Mitogen-activated protein kinase inhibitors reduce the nuclear accumulation of phosphorylated Smads by inhibiting Imp 7 or Imp 8 in HepG2 cells

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Abstract. The transforming growth factor (TGF)-β/Smad signaling pathway is involved in hepatocellular carcinoma development. Smad2 and Smad3 are phosphorylated following TGF-β1 stimulation and subsequently oligomerize with Smad4 to form the Smad2/3/4 complex, which translocates into the nucleus and regulates target genes, including plasminogen activator inhibitor type 1 (PAI1). Importin (Imp)7 and Imp8 are responsible for transporting phosphorylated (p)Smad2/3 and Smad4 into the nucleus. In our previous study, it was demonstrated that mitogen-activated protein kinase (MAPK) inhibitors, including inhibitors of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 could inhibit the transcription of PAI1, but ERK inhibitor had no significant effect on the phosphorylation of Smad2/3, and the formation of Smad2/3/4 complexes, which was different from the effect of JNK or p38 inhibitor. We hypothesized that MAPK inhibitors, particularly ERK inhibitor, reduced the transport of Smads into the nucleus by affecting Imp7 and Imp8. To confirm this hypothesis, HepG2 cells were incubated with different MAPK inhibitors for 5 h and subsequently stimulated with TGF-β1 for 1 h. Next, the intracellular locations of Smads (pSmad2C, pSmad2L, pSmad3C, pSmad3L and Smad4) and Imp7/8 were detected using immunofluorescence staining assays, and the expression of Imp7/8 was investigated using immunoblotting. It was revealed that JNK or p38 inhibitor decreased the phosphorylation of Smad2C, Smad2L and Smad3L, and affected their nuclear accumulation. Although only inhibiting the phosphorylation of Smad2C, ERK inhibitor affected the nuclear accumulation of pSmad2C, pSmad2L, pSmad3C and pSmad3L. The three MAPK inhibitors attenuated the nuclear distribution of Smad4, and the expression and nuclear accumulation of Imp7. ERK and JNK inhibitors attenuated the expression and nuclear accumulation of Imp8. Thus, the results of the present study suggest that MAPK inhibitors, particularly ERK inhibitor, modulate the nuclear accumulation of Smads via the inhibition of Imp 7/8.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortalities worldwide (1). The transforming growth factor-β1 (TGF-β1)/Smad signaling pathway serves an important role in HCC development. In the canonical TGF-β1/Smad signaling pathway, TGF-β receptor type I (TβR-I) activated by TGF-β1 phosphorylates receptor-regulated (R)-Smads, including Smad2 and Smad3 at the C-terminal, producing phosphorylated (p)Smad2C and pSmad3C (2). Subsequently, pSmad2C and pSmad3C oligomerize with Smad4 to form the Smad2/3/4 complex, which then translocates into the nucleus and regulates target gene expression (2).

The canonical Smad signaling pathway is integrated into a complex network of cross-talks with other signaling pathways, particularly the mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase pathways. In the cytoplasm, MAPKs phosphorylate R-Smads at the linker region, producing pSmad2L and pSmad3L. This regulatory phosphorylation at the linker region inhibits the translocation into the nucleus and the transcriptional activity of R-Smads that have been phosphorylated at the C-terminal (3). JNK/pSmad3L upregulates the expression of plasminogen activator inhibitor type 1 (PAI1), which is a downstream target gene of the TGF-β1/Smad signaling pathway (4,5). This has been demonstrated to facilitate hepatocytic cell invasion and reduce the tumor suppressive activity of pSmad3C in liver cancer (6,7). It has been suggested that the phosphorylated isoforms of R-Smad are essential for the interaction between MAPKs and the TGF-β1/Smad signaling pathway (8).

