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HSP70 decreases receptor-dependent phosphorylation of Smad2 and blocks TGF-β-induced epithelial-mesenchymal transition

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Abstract

Smad2 and Smad3, the intracellular mediators of transforming growth factor β (TGF-β) signaling, are directly phosphorylated by the activated type I receptor kinase, and then shuttle from the cytoplasm into the nucleus to regulate target gene expression. Here, we report that the 70-kDa heat-shock protein (HSP70) interacts with Smad2 and decreases TGF-β signal transduction. Ectopic expression of HSP70 prevents receptor-dependent phosphorylation and nuclear translocation of Smad2, and blocks TGF-β-induced epithelial-mesenchymal transition (EMT) in HaCat cells. Our findings reveal an essential role of HSP70 in TGF-β-induced epithelial-mesenchymal transition (EMT) by impeding Smad2 phosphorylation.

Keywords: TGF-β; HSP70; Smad2; EMT

1. Introduction

The transforming growth factor β (TGF-β) superfamily are a group of secreted polypeptides that regulate a diverse array of developmental and biological processes, including cell proliferation, differentiation, apoptosis, and specification of developmental fate. TGF-β ligands initiate signaling by binding to and bringing together type I and type II receptors. This allows type II receptor to phosphorylate and activate type I receptor. Smad2 and Smad3 are directly phosphorylated by the activated type I receptor, and then translocated into the nucleus to regulate the transcription of target genes (Shi and Massague, 2003).

The Smad2 protein includes a linker region, and two conserved domains: the MH1 domain and MH2 domain, which provide a number of Smad2 interaction sites (Massague et al., 2005). When TGF-β signaling is activated, phosphorylated Smad2 functions in the nucleus as a transcription factor. Smad2 interacts with other cofactors in the nucleus to influence transcriptional activation. Indeed, a large number of Smad2 cofactors have been confirmed, such as FAST/FoxH1, Sp1, and Milk/Mixer (Kunwar et al., 2003; Feng and Derynck, 2005). At steady state, Smad2 is predominantly cytoplasmic, and Smad2 binding proteins in the cytoplasm are also involved in modulating TGF-β signaling. For example, the purified Smad-binding domain (SBD) of the cytoplasmic Smad anchor for receptor activation protein (SARA) inhibits nuclear accumulation of the Smad2 MH2 domain (Xu et al., 2000). In normal tissues and primary epithelial cells, SnoN also antagonizes TGF-β signaling by sequestering Smad proteins in the cytoplasm (Krakowski et al., 2005).

Heat-shock proteins (HSPs) are a family of conserved proteins whose expression increases when cells suffer physiological and environmental stress. This stress includes elevated temperatures, heavy metals, or chemicals (Sherman and Multhoff, 2007). As a core member of the HSP family, HSP70 assists the folding of a wide range of newly synthesized proteins and refolding of misfolded proteins (Neupert and Brunner, 2002). The physiological function of HSP70 allows cells to cope with proteins denatured by deleterious

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conditions. In contrast to the low expression level in normal cells, HSP70 is overexpressed in various tumor cells. High levels of HSP70 promote tumorigenesis and increase the tolerance of tumor cells (Calderwood et al., 2006). In addition, HSP70 chaperones numerous signaling moleculars, such as p53 and cystic fibrosis transmembrane conductance regulator (CFTR), which can modulate cell growth, differentiation, and specification (Yang et al., 1993; Meacham et al., 2001).

In this study, we adopted a glutathione-S-transferase (GST) pull down assay using zebrafish Smad2 (zSmad2) as the bait to detect potential Smad2 binding proteins. HSP70 was identified to interact with Smad2, and its ectopic expression suppressed the receptor-dependent phosphorylation and nuclear translocation of Smad2. Knocking down the endogenous HSP70 led to a considerable boost of TGF-β signal. Furthermore, TGF-β-induced epithelial-mesenchymal transition (EMT) in HaCaT cells was reduced by HSP70 overexpression.

2. Materials and methods

2.1. Construction of expression plasmids

The GST cDNA was amplified from pGEX-4T-1 and subcloned into vector pCS2. The full-length zSmad2 cDNA was inserted downstream of GST to allow expression of a GST-zSmad2 fusion protein. The human HSP70 cDNA was digested from pCMV5-HSP70 (ORIGENE) and subcloned into the pCS2-HA vector. All these constructs were confirmed by DNA sequencing.

