

MAPK inhibitors differently modulate TGF- β /Smad signaling in HepG2 cells

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Abstract The aim of this study was to investigate the mitogen-activated protein kinase (MAPK) pathway, which crosstalk with TGF- β /Smad signaling via linker phosphorylation of Smad2/3 to promote hepatocarcinogenesis. After DEN-induced hepatocellular carcinoma (HCC) in rats showed increased phosphorylation of JNK1/2, p38, and ERK1/2, we next antagonized TGF- β ₁-induced phosphorylation of JNK1/2, p38, ERK1/2, Smad2/3 signaling in HepG2 cells using SP600125, SB203580, and PD98059, respectively. Cell proliferation and invasion were assessed by MTT assay and transwell invasion chambers, respectively. Smad2/3, Smad4, and Smad7 expressions and PAI-1 messenger RNA (mRNA) transcription were measured by using immuno-precipitation/immuno-blotting and real-time RT-PCR, respectively. All the MAPK-specific inhibitors suppressed cell invasion, while all but PD98059 suppressed cell proliferation. Both SP600125 and SB203580 blocked pSmad2C/L and oncogenic pSmad3L. PD98059 blocked pSmad2L but had no effect on elevated pSmad2C and oncogenic pSmad3L. All but PD98059 blocked Smad2/3/4 complex formation and restored Smad7 expression, while all the three MAPK-specific inhibitors repressed PAI-1 mRNA transcription. Both SP600125 and SB203580 inhibited HepG2 cells' proliferation and invasion by blocking oncogenic pSmad3L and Smad2/3/4 complex formation. PD98059 repressed PAI-1 mRNA by an unknown mechanism.

Keywords Hepatocellular carcinoma · MAPK-specific inhibitors · Smad · TGF- β ₁

Introduction

Derangements of several cell signaling pathways have been implicated in the initiation, progression, and development of hepatocellular carcinoma (HCC). One of such pathways is the mitogen-activated protein kinase (MAPK)-regulated TGF- β /Smad pathway which in its dysregulated state has been shown to promote tumorigenesis and metastasis [1, 2] in late phase of HCC either through canonical Smad signaling [3] or non-Smad pathways via MAPK-dependent linker phosphorylation of Smad2 and Smad3 [4, 5]. As results of the many pathways implicated in HCC, efforts to find therapeutic targets for effective therapy against HCC have always proved cumbersome. Further, compounding an already difficult problem is the ability of hepatoma cells to reactivate hitherto quiescent signaling pathways to compensate for the inhibited ones. It is therefore not surprising that therapies targeting inhibition of a single pathway in HCC in clinical trials such as phosphoinositol 3-kinase (PI3K) inhibitors, AKT inhibitors, mammalian target of rapamycin (mTOR) inhibitors, and TGF- β -inhibitors as a single therapy have all proved ineffective. Certainly, this stimulates dire need of multi-target signal inhibitors such as the MAPK-specific inhibitors, which has the potential to target the MAPK pathway as well as the oncogenic arm of the TGF- β /Smad signaling in almost all known cancer subtypes including HCC. Importantly, MAPK-dependent linker phosphorylation of Smad2/3 and their subsequent nuclear import are crucial for MAPK-regulated TGF- β /Smad signaling in cancer [6–8]; therefore, targeting

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this pathway through rigorous investigations will not only expand our understanding of cancer biology but could also offer an important therapeutic avenue for future development of anti-cancer drugs. We investigated the MAPK-regulated TGF- β signaling by using HepG2 cell line (a kind of human hepatoma cell line) which is a well-accepted cell line for studying cancer phenotypic hallmarks of HCC in particular as well as signaling pathways implicated in HCC.

In effect, we tested the hypothesis that targeted inhibition of the MAPK-regulated TGF- β /Smad signaling pathway in HCC by MAPK-specific inhibitors (SP600125 [JNK-specific inhibitor], SB203580 [p38-specific inhibitor], and PD98059 [ERK-specific inhibitor]) may abrogate HCC progression, particularly the two important phenotypic hallmarks (cell proliferation, migration, and invasion) of HCC. Previously, we have demonstrated the involvement of TGF- β /Smad signaling in proliferation and invasion of HepG2 cells [9–11] and subsequently we demonstrated abrogation of the crosstalk between TGF- β /Smad and the JNK-dependent MAPK pathway in keloid fibroblast cells using SP600125, SB203580, and PD98059 [12], but the pleiotropic and cell type-specific signaling of TGF- β and its signaling collaborators provides a strong reason to investigate this pathway in many cancer cell lines including HepG2 cells. To pursue this, we used both *in vivo* and *in vitro* models of HCC to investigate the MAPK-regulated TGF- β /Smad signaling as a means to prospect for new therapeutic targets.