MAPK signaling regulates the subcellular distribution of pSmads, in addition to regulating the phosphorylation of Smads (9). This regulation can be context-dependent. The

data of previous studies revealed that ERK activation inhibits Smad2/3 nuclear translocation and that the ERK inhibitor, U0126, can restore the accumulation of Smad2/3 in the nucleus (10,11). Furthermore, ERK or p38 inhibitors are able to inhibit the nuclear translocation of pSmad2/3, which is induced by cyclosporine A (12). Inhibition of JNK1 activation prevents TGF-β1-induced Smad3 activation and nuclear translocation (13). However, this regulation is also cell-type specific. The p38 inhibitor SB203580 inhibits TGF-β-dependent translocation of Smad2/3 to the nucleus in myofibroblasts or human dental pulp cells, but not in Burkitt lymphoma cells (14-16). Our previous study reported that in keloid fibroblasts, the ERK inhibitor PD98059 inhibited the nuclear accumulation of pSmad2/3 (17). However, the effect of MAPK inhibitors on the translocation of R-Smads in HepG2 cells remains unclear.

Our previous data demonstrated that MAPK specific inhibitors, including ERK inhibitor (PD98059), JNK inhibitor (SP600125) or p38 inhibitor (SB203580), could inhibit the transcription of PAI1 in HepG2 cells (18). The phosphorylation of R-Smads was decreased following treatment with SP600125 and SB203580, but was scarcely affected upon PD98059 treatment, indicating that PD98059 affects the expression of PAI1 via interfering with the nuclear accumulation of pSmad2/3 (18). Thus, the present study aimed to investigate the effect of MAPK signaling on the nucleocytoplasmic distribution of pSmad2/3 in HepG2 cells.

There are four distinct mechanisms that may explain the nuclear accumulation of Smads in response to TGFβ: Increase in nuclear import, decrease in nuclear export, release from cytoplasmic anchoring or establishment of nuclear anchoring (19). In the current study, the nuclear import activity of Smads was investigated. In the conventional nuclear import progress, cargo proteins bind different importin (Imp)β proteins to form complexes, with or without the aid of Impα protein, which then translocate into the nucleus. Imp7 or 8 are two members of the Impβ family, responsible for transporting pSmad2/3 and Smad4 (20,21). The role of Imp7 or 8 in the regulation of the MAPK signaling pathway associated with the translocation of activated R-Smads was also investigated in the present study. In order to confirm this hypothesis, the intracellular distribution of pR-Smads and Smad4, and the subcellular localization of Imp7/8 were determined in HepG2 cells treated with three different MAPK inhibitors and TGF-β1.

Materials and methods

Cell culture. The human HCC HepG2 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HepG2 cells were grown as sub-confluent monolayer cultures in Dulbecco's modified Eagle's medium (DMEM; cat. no. SH30022.01; Hyclone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (cat. no. 11011-8611; Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China). Cells were incubated at 37˚C with 5% CO_2. The experiment was performed at the log phase of growth after the cells had been plated for 24 h. HepG2 cells were starved overnight in a humidified 5% CO_2 incubator at 37˚C in serum-free DMEM, in the absence or presence of 10 μM ERK inhibitor (PD98059), JNK inhibitor (SP600125) or p38 inhibitor (SB203580) (all EMD Millipore, Billerica, MA, USA) for 5 h in a humidified 5% CO_2 incubator at 37˚C; subsequently cells were treated with 9 pM TGF-β1 (R&D Systems, Inc., Minneapolis, MN, USA) for 1 h in a humidified 5% CO_2 incubator at 37˚C. The cells in the control groups were added to an equal volume of serum-free medium.