2.2. Cell culture and transfection

HEK293T and HaCaT cells were cultured in DMEM (GIBCO, USA), containing 10% fetal bovine serum (FBS). Transcient transfection of HEK293T and HaCaT cells was achieved using the cationic polymer polyethylenimine (PEI) or lipofectamine 2000 (Invitrogen, USA).

2.3. GST pull down and mass spectrometry

GST and GST-zSmad2 expression plasmids were separately transfected into HEK293T cells, and harvested after 48 h. Whole cell lysates were prepared using TNE lysis buffer (25 mmol/L Tris−HCl, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, and 0.5% NP-40) containing a protease inhibitor mixture. After a "pre-clear procedure", the samples were incubated with glutathione-sepharose beads at 4 °C for 4 h. The beads were washed four times with TNE buffer. The bound proteins were separated by SDS-PAGE, visualized by silver staining, and subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) sequencing.

2.4. In vitro translation assays

HSP70, GST and GST-zSmad2 were translated *in vitro* using Promega TNT Sp6 quick coupled reticulocyte lysate systems. The procedure is according to the recommendation of the manufacture, then followed by GST binding assay, and the proteins were separated by SDS-PAGE and analyzed by Western blotting.

2.5. Immunoprecipitation

HEK293T cells were transfected with indicated vectors, and harvested after 48 h. Whole cell lysates were prepared using TNE lysis buffer. Cellular extracts were incubated with anti-myc or anti-HA antibodies at 4 °C for 1 h, followed by addition of protein A beads and rocking for 4 h. Then beads were washed with TNE buffer, and the immune complexes were subjected to SDS-PAGE, followed by Western blotting.

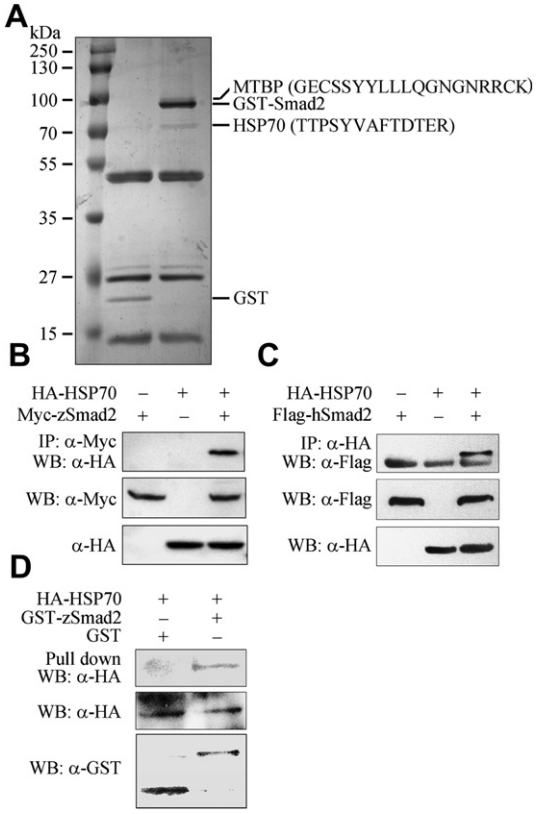

Fig. 1. HSP70 associates with Smad2. **A:** affinity purification of zSmad2 binding proteins. Cellular extracts from HEK293T cells expressing GST or GST-zSmad2 were affinity purified with glutathione-sepharose beads. The eluates were resolved by SDS-PAGE and silver stained. The proteins bands were retrieved and analyzed by mass spectrometry. **B:** HSP70 coimmunoprecipitates (co-IP) with zSmad2. HEK293T cells were transfected with HA-tagged HSP70 and Myc-tagged zSmad2, as indicated. At 40 h post-transfection, the cells were harvested for anti-Myc immunoprecipitation (IP). zSmad2 associated HSP70 was revealed by anti-HA immunoblotting (upper panel). The amount of HA-HSP70 or Myc-zSmad2 in 5% of cell lysates input was assessed by Western blotting (WB) (middle and lower panels). **C:** human Smad2 (hSmad2) can coimmunoprecipitate (co-IP) with HSP70. HEK293T cells were transfected with HA-tagged HSP70 and Flag-tagged hSmad2 as indicated. At 48 h post-transfection, the cells were harvested for anti-HA immunoprecipitation. **D:** HSP70 directly interacts with zebrafish Smad2. HSP70, GST and GST-Smad2 were *in vitro* translated, and the interaction of these proteins was detected by GST pull down.