Materials and methods

Animal model of HCC

Mature and healthy male Sprague–Dawley rats of body weight (180–200 g) were purchased from Xipuer-Bikai Company (Shanghai, China). The rats were housed in conventional cages at 20–22 °C, supplied with standard laboratory chow and water *ad libitum*, and kept at a 12-h light/dark cycle. The rats were maintained under these conditions for at least 1 week for acclimatization before the commencement of experiments. The handling and use of the rats in the study was carried out in accordance with the guidelines for the humane treatment of animals set out by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University. The rats were randomly divided into two groups: the normal group and the DEN treatment group. The rats in the normal group were given normal animal chow and water, while the rats in the DEN group were treated with 0.2 % DEN in 0.5 % CMC-Na by gavage 5 times a week for 14 weeks to induce HCC. The rats were sacrificed two weeks after DEN administration (16th week). One lobe of liver from each rat was harvested and stored at a temperature of –80 °C until use. Liver tissue specimens were extracted

with TNE buffer containing 10 mM Tris–HCl (pH 7.5), NaCl (150 mM), ethylene diamine tetraacetic acid (1 mM), 1 % Nonidet P-40, and phenylmethylsulfonyl fluoride (100 mM). Protein concentration was determined by using the bicinchoninic acid protein assay kit (Pierce, IL, USA). The extracts were examined for phosphorylation of the MAPK pathway by immuno-blot analysis.

Cell culture

The human HCC HepG2 cell line was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). HepG2 cell lines were grown as sub-confluent monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). The cells were kept in 95 % air and 5 % CO₂ at 37 °C. The experiments were performed at the log phase of growth after the cells had been plated for 24 h.

Cell proliferation assay

HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and cultured in DMEM supplemented with 10 % FBS. Following starving in a serum-free medium overnight, in the absence or presence of JNK inhibitor (SP600125), p38 inhibitor (SB203580 or ERK inhibitor (PD98059) all obtained from Calbiochem (San Diego, CA, USA) for 24 h. Subsequently, the cells were treated with exogenous TGF- β_1 (40 pM, R&D Systems, Minneapolis, MN, USA) for 12 h. The cells of the control group were treated with an equal volume of serum-free medium. Cell proliferation was assessed by using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Absorbance was measured in an enzyme-linked immuno-sorbent assay plate reader (MK3 Instruments, Thermo Fisher USA) at 570 nm. The data were expressed as a mean from at least three independent experiments.

Cell invasion assay

A cell invasion assay was conducted as described previously [13]. Briefly, transwell invasion chambers with 8- μ m membrane pores coated with Matrigel (BD Biosciences, San Jose, CA, USA) were placed in 24 well plates. HepG2 cells were starved overnight in serum-free DMEM, and the cells (2×10^4 /well) were then seeded in the upper compartment of the chamber and incubated in the absence or presence of SP600125, SB203580, or PD98059 for 24 h and with TGF- β_1 (40 pM) for 12 h was placed in the lower compartment of the chamber, while serum-free medium was used for the control study. After incubation, cells remaining on the upper surface of

the membrane were completely removed by a cotton swab. Cells which crossed the Matrigel barrier and migrated to the lower side of the chamber were fixed and stained with hematoxylin and eosin. The number of invading cells was counted in five fields using a microscope at a magnification of $\times 100$, and the mean count for each chamber was determined.

Immuno-precipitation and immuno-blot analysis

HepG2 cells were seeded at a density of 1×10^6 cells/10 cm dish and cultured in DMEM supplemented with 10 % FBS. Following starving in the serum-free medium overnight, in the absence or presence of SP600125 (10 μ M), SB203580 (10 μ M) or PD98059 (10 μ M), all obtained from Calbiochem (San Diego, CA, USA) for 5 h, the cells were subsequently treated with exogenous TGF- β_1 (9 pM) (R&D Systems, Minneapolis, MN, USA) at varied times depending on the different purposes. The cells of the control group were treated with an equal volume of serum-free medium.