Immunofluorescence analysis. To detect the intracellular localization of Smads, HepG2 cells were grown on slides in 24-well plates and then treated under the aforementioned conditions. After fixing with 4% paraformaldehyde for 30 min at room temperature, the cells were permeabilized, and blocked with 0.1% saponin and 0.5% bovine serum albumin in PBS for 30 min at 4˚C. Subsequently, samples were incubated with the specified primary antibody (dilution, 1:50) overnight at 4˚C. The primary antibodies used were as follows: Domain-specific antibodies directed against pR-Smads [αpSmad2C (Ser465/467) (cat. no. 3108) and αpSmad3C (Ser423/425) (cat. no. 9520) (both from Cell Signaling Technology, Inc., Danvers, MA, USA); αpSmad2L (Ser249/254) and αpSmad3L (Ser207/212) (both provided by Dr K. Matsuzaki, Kansai Medical University, Osaka, Japan)]; mouse monoclonal anti-Smad4 antibody (cat. no. sc-7966; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); rabbit anti-Imp 7 (cat. no. ab99273) and rabbit anti-Imp 8 antibodies (cat. no. ab72109) (both from Abcam, Cambridge, UK). Subsequently, cells were incubated with fluorescein isothiocyanate-conjugated goat immunoglobulin G (IgG) anti-rabbit (cat. no. ZF-0311) or anti-mouse (cat. no. ZF-0312) (1:100 dilution; both from OriGene Technologies, Inc., Beijing, China) antibody for 2 h at room temperature. Next, slides were mounted with 80% phosphoglycerol, and images were captured under a fluorescence microscope (Olympus, Tokyo, Japan). In a single experiment, ≥100 stained cells/sample were analyzed.

Immunoblot analysis. To determine Imp7 and Imp8 expression, immunoblotting was performed using the aforementioned rabbit anti-Imp 7 and rabbit anti-Imp 8 primary antibodies diluted in Tris-buffered saline solution/0.1% Tween 20 (TBST) (1:10,000), and mouse anti-GAPDH (cat. no. 97166; Cell Signaling Technology, Inc.). Total protein was extracted from the HepG2 cells using Western blot and IP Cell Lysis reagent (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China). The samples were subjected to 12% SDS-PAGE (10 μg/lane) and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore). Non-specific antibody binding was blocked using 5% skimmed milk powder dissolved in TBST. PVDF membranes were incubated with the primary antibodies overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated affinipure goat IgG anti-rabbit (cat. no. ZB-2301) or anti-mouse (cat. no. ZB-2305) (both from OriGene Technologies, Inc.) diluted in TBST (1:10,000) for 1 h at room temperature. After being washed three times with TBST, the immunoreactive proteins were visualized using an enhanced chemiluminescence reagent (GE Healthcare, Chicago, IL, USA) and autoradiography. Densitometric analysis was performed using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All cell experiments were performed three times. All data are presented as the mean ± standard

deviation. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Experimental and control values were compared using the unpaired Student's t-test or one-way analysis of variance followed with the post-hoc Fisher's least significant difference test for multiple comparisons where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of three MAPK-specific inhibitors on TGF-β1-stimulated nuclear translocation of pSmad2/3 and Smad4 in HepG2 cells. Immunofluorescence analysis was used to detect the intracellular localization of pSmad2/3 at the C-terminal and linker region using corresponding anti-pSmad2/3 antibodies. Per sample, ≥100 stained HepG2 cells were analyzed.

Intracellular location of pSmad2/3 and Smad4 expression in HepG2 cells is presented in Fig. 1. pSmad2C, pSmad2L, pSmad3C and pSmad3L expression increased and was translocated into the nuclei following TGF-β1 treatment. The presence of ERK inhibitor PD98059 only inhibited the phosphorylation of Smad2C and had no notable influence on the phosphorylation of the other three pR-Smads. However, it affected the nuclear accumulation of pSmad2C, pSmad2L, pSmad3C and pSmad3L. The presence of JNK inhibitor SP600125 inhibited the phosphorylation of Smad2C, Smad2L and Smad3L, and affected their nuclear accumulation, but had no notable influence on the phosphorylation of Smad3C, despite affecting its nuclear accumulation. The p38 inhibitor SB203580 inhibited the phosphorylation of Smad2C and Smad2L, but had no notable influence on the phosphorylation of Smad3C, despite affecting its nuclear accumulation. The phosphorylation of Smad3L was inhibited, but the distribution in the cytoplasm and nuclei was scarcely affected in the presence of p38 inhibitor. Nuclear accumulation of Smad4 increased following stimulation with TGF-β1. All three MAPK inhibitors inhibited the translocation of Smad4 into the nucleus.