2.6. Luciferase assay

HaCaT cells were cultured in 24 well plates, and transfected with the indicated constructs and the internal control, pRenilla-TK vector. Cells were serum-starved for 8 h before being treated with TGF-β1 (5 ng/mL). Luciferase activity was quantified about 16 h later using the dual luciferase assay (Promega, USA). Each experiment was performed in triplicate and the data represent the mean ± SD of three independent experiments after normalization to Renilla activity.

2.7. RNA interference

The small interfering RNAs (siRNA) against HSP70 were designed by inserting oligos with a haripin loop into pLenti Lox 3.7 vector. We introduced two oligos targeting the following sequences: siRNA1, GAGAACGACATGTCCTGA; siRNA2, ACATTGATGCCAATGGTA. In addition, we used a siRNA targeting GFP as control.

2.8. Immunofluorescence

HaCaT cells grown on cover slips and transfected with the indicated constructs using lipofectamine 2000. Cells were fixed with 4% formaldehyde for 30 min, followed by 0.5% Triton X-100 treatment for 30 min, and subsequently washed in PBS three times. The cells were incubated with anti-HA antibody, followed by incubation with rhodamine-conjugated anti-mouse secondary antibody (Sigma, USA). Samples were stained by Hoechst or phalloidin before taking images under a confocal microscope (Zeiss LSM 510 Meta, Germany).

3. Results

3.1. HSP70 interacts with Smad2

The activities of Smads are regulated positively or negatively by a variety of protein partners (Schmierer and Hill, 2007). In an effort to better understand the regulatory mechanism of Smad activity, we performed GST pull down assays and mass spectrometry to identify proteins that interact with Smad2. GST-zSmad2 was transiently expressed in HEK293T cells as the bait. After GST affinity purification and SDS-PAGE separation, two significantly zSmad2-enriched bands at 93 kDa and 70 kDa, respectively, were identified (Fig. 1A). Subsequently, mass spectrometry analysis indicated that these two proteins were the Mdm2-binding protein (MTBP) and HSP70. The interaction of human HSP70 with zSmad2 was further confirmed by immunoprecipitation assays (Fig. 1B). In addition, human Smad2 could also be coimmunoprecipitated by HSP70 (Fig. 1C). To confirm the direct interaction between HSP70 and Smad2, GST pull down assays were carried out by using *in vitro* translated proteins. As shown in Fig. 1D, HSP70 could