The supernatants of pretreated cell lysates were subjected to IP with mouse anti-Smad2/3 antibody (BD Bioscience, San Jose, CA, USA), followed by adsorption onto protein-G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA). The phosphorylation of Smad2/3 at C-terminal (pSmad2C and pSmad3C) and linker region (pSmad2L and pSmad3L) were then monitored by IB analysis using each domain-specific antibody against the phosphorylation site: α pSmad2C (Ser^{465/467}), α pSmad3C (Ser^{423/425}), (Cell Signaling, Beverly, MA, USA); α pSmad2L (Ser^{249/254}) or α pSmad3L (Ser^{207/212}) (a present from Prof. K Matsuzaki). After IP, Smad2/3/4 complex expression was monitored using mouse monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology); the endogenous Smad2 and Smad3 expression in HepG2 cells was monitored using rabbit polyclonal anti-Smad2/3 (FL-425) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Other cell lysates immuno-blot were made using the following primary antibodies: mouse monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology), goat polyclonal anti-Smad7 antibody (Santa Cruz Biotechnology), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To examine the phosphorylation of MAPK pathway, the following primary antibodies were used: rabbit anti-phospho-JNK1/2 antibody, rabbit anti-JNK1/2 antibody, rabbit anti-phospho-p38 kinase antibody, rabbit anti-p38 kinase antibody, and rabbit anti-phospho-ERK1/2 antibody, and rabbit anti-ERK1/2 antibody (Promega Corp., Madison, WI, USA). The samples were boiled for 5 min in SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4 % SDS, 12 % β -mercaptoethanol, 20 % glycerol, and 0.01 % bromophenol blue]. Subsequently, the samples

were subjected to SDS-PAGE and then were transferred onto polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, Mass). Non-specific antibody binding was blocked in Tris-buffered saline solution/0.1 Tween 20 (TBST) containing 5 % skimmed milk. PVDF membranes were incubated with primary antibodies overnight at 4 °C, followed by the appropriate secondary antibodies for 1 h at room temperature. After washing three times with TBST, the immuno-reactive proteins were visualized by ECL (RPN2106; Amersham Pharmacia Biotech) and also by autoradiography. Densitometric analysis was carried out by using ImageJ software.

Real-time reverse transcriptase-polymerase chain reaction

The total RNA of pretreated cells was extracted using RNAiso Reagent (TaKaRa, Japan) and quantified by spectrophotometry. Real-time RT-PCR analysis was performed in an iCycler iQ Multicolor Detection System (Bio-Rad) according to the manufacturer's instruction. For each sample, 200 ng of the total RNA was reverse-transcribed to cDNA and amplified using the SYBR®PrimeScript™ RT-PCR kit (TaKaRa, Japan) with specific oligonucleotide primers for human PAI-1 target sequences and human β -actin (for normalization); ddH₂O was the negative control. The primers were designed and synthesized by TaKaRa Biotechnology, Japan. Primers sequences were the following:

β -actin: forward 5'-TGGCACCCAGCACAATGAA-3'
Reverse 5'-CTAAGTCATAGTCCGCTAGAAGCA-3'
PAI-1: forward 5'-TCATCATCAATGACTGGGTGAA
GAC-3'
Reverse 5'-TTCCACTGGCCGTTGAAGTAGAG-3'

Thermal cycling parameters were as follows: 95 °C for 10 s, 40 cycles of denaturing at 95 °C for 5 s, and annealing/extension for 30 s at 60 °C. Threshold cycles (Ct) at which emission rises above baseline were automatically calculated by the real-time RT-PCR system. Each Ct value was normalized to the housekeeping gene β -actin Ct value and a control sample. Relative quantization was expressed as fold induction compared to control conditions. Melting curves were generated after each run to confirm amplification of specific transcripts.