Effects of three MAPK-specific inhibitors on TGF-β1-stimulated nuclear translocation and expression of Imp7/8 in HepG2 cells. Intracellular localization of Imp7 and Imp8 was examined through immunofluorescence microscopy. Per sample, ≥100 stained HepG2 cells were analyzed.

As presented in Fig. 2, Imp7 and Imp8 expression increased and was translocated into the nuclei under TGF-β1 treatment in HepG2 cells. The presence of ERK, JNK and p38 inhibitors inhibited the expression of Imp7 and Imp8. Nuclear accumulation of Imp7 was affected in the presence of three inhibitors, particularly p38 inhibitor. The presence of ERK or JNK inhibitors decreased the nuclear accumulation of Imp8, and the p38 inhibitor had no notable influence on the nuclear accumulation of Imp8.

Immunoblotting results revealed that the expression of Imp7/8 was significantly upregulated following TGF-β1 treatment compared with untreated control HepG2 cells. In the presence of SB203580, PD98059 and SP600125, the expression of Imp7/8 was significantly inhibited compared with the TGF-β1-treated group (Fig. 3).

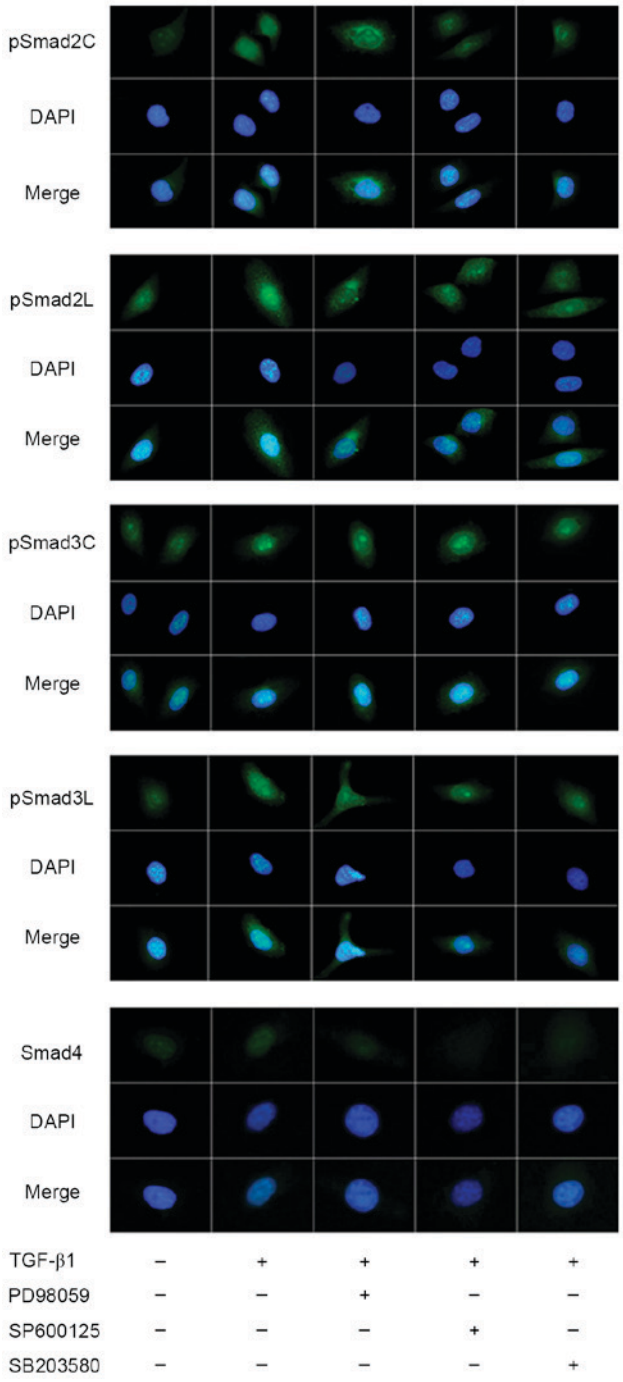

Figure 1. Effect of three mitogen-activated protein kinase-specific inhibitors on TGF-β1-stimulated nuclear translocation of pSmad2/3 and Smad4 in HepG2 cells. HepG2 cells were starved overnight in serum-free medium, in the absence or