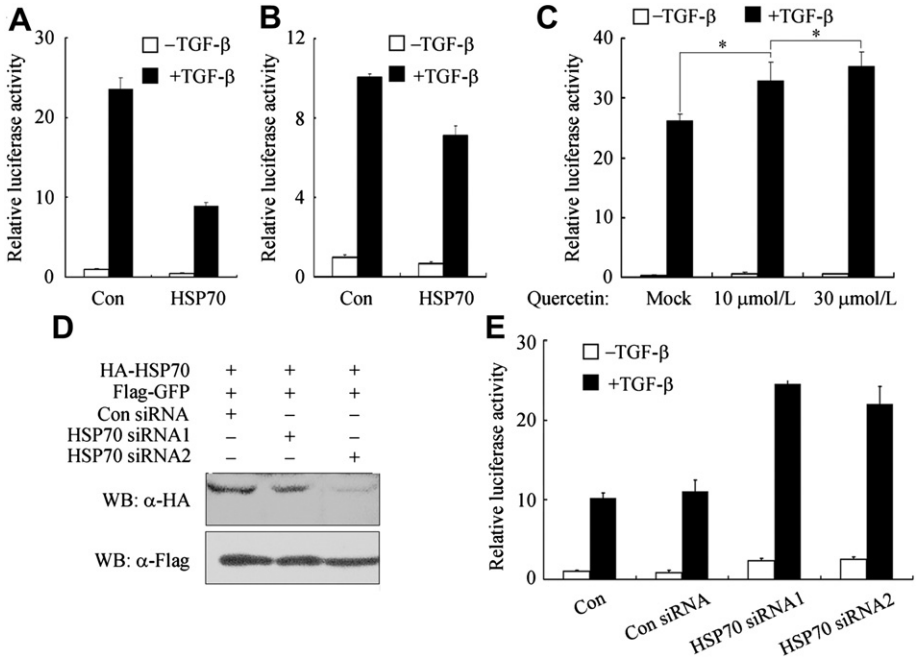

Fig. 2. HSP70 blocks TGF-β signaling. HaCat cells (**A**) or HEK293 cells (**B**) were cotransfected with ARE-luciferase and Fast2 along with HA-HSP70 as indicated, and treated with TGF-β1 (5 ng/mL) for 16 h before harvesting for luciferase assay. **C:** flavanoid quercetin treatment promotes expression of the ARE-luciferase reporter in a dose-dependent manner. HaCat cells were cotransfected with ARE-luciferase and Fast2 in the presence of TGF-β1 (5 ng/mL), and treated with flavanoid quercetin (0−30 μmol/L) for 16 h before harvesting for luciferase assay. **D:** western blotting analysis revealed that both of HSP70 siRNA1 and 2 could effectively knock down the ectopic expression of HSP70. **E:** knock down of endogenous HSP70 results in enhanced expression of the ARE-luciferase reporter. HaCaT cells were transfected with ARE-luciferase and siRNAs, with or without stimulation of TGF-β1 for (5 ng/mL) 16 h. Reporter assays were performed in triplicate, and the data represent the mean ± SD of three independent experiments after normalization to Renilla luciferase activity. Single asterisk demonstrates the signal-intensity differences ($P < 0.05$).

directly bind to GST-Smad2 fusion protein. These experiments strongly indicate that HSP70 physically associates with Smad2, and the association of these two proteins is conserved between different species.

3.2. HSP70 downregulates Smad2 mediated TGF-β signal transduction

HSP70 has various functions during folding, unfolding, and assembly of proteins, as well as in the control of cell signaling (Li and Srivastava, 2004). The interaction of HSP70 with Smad2 suggested that HSP70 may also function in TGF-β signal transduction. Therefore, we analyzed the effect of HSP70 on the transcriptional responses of TGF-β using an activin/TGF-β-responsive reporter, activin response element (ARE)-luciferase, which is Smad2/4-specific (Chen et al., 1997). Ectopic expression of HSP70 significantly suppressed TGF-β-induced expression of the ARE reporter, both in HaCat (Fig. 2A) and in HEK293 cells (Fig. 2B). More importantly, following treatment with the flavanoid quercetin, which is chemoprotective and acts as a sensitizer for anti-cancer drug therapy through inhibition of *hsp70* gene expression (Ranelletti et al., 1992; Jung et al., 2010), the TGF-β-induced activity of the ARE reporter was induced in proportion to the different drug concentrations (Fig. 2C). To examine the activity of endogenous HSP70 on TGF-β signaling, we constructed siRNAs against human HSP70, which could specifically knock down human HSP70 expression but had no effect on GFP control (Fig. 2D). Down-regulation of endogenous HSP70 by siRNA1 or siRNA2 not only led to a higher basal expression of the ARE-luciferase reporter, but also increased TGF-β-promoted expression of this reporter, whereas a siRNA against GFP had no effect (Fig. 2E). These observations support the conclusion that HSP70 has a repressive function on TGF-β/Smad2 signal transduction.

3.3. HSP70 perturbs receptor-dependent Smad2 phosphorylation

To investigate whether the repressive function of HSP70 in TGF-β signal transduction acts *via* its interaction with Smad2, we first examined the cellular localization of HSP70 with or without TGF-β stimulation. HSP70 was localized exclusively in the cytoplasm in HaCat cells in the absence of TGF-β1 and its cellular localization did not change upon TGF-β1 treatment (Fig. 3A). Moreover, the association of these two proteins did not significantly change after activating TGF-β signaling by expressing the constitutively active type I TGF-β receptor, caALK5 (Fig. 3B). These results indicate that HSP70 exerts its repressive activity in the cytoplasm, possibly by sequestering Smad2 in the cytoplasm. Subsequently, we found that the receptor-dependent phosphorylation of Smad2 on serines 465 and 467 was markedly decreased by HSP70 expression (Fig. 3C). Furthermore, the TGF-β-induced Smad2 nuclear translocation was evidently disturbed by overexpression of HSP70 (Fig. 3D). Taken together, these results suggest that HSP70 sequesters Smad2 in the cytoplasm, perhaps by preventing the recruitment of Smad2 by the type I receptor located on cell membrane, and decreases receptor-dependent Smad2 phosphorylation.

