# MAPK–Dependent Smad2/3 Phosphorylation in HepG2 Cells

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**Abstract** **Objective** To study the effect of MAPKs inhibitors on Smad2/3 phosphorylation induced by TGF−β1 in HepG2 cells. **Methods** The cells were stimulated by TGF−β1 and treated with three inhibitors specific to JNK, p38 and ERK separately. The proliferation of HepG2 cells were analyzed by MTT, and the effect of inhibitors on Smad2/3 phosphorylation were analyzed by Western blot. **Results** The proliferation of the cells was not remarkably stimulated by TGF−β1 (9 pM)for 0.5 and 1h, but significantly for 2, 4, 8, 16h; the phosphorylation level of Smad2C, Smad2L and Smad3L increased in the cells induced by TGF−β1 (9 pM) for 0.5 h; the phosphorylation of Smad2C and Smad3L were inhibited by JNK inhibitor (3, 10μM) and p38 inhibitor (1, 3, 10μM); and ERK inhibitor (1, 3, 10μM) had no apparent effect on the phosphorylation of Smad2/3. **Conclusions** The phosphorylation of Smad2/3 might be induced by TGF−β1 via activated JNK and p38 in HepG2 cells.

**Key words** HepG2 cell; MAPKs; inhibitor; Smad2/3; phosphorylation

The transforming growth factor β (TGF−β) proteins are a family of multifunctional cytokines that participates not only in tumor suppressor activities such as growth inhibition and apoptosis, but also in oncogenic activities such as growth stimulation, increased motility and invasiveness [1]. TGF−β appears to be an important regulator in both normal and pathological conditions in the liver [2]. Progress over the past several years has disclosed some details of how TGFβ elicits responses [3]. TGF−β initiates its signaling by binding and activating TGF−β receptor types I (TβR I) and II (TβR II), which then form heterocomplexes and activate downstream components[4, 5]. The binding of TGF−β to TGF−β receptor complexes induces the phosphorylation of receptor−activated Smads, including Smads1, 2, 3 [6, 7]. The phosphorylated receptor−activated Smads form a heteromeric complexe with the co −Smad Smad4. The complex translocates to the nucleus, where they may either directly bind the promoters of its target genes, or associate with other transcription factors to induce gene transcription[8, 9]. The mitogen−activated protein kinases (MAPKs) represent another major type of signaling intermediate for TGF−β. Notably, TGF−β induces activation of MAPKs through the upstream mediators Ras, RhoA, and TGF−activated kinase 1 (TAK1)[10−12]. Recent reports demonstrated the activated MAPKs phosphorylated Smad2/3 at linker region downstream of TGF−β [13, 14]. However, the cellular events targeted by theses TGF−β−mediated kinase activation events have not been widely studied in liver carcinogenesis. In this study, we aim to investigate the effect of MAPK inhibitors on Smad2/3 phosphorylation induced by TGF−β1 in HEPG2 cells.

## *MATERIALS AND METHODS*

### Cell Cultures and Reagents

HEPG2 were cultured in Dubecco´s modified Eagle´s medium (DMEM) supplemented with 10%FCS, 100 unit/ml penicillin G, and 0.1mg/ml streptomycin. Cell cultures were matained at 37℃ in a humidified atmosphere of 5% $CO_2$. Anti−pSmad2C Ab and anti−pSmad3C Ab were purchased from Sigma. Anti−pSmad2L Ab and anti−pSmad3L Ab were obtained from Prof Okazaki in Kansai Medical University, MAPK inhibitors PD98059, SP600125, and PD169316 were from Sigma Company.

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**TGFβ1–induced proliferation of HEPG2**

HEPG2 were seeded at $1\times10^5$ cells per well in 96–well dishes and cultured in 100μl of Dulbecco's modified Eagle medium supplemented with 10% FCS. Twelve hours later, the medium were replaced with serum–free medium. Then the cells were incubated at 37℃in a humidified atmosphere of 5% $CO_2$ for 24h and treated with TGF–β1 for 30min, 1h, 2h, 4h, 8h, 16h separately before adding MTT.