presence of 10 μM extracellular signal-regulated protein kinase inhibitor (PD98059), 10 μM c-Jun N-terminal kinase inhibitor (SP600125) or 10 μM p38 inhibitor (SB203580) for 5 h, respectively; they were subsequently treated with 9 pM TGF-β1 for 1 h. Counterstaining using DAPI was performed and then immunofluorescence staining assays were performed using the corresponding antibodies. TGF-β1, transforming growth factor-β1; p, phosphorylated.

Discussion

Following stimulation with TGF-β1, R-Smads can be phosphorylated at the C-terminal by TβR-I or at the linker region by MAPK, to produce different phosphorylated isoforms, including pSmad2C, pSmad2L, pSmad3C and pSmad3L, with

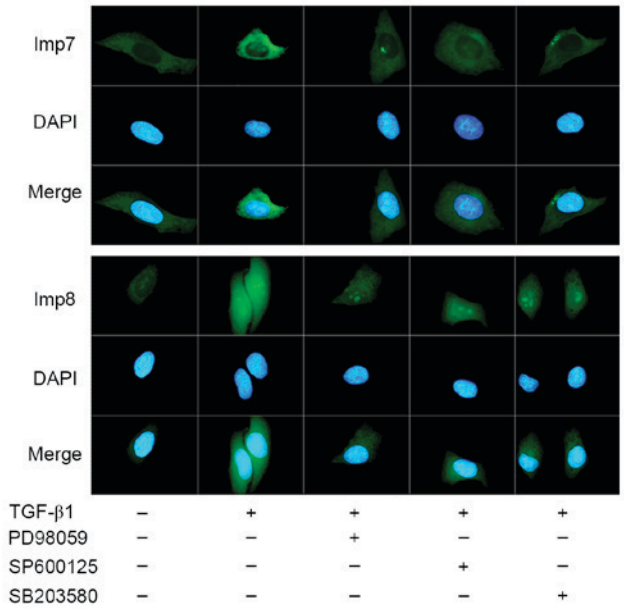

Figure 2. Effect of three mitogen-activated protein kinase-specific inhibitors on nuclear distribution of Imp7 and Imp8 in HepG2 cells. HepG2 cells were starved overnight in serum-free medium, in the absence or presence of 10 μM extracellular signal-regulated protein kinase inhibitor (PD98059), 10 μM c-Jun N-terminal kinase inhibitor (SP600125) or 10 μM p38 inhibitor (SB203580) for 5 h, respectively; they were subsequently treated with 9 pM TGF-β1 for 1 h. Counterstaining using DAPI was performed and then immunofluorescence staining assays were performed using corresponding antibodies. TGF-β1, transforming growth factor-β1; Imp, importin.