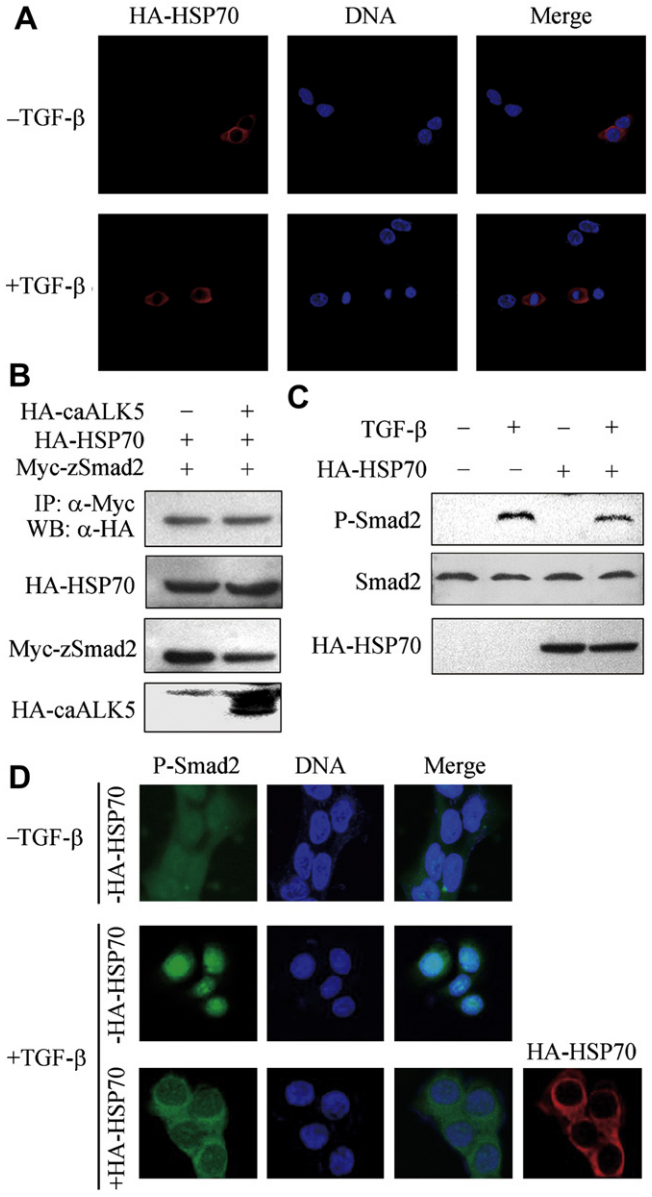

Fig. 3. Cytoplasmic HSP70 decreases P-Smad2 level. **A:** HSP70 is localized in the cytoplasm in the absence or presence of TGF-β stimulation. HaCaT cells transfected with HA-HSP70 for 36 h were treated with or without TGF-β1 (5 ng/mL) for 2 h, and then fixed for anti-HA immunofluorescence (red). The nuclei are marked by Hoechst (blue). **B:** HEK293T cells were cotransfected with HA-tagged HSP70 and Myc-tagged zSmad2, with or without HA-caALK5. At 48 h post-transfection, the cells were harvested for anti-Myc immunoprecipitation. **C:** HEK293 cells transfected with the indicated vectors after 36 h were treated with or without TGF-β1 (5 ng/mL) for 2 h, and then harvested for anti-phospho-Smad2 Western blotting analysis. The expression of endogenous Smad2 and HA-HSP70 was revealed by anti-Smad2 and anti-HA immunoblotting. **D:** HSP70 blocks the nuclear accumulation of Smad2 upon TGF-β stimulation. HaCaT cells treated with or without TGF-β1 (5 ng/mL) for 1 h, followed by immunofluorescence for P-Smad2 (green), Hoechst (blue) and HA epitope (red).