**Immunoprecipitation and immunoblotting**

Cells were starved for 18h in serum–free medium, then were treated with PD169316, SP600125, or PD98059 for 2h and 9pM TGF–β1 for 30 min. After treated with 9pmol/L TGF–β1 for 30 min, HEPG2 cells were lysed in lysis buffur (25mM HEPEPS,150mM NaCl, 50mM NaF, 0.5mg/ml 4–(2–aminoethyl) benzenesulfonyl fluoride, and 1μm/ml pepstatin, 10% glycerol, 5mM EDTA, 1% Triton–X–100, 5mM sodium orthovanadate), and the whole cell lysates were subjected to immunoprecipitation with monoclonal anti–Smad2/3 Ab, followed by adsorption to protein G–sepharose. The proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The primary Abs (rabbit polyclonal anti–pSmad2C, rabbit polyclonal anti–pSmad2L, rabbit polyclonal anti–Smad3C, and rabbit polylonal anti–Smad3L) were added for 12h at 4℃. The appropriate secondary Ab was added for 1h at room temperature. The membranes were visualized with horseradish peroxidase–conjugated rabbit IgG antibodies using the enhanced chemiluminescence Western blotting system (ECL).

**Table 1** The effect of TGF–1β on proliferation of HepG2 cells

| Group | Stimulus duration (h) | A490(OD) |
|---|---|---|
| medium | – | 0.54±0.02 |
| TGF–β1(9 pM) | 0.5 | 0.57±0.04 |
| | 1 | 0.64±0.11 |
| | 2 | 0.70±0.07★ |
| | 4 | 0.71±0.02★★ |
| | 8 | 0.73±0.06★★ |
| | 16 | 0.85±0.03★★ |

★$P$<0.05, ★★ $P$<0.01. OD values were measured at 490nm.

**Statitical Analysis**

All data were expressed as mean± SD, experimental and control values were compared using the unpaired student´s $t$–test. $P$<0.05 was considered to be statisticlly significant.

## *RESULTS*

**TGF–β1 accelerate HEPG2 cells growth**

We studied the effect of TGF–1β on cellular proliferation by MTT, and the result demonstrated that TGFβ stimulated cell growth(Table 1).

**TGF –β1 –induced phosphoralation of Smad2 and Smad3**

The phosphorylation level of Smad2C, Smad2L and Smad3L increased in the cells treated with TGF–β1(9 pM) for 0.5h, basal phosphoralation of Smad2 and Smad3 linker regions were oberserved in HEPG2 cells. (Figure 1).

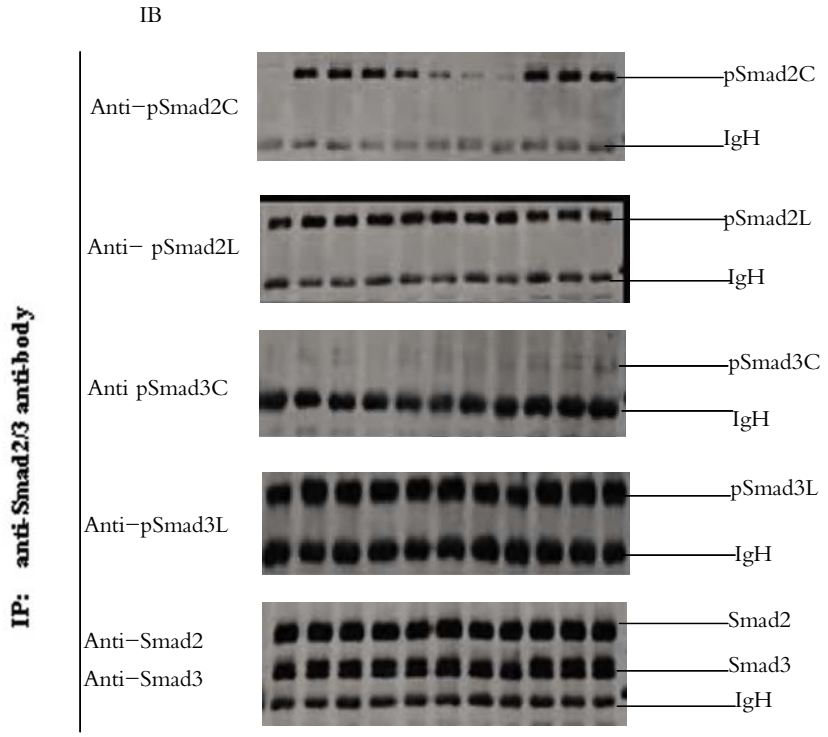
**MAPKs inhibitors result in a decrease of Smad2/3 phosphoralation at the linker region**