Statistics

Data were expressed as mean \pm standard deviation (SD). Statistical analyses were performed by SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). Experimental and control groups were compared by one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Phosphorylation of pJNK1/2, pp38, and pERK1/2 in vivo and in vitro

JNK1/2, p38, and ERK1/2 were expressed in DEN-induced HCC, specifically ERK1/2 was the most phosphorylated and p38 the least (Fig. 1a). Subsequently, in an in vitro experiment involving TGF- β_1 -stimulated HepG2 cells, we investigated TGF- β_1 -induced phosphorylation of JNK1/2, p38, and ERK1/2 by blocking them with their respective domain-specific inhibitors. Though all the MAPKs were expressed

in TGF- β_1 -stimulated HepG2 cells, comparatively, it was pERK1/2 which was most expressed (Fig. 1b). All the phosphorylated MAPKs (pJNK1/2, pp38, and pERK1/2) were blocked by their respective inhibitors (SP600125, SB203580, and PD98059, respectively) (Fig. 1b).

MAPK-specific inhibitors suppressed TGF- β_1 -induced HepG2 cell proliferation and invasion

Treatment of HepG2 cells with TGF- β_1 (40 pM) led to increase in cell proliferation (Fig. 2a) and invasion (Fig. 2b). However, prior treatment of HepG2 cells with