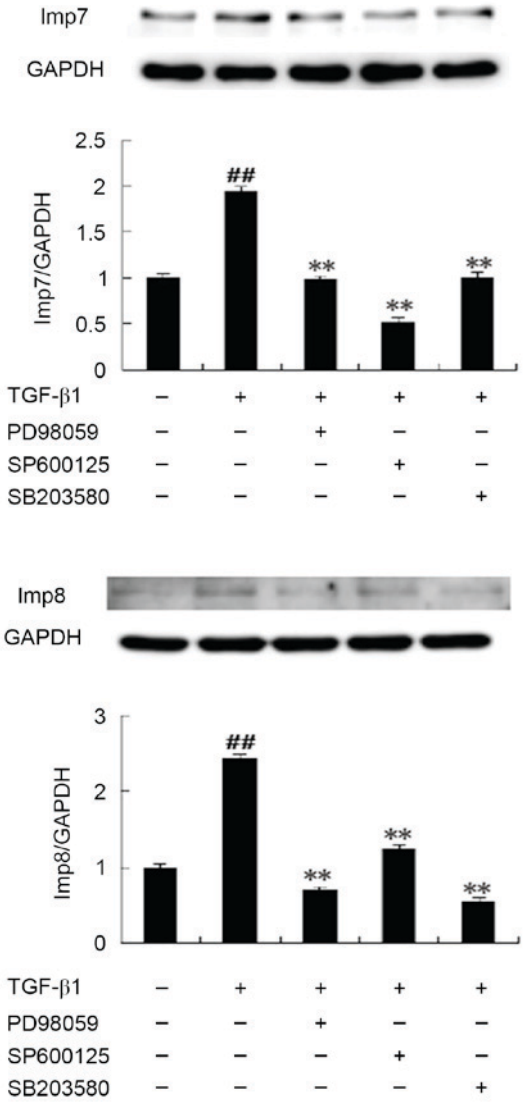

Figure 3. Effects of three mitogen-activated protein kinase-specific inhibitors on the expression of TGF-β1-mediated Imp7/8 in TGF-β1-activated HepG2 cells. HepG2 cells were starved overnight in serum-free medium, in the absence or presence of 10 μM extracellular signal-regulated protein kinase inhibitor (PD98059), 10 μM c-Jun N-terminal kinase inhibitor (SP600125) or 10 μM p38 inhibitor (SB203580) for 5 h, respectively; they were subsequently treated with 9 pM TGF-β1 for 1 h. The expression levels of Imp7/8 were analyzed using immunoblotting. Intensities of Imp7/8 bands were normalized to those of GAPDH of the corresponding treatment groups. The ratio of the Imp7/8 protein to GAPDH without exogenous TGF-β1 was assigned a value of 1. The presented data are based on ≥3 independent experiments. $^{\#\#}$P<0.05 vs. control; **P<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; Imp, importin.

distinct transcriptional responses to regulate different physiological and pathological processes (22).

MAPK/pSmad3L conveys mitogenic signals, while TβR-I/pSmad3C conveys cytostatic signals. pSmad3L and pSmad3C signals oppose each other, regulating the balance between cell growth and inhibition. In tumor cells, pSmad3L signaling impairs tumor-suppressive pSmad3C signaling, transmitting oncogenic TGF-β1 signaling (6,23-25). Similarly, in HepG2 cells, TGF-β1 promotes cell proliferation and increases the phosphorylation of oncogenic Smad3L, demonstrating an oncogenic effect (18). In a previous study, the increased cell proliferation rate was markedly inhibited by JNK inhibitor or p38 inhibitor, which also inhibited pSmad3L expression. However, ERK inhibitor did not affect the proliferation of HepG2 cells and pSmad3L expression (18). In the current study, the change in fluorescence intensity of pSmad3L was consistent with the aforementioned results in previous study mentioned above (18). JNK inhibitor or p38 inhibitor were demonstrated to inhibit pSmad3L distribution in the nucleus. Furthermore, ERK inhibitor reduced the nuclear accumulation of pSmad3L, while reducing the nuclear accumulation of pSmad3C. The effect of ERK inhibitor on the nuclear accumulation of pSmad3L or pSmad3C may reach a balance, without the functional effect.

In the advanced stage of tumor progression, the role of TGF-β1 switches to tumor promotion, inducing tumor cell invasion. PAI1, a downstream target of the TGF-β/Smad signaling pathway (4,5), facilitates cell migration and invasion by enhancing cell adhesion (7). PAI1 induction requires pSmad2L (Ser-245/250/255)/C and pSmad3L (Ser-213) activity (15,26-28). We previously demonstrated that TGF-β1 induced the expression of pSmad2C, pSmad2L and pSmad3L, and increased PAI1 expression and the invasiveness of HepG2 cells (18). All three MAPK inhibitors suppressed the invasiveness of the cells and PAI1 expression. Among them, JNK or p38 inhibitor inhibited the expression of pSmad2C, pSmad2L and pSmad3L, but ERK inhibitor only inhibited the expression of pSmad2L, and did not affect the expression of pSmad2C or pSmad3L (18). In the present study, the immunofluorescence results demonstrated that the nuclear distribution of pSmad2C and pSmad3L decreased following treatment with PD98059, although the expression of pSmad2C and pSmad3L remained unchanged, suggesting that ERK inhibitor may inhibit the

