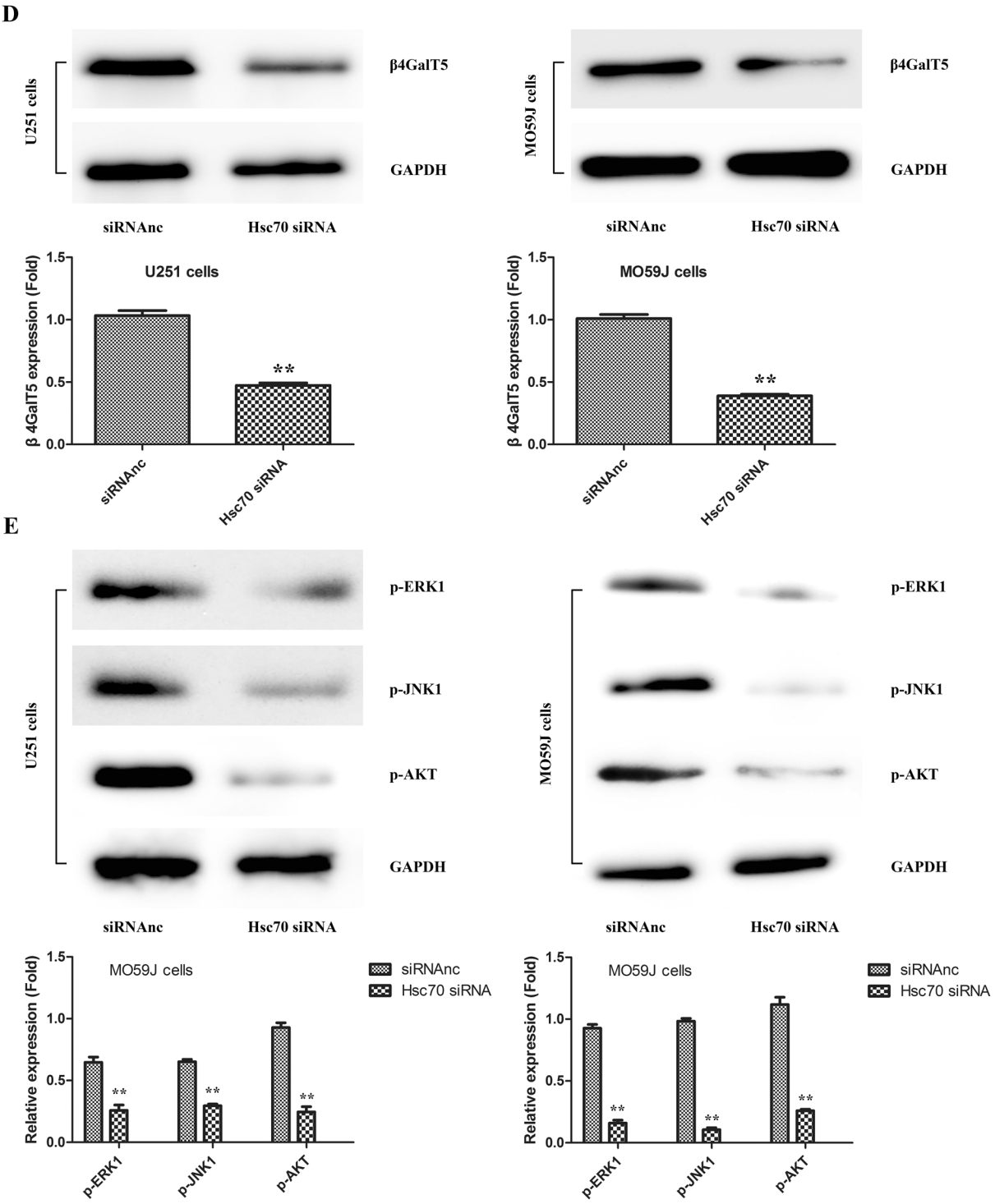
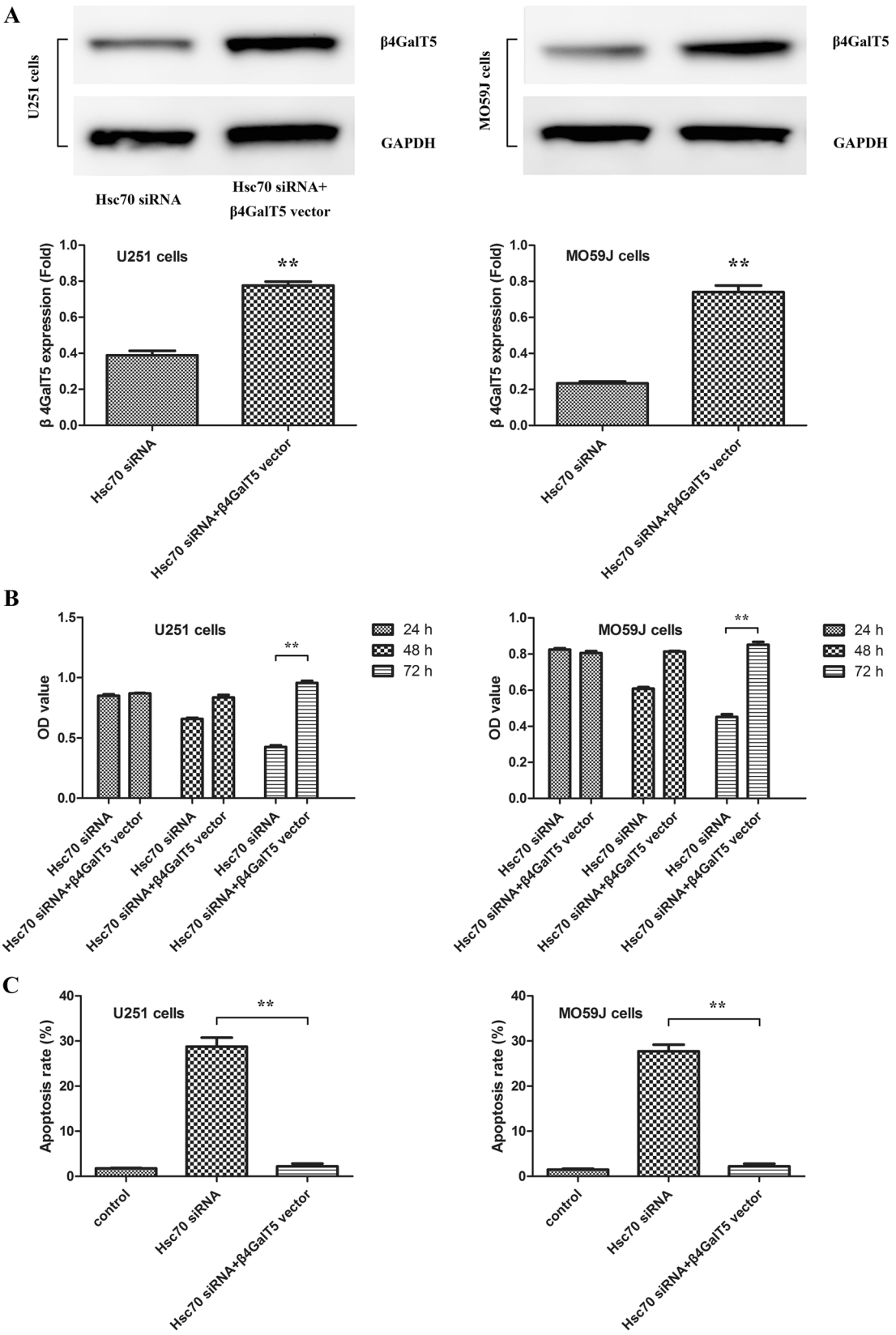