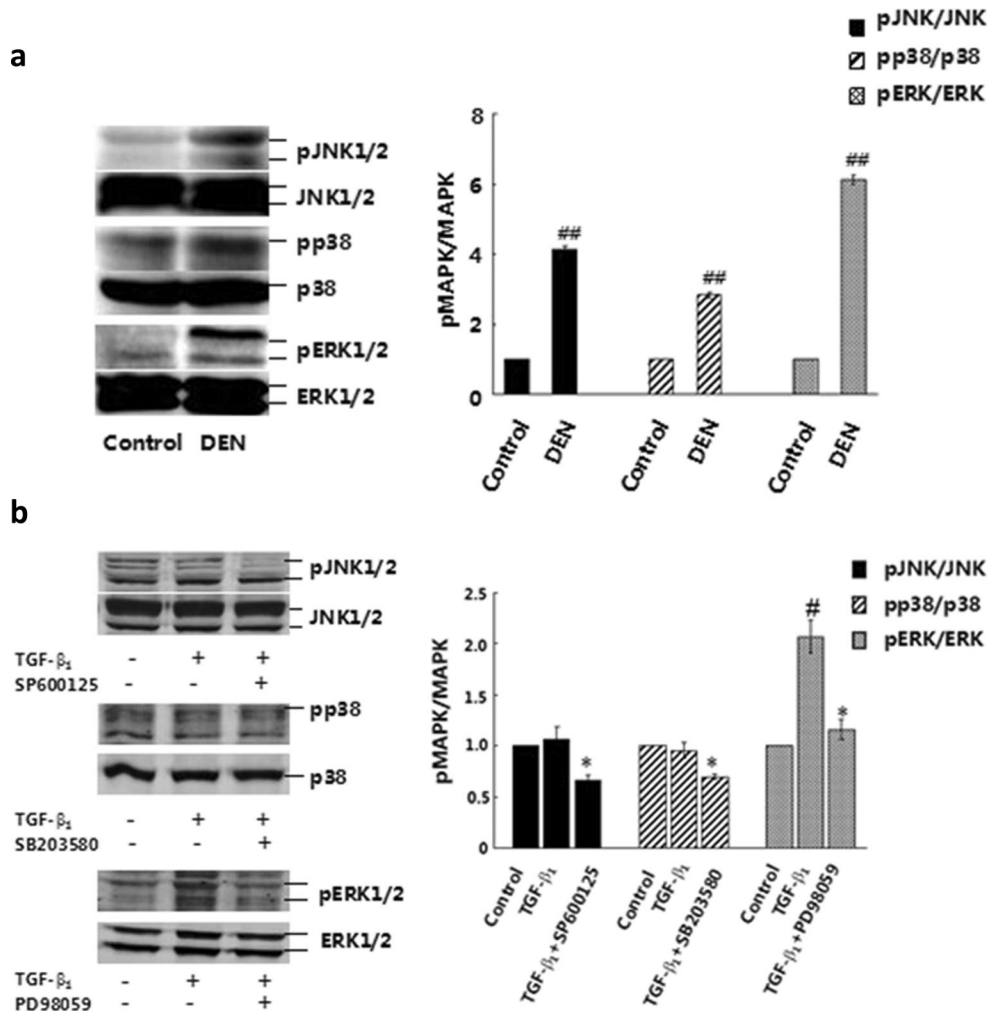


Fig. 1 MAPK phosphorylation in DEN-induced HCC in rats and the effects of SP600125, SB203580, and PD98059 on TGF- β_1 -induced MAPK phosphorylation in HepG2 cells. **a** Following protein extraction from homogenized livers, homogenates were incubated with each antibody of the respective MAPKs. Intensities of phosphorylated (p) pJNK1/2, pp38, and pERK1/2 bands were normalized to those of JNK1/2, p38, or ERK1/2 of the corresponding treatment groups. The ratio of the phosphorylated protein: total protein of control group was assigned a value of 1. The data presented is based on at least three independent experiments ([#] $P < 0.05$ compared with control group; ^{*} $P < 0.05$ compared with DEN group) ($n = 8$). **b** HepG2 cells were starved overnight in serum-free medium, in the absence or presence of

SP600125 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 5 h, in each case. The cells were subsequently treated with exogenous TGF- β_1 (9 pM) for 15 min (for JNK and ERK) or 1 h (for p38) and were then harvested and lysed; the blots were incubated with each respective antibody. Intensities of pJNK1/2, p-p38, and pERK1/2 bands were normalized to those of total JNK1/2, p38, or ERK1/2 of the corresponding treatment groups. The ratio of the phosphorylated protein to total protein without exogenous TGF- β_1 was assigned a value of 1. The data presented is based on at least three independent experiments ([#] $P < 0.05$ compared with control group; ^{*} $P < 0.05$ compared with TGF- β_1 group) ($n = 3$)

SP600125, SB203580, and PD98059 at 10 μM in each case before TGF- β_1 (40 pM) led to significant blockade of cell proliferation by SP600125 and SB203580 but minimal in the case of PD98059 (Fig. 2a). Out of the three MAPK-specific inhibitors, SP600125 produced the most significant inhibition of cell invasion followed by SB203580. Although, PD98059 blocked HepG2 cell invasion compared to TGF- β_1 group, it was not much different from the control group (Fig. 2b).

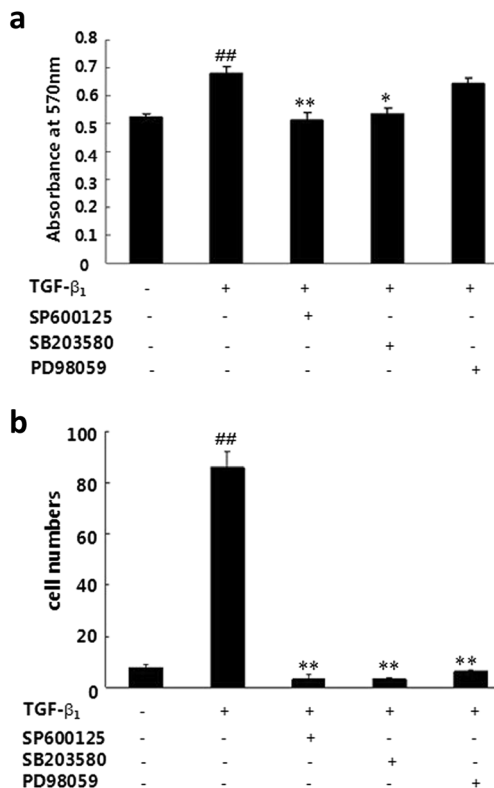


Fig. 2 Effects of SP600125, SB203580, and PD98059 on TGF- β_1 -induced HepG2 cell proliferation and invasion. **a** HepG2 cells were seeded in 96-well plates at a density 1×10^4 cells/well and cultured to sub-confluence. After starving the cells overnight in serum-free medium, the cells were incubated in the medium for 24 h in the presence or absence of SP600125 (10 μM), SB203580 (10 μM), or PD98059 (10 μM); subsequently, they were each treated with exogenous TGF- β_1 (40 pM) for 12 h before the end of incubation. Cell proliferation was assessed by using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Absorbance was measured by enzyme-linked immunosorbent assay plate reader at 570 nm. **b** The cells (2×10^4) were seeded in the upper compartment of a transwell chamber in each well 24-well dishes and incubated in the presence or absence of SP600125 (20 μM), SB203580 (10 μM), or PD98059 (20 μM) for 24 h and with TGF- β_1 (40 pM) for 12 h. After incubation, cells remaining on the upper surface of the membrane were completely removed by cotton swab. Cells that cross the Matrigel barrier and migrated to the lower side of the chamber were fixed and stained using hematoxylin and eosin. The number of invading cells was counted in five fields under a microscope at a magnification of $\times 100$, and the mean for each chamber was determined. All the values were expressed as mean \pm SD; $n=3$. The data presented is based on at least three independent experiments (^{##} $P < 0.01$ compared with control group; ^{**} $P < 0.01$ compared with TGF- β_1 group)