invasiveness of HepG2 cells and PAI1 expression via regulation of pR-Smad transport.

R-Smads-Smad4 complexes are essential for TGFβ1 signaling, and Smad4 is an essential partner in these complexes (2). Following the formation of heterocomplexes with Smad4, pSmad3L and pSmad3C enter the nucleus to transduce signaling (29). pSmad2C/L undergoes translocation to the nucleus where it binds to the pSmad3L/Smad4 complex (15,30). The cell invasion-induced effect of pSmad2C/L and pSmad3L requires Smad4 and complex formation. In addition, the nuclear accumulation of Smad4 is dependent on R-Smad accumulation (31-33). Furthermore, the intracellular distribution of Smad4 reflects the distribution of pR-Smads or R-Smads-Smad4 complexes. In the present study, following treatment with JNK or p38 inhibitor, the formation of Smad2/3/4 complexes was inhibited as pR-Smad expression was inhibited, subsequently the nuclear accumulation of Smad4 decreased. Although ERK inhibitor did not inhibit the Smad2/3/4 complexes, it reduced the nuclear distribution of Smad4, further confirming the effect of ERK inhibitor on the translocation of Smad2/3/4 complexes.

Smads are continuously shuttling between the cytoplasm and the nucleus even in unstimulated cells. TGF-β promotes Smad2, 3 and 4 accumulating in the nucleus, reaching a maximum concentration after ~45 min (34), as was observed in the current study. C-terminal phosphorylation is a prerequisite for Smads to accumulate in the nucleus. The data of *in vitro* experiments demonstrated that Smad3 is imported into the nucleus more efficiently following phosphorylation (35). Schmierer *et al* (36) proposed a mathematical model to understand the mechanism of nucleocytoplasmic shuttling of Smads, which requires that the import of Smad complexes into the nucleus should be ~5 times faster compared with the import of monomeric Smads. It has been suggested that the phosphorylation of R-Smads and the formation of Smad2/3/4 complexes are important to the nuclear import of Smads, which may explain why JNK or p38 inhibitors inhibit the translocation of Smads into the nucleus. However, ERK inhibitor also inhibited the nuclear accumulation of Smads, with little influence on the phosphorylation of R-Smads and the formation of Smad2/3/4 complexes.

It has been reported that the subcellular distribution of representative cargo proteins is similar to that of Impβ (37). In the present study, it was observed that the nuclear accumulation of Imp7 or 8 was impaired by MAPK inhibitors, similar to that of Smads, suggesting that MAPK inhibitors regulate Smads import by affecting Imp7 or 8.

Previous studies have reported that the knockdown of Imp7 and Imp8 inhibits TGF-β-induced Smad2/3 nuclear translocation, while overexpression of Imp8 increases the concentration of Smad3 or 4 in the nucleus (20,21). The expression levels of Imp7 or Imp8 directly affect the nuclear translocation of Smads. The data of the present study demonstrated that all three inhibitor types were able to significantly decrease the expression of Imp7 or Imp8. Thus, this suggests that inhibiting Imp7 or Imp8 is an important mechanism in regulating Smad translocation by MAPK inhibitors.

In conclusion, the results of the present study demonstrated that MAPK inhibitors, particularly ERK inhibitor, regulate the TGF-β1/Smad signaling pathway by reducing the nuclear accumulation of Smads. Inhibiting Imp7 or Imp8 is an important mechanism in regulating Smad translocation by MAPK inhibitors.

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