Recently, MAPKs pathways were showed to be activated in response to TGF–β1. We investigated the roles of P38, JNK and ERK in cultured HEPG2 using PD169316, SP600125 and PD98059. The results showed that the phosphorylation of Smad2C and Smad3L were inhibited by JNK inhibitor (3, 10 μM) and p38 inhibitor (1, 3, 10μM),; and ERK inhibitor (1, 3, 10μM) had no apparent effect on the phosphorylation of Smad2/3 (Figure 1)

## *DISCUSSION*

Transforming growth factor beta (TGF–β) is a multifunctional cytokine involved in the regulation of cell proliferation, differentiation and survival/or apoptosis of

| Inhibitor (pmol) | | JI | PI | EI |
|---|---|---|---|---|
| | − − | 1 3 10 | 1 3 10 | 1 3 10 |
| TGF−β1(9pmol) | − + | + + + | + + + | + + + |




many cells. Some reports that TGF −β1 accelerates HCC −M and HCC −T celll growth in an autocrine fashion [15], and may contribute to tumour pathogenesis by direct support of tumour growth and influence on local microenvironment, which resulted in immunosuppression, angiogenesis, and modification of the extracellular matrix [16, 17]. The results of this study suggested that TGF−β1 may be an important cytokine contributing to liver tumor pathogenesis.

The phosphorylation of R −Smad is a key step in Smad signaling, but how will Smad2/3 change in HepG2 cells activated by TGF −β1? We cultured the HepG2 cells with 9 pM TGF−β1 for 0.5 hour, which did not stimulate cells growth and the change of endogenous Smad2/3 quantity. The phosphorylation level of Smad2/3 were analyzed by four specific Abs. The result showed that the phosphorylation level of Smad2C, Smad2L and Smad3L increased, which suggeseted the phosphorylation of Smad2C, Smad2L and Smad3L might be induced by TGF −β1. TGF −β1 expression and secretion into medium increases significantly in human hepatocellular carcinoma[15]. Our result showed that pSmad2L/3L were expressed partly in control group, which demonstrated TGF −β1 might be secreted by HepG2 cells.

The phosphorylation Smad2/3 are induced by TGF −β1 through multiple intracellular pathways, MAPKs pathways have been reported in some cell types. For example, TGF −β1 activated p38 MAPK pathway, further leading to Smad3 phosphoylation at linker region in cultured rat myofibroblast[18]; ERK−dependent Smad2/3 linker region phosphorylation enhances collagen I synthesis in human mesangial cells, however, ERK −dependent activation of Smad2/3 occurs only in certain cell types [19]; TGF−β1 treatment of normal stomach−origin cells activated the JNK pathway,

thereafter inducing endogenous R−Smads phosphorylation at linker regions [20]. To investigate the effect of MAPKs on Smad2/3 phosphorylation in HepG2 cells by TGF−β1, we used three specific inhibitors to JNK, p38 and ERK. As figure1 showed, the phosphorylations of Smad2C and Smad3L were suppressed remarkabaly by JNK inhibitor (3, 10μM) and p38 inhibitor (1, 3, 10μM), and ERK inhibitor (1, 3, 10μM) had no apparent effect on the phosphorylation of Smad2/3. All the results approved that the phosphorylation of Smad2/3 might be induced by TGF−β1 via activated JNK and p38 in HepG2 cells.

In Conclusion, the pathway that the phosphorylation of Smad2/3 might be induced by TGF−β1 via activated JNK and p38 might contribute to HepG2 cells proliferation caused by TGF−β1. The finding may provide targets for new drug development.

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