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Journal of Genetics and Genomics 38 (2011) 111−116
JOURNAL OF GENETICS AND GENOMICS
www.jgenetgenomics.org

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

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Received 29 November 2010; revised 16 January 2011; accepted 17 January 2011

**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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doi:10.1016/j.jgg.2011.02.001

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.

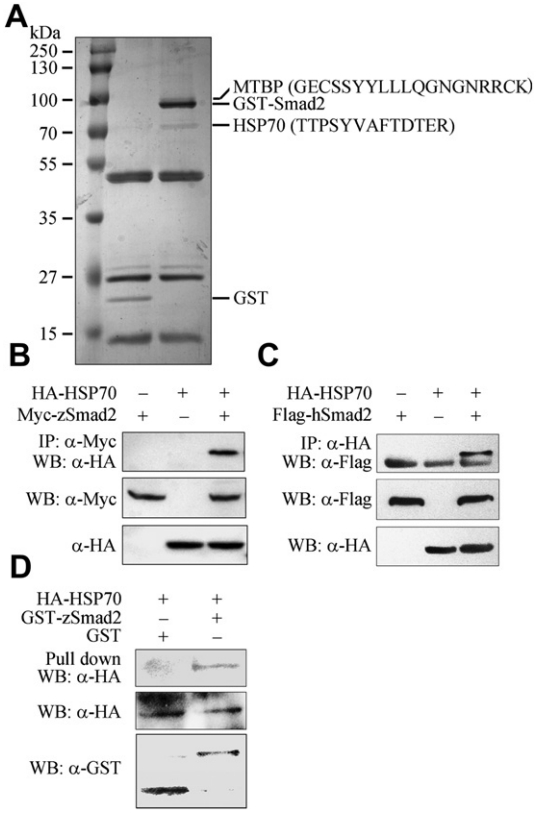


Fig. 1. HSP70 associates with Smad2. **A:** affinity purification of zSmad2 binding proteins. Cellular extracts from HEK293T cells expressing GST or GST-zSmad2 were affinity purified with glutathione-sepharose beads. The eluates were resolved by SDS-PAGE and silver stained. The proteins bands were retrieved 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.