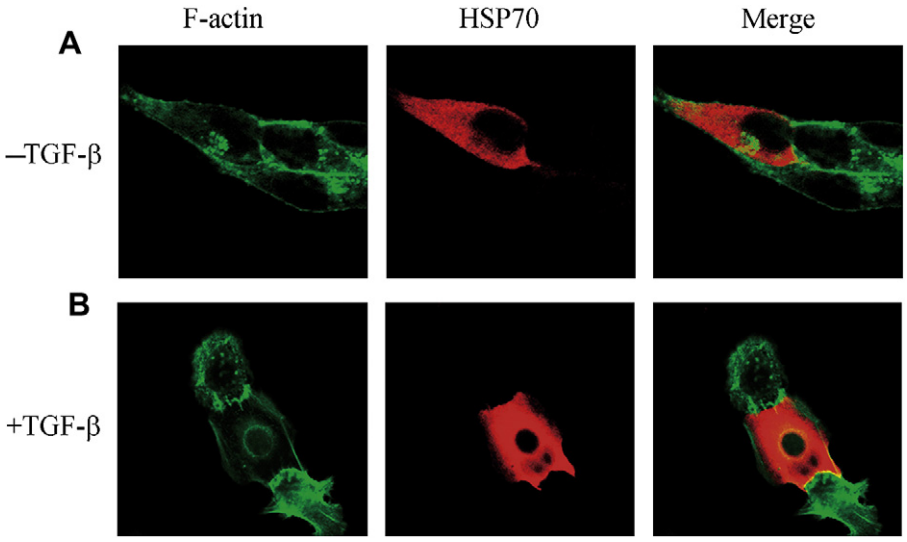

Fig. 4. TGF-β-induced EMT is inhibited by HSP70 overexpression. HaCaT cells transfected with HA-HSP70 were treated without (**A**) or with (**B**) TGF-β1 for 36 h. The cells were then fixed, and subjected to immunofluorescence with anti-HA antibody (red) and phalloidin-FITC to stain F-actin (green).

3.4. HSP70 inhibits TGF-β-induced EMT in HaCaT cells

TGF-β promotes EMT in epithelial cells, which is characterized by loss of E-cadherin from the plasma membrane and replacement of filamentous actin (F-actin) (Schmierer and Hill, 2007). In the absence of TGF-β, HaCaT cells, with or without HSP70 overexpression, appeared as typical epithelial cells with cortical actin architectures at cell-cell junctions (Fig. 4A). Treatment with TGF-β in HSP70-non-overexpressing cells induced an obvious EMT, as evidenced by the formation of stress fibers. In contrast, in HSP70-overexpressing cells, the TGF-β-induced EMT was blocked. Their morphology remained epithelial, and appeared to be limited stress fiber formation (Fig. 4B). These results indicated that HSP70 could disrupt the process of TGF-β-induced EMT.

4. Discussion

The activation and termination of Smad activity is a key step in TGF-β signal transduction. The positive and negative controls of signaling are accompanied by competition and cooperation between Smad proteins and their binding partners. Perturbation of this balance can lead to tumorigenesis and other disruption in cells (Takaku et al., 1998; Taylor and Wrana, 2008). At steady state, R-Smads are predominantly cytoplasmic, and upon ligand stimulation, R-Smads and Smad4 accumulate in the nucleus (Chen et al., 2006). The mechanisms of regulation of Smad2 cellular localization are not sufficiently resolved. In the present study, we have confirmed that HSP70 interacts with Smad2 in the cytoplasm, preventing its receptor-dependent phosphorylation, and TGF-β-stimulated nuclear translocation.

In premalignant cells, TGF-β maintains their homeostasis and suppresses spontaneous tumorigenesis. Lack of TGF-β pathway activity induces specific tumor growth and invasion. It also boosts the evasion of immunity in cancer cells (Gorelik and Flavell, 2000; Thomas and Massague, 2005). HSP70 is highly expressed in cancer cells, and has a significant function for increasing cell survival and preventing cell apoptosis (Nylandsted et al., 2000). According to our findings, high expression of HSP70 in cells under certain pathological conditions efficiently blocks TGF-β-induced phosphorylation of Smad proteins, leading to resistance to TGF-β stimulus and promotion of tumorigenesis.

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