Fig. 3 (continued)


A
U251 cells
β4GalT5
GAPDH
MO59J cells
β4GalT5
GAPDH
Hsc70 siRNA
Hsc70 siRNA+
β4GalT5 vector
U251 cells
β 4GalT5 expression (Fold)
**
Hsc70 siRNA
Hsc70 siRNA+β4GalT5 vector
MO59J cells
β 4GalT5 expression (Fold)
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Hsc70 siRNA
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B
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OD value
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24 h
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MO59J cells
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U251 cells
Apoptosis rate (%)
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control
Hsc70 siRNA
Hsc70 siRNA+β4GalT5 vector
MO59J cells
Apoptosis rate (%)
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control
Hsc70 siRNA
Hsc70 siRNA+β4GalT5 vector

◂**Fig. 4** Effects of β4GalT5 overexpression on the anti-tumor effects of Hsc70 siRNA. **a** The expression of β4GalT5 in U251 and MO59J cells after Hsc70 siRNA and/or β4GalT5 overexpressed vector transfection was analyzed by western blot. **b** Cell viability of U251 and MO59J cells transfected with Hsc70 siRNA and/or β4GalT5 overexpressed vector was measured by MTT assay. **c** Cell apoptosis of U251 and MO59J cells transfected with Hsc70 siRNA and/or β4GalT5 overexpressed vector was measured by Annexin V/PI stain detection

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Compliance with Ethical Standards

Conflict of interest The authors declare no potential conflicts of interest.

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