Effect of MAPK-specific inhibitors on Smad2/3 phosphorylation at both C- and linker phospho-domains and the formation of Smad2/3/4 complex

Treatment of HepG2 cells with TGF- β_1 (9 pM) resulted in increased phosphorylation of Smad2C, Smad2L, and oncogenic Smad3L, while Smad3C phosphorylation remained unaffected. Upon treatment of HepG2 cells with SP600125, SB203580, and PD98059 followed by TGF- β_1 (9 pM) led to varied inhibitory effects on TGF- β_1 -induced phosphorylation of Smad2L/C, Smad3L, and also Smad2/3/4 complex formation. Remarkably, both SP600125 and SB203580 blocked phosphorylation of Smad2C/L and oncogenic Smad3L yet at varying degrees, but the two had no effect on the phosphorylation of tumor suppressor Smad3C; however, PD98059 only blocked phosphorylation of Smad2L but had no effect on elevated phosphorylation of Smad2C and oncogenic Smad3L (Fig. 3a). All the three MAPK-specific inhibitors had no effect on Smad3C phosphorylation (Fig. 3a).

Similarly, TGF- β_1 (9 pM) led to an increase in the expression of Smad2/3/4 complex shown by increased expression of Smad2/3 and Smad4 (Fig. 3b), but with the exception of PD98059, all the MAPK-specific inhibitors blocked the increased expressions of Smad2/3 and Smad4. Importantly, SB203580 and SP600125 potently blocked pSmad2/3 and Smad4 expression (Fig. 3b).

MAPK-specific inhibitors restored Smad7 expression

TGF- β_1 (9 pM) treatment decreased the expression of Smad7 in HepG2 cells, but prior treatment of HepG2 cells with the MAPK-specific inhibitors at a concentration of 10 μM in each case restored Smad7 expression, though it was minimal in the case of PD98059 (Fig. 3c).

MAPK-specific inhibitors repressed PAI-1 mRNA transcription

Upon treatment of HepG2 cells with TGF- β_1 (9 pM) for 12 h, the transcripts of PAI-1 messenger RNA (mRNA) increased (Fig. 4); however, prior treatment of HepG2 cells with SP600125, SB203580, and PD98059 at 10 μM in each case decreased hitherto up-regulated TGF- β_1 -induced transcription of PAI-1 mRNA. All the three MAPK-specific inhibitors significantly repressed the transcription of PAI-1 mRNA compared to the TGF- β_1 group (Fig. 4). Out of the three MAPK-specific inhibitors, SB203580 potently repressed PAI-1 mRNA transcription followed by PD98059. SP600125 reduced TGF- β_1 -induced PAI-1 mRNA transcription when compared to the model group, but it was not comparable to that of the control group.

Discussion

We herein present a study to highlight the modulation of the MAPK pathway and the abrogation of the MAPK-regulated TGF- β /Smad signaling in HepG2 cells by three MAPK-specific inhibitors (SP600125, SB203580, and PD98059). The MAPK pathway crosstalks with the TGF- β /Smad signaling by regulating Smad phosphorylation at the linker region to increase nuclear translocation of pSmad2/3 and also to enhance non-canonical TGF- β signaling [14]. Indeed, linker phosphorylation of pSmad2/3 by JNK1/2 [4], p38 MAPK [15, 16], and ERK1/2 [17] arguably has been shown to augment the overall oncogenic role of TGF- β_1 signaling in HCC. Thus, effective abrogation of the crosstalk between the MAPK pathway and the TGF- β /Smad signaling, particularly linker-specific phosphorylation of oncogenic mediator (Smad3) in HCC may represent an important target in finding an effective therapy for HCC. We investigated the MAPK-regulated TGF- β /Smad signaling in HepG2 cell lines because it is a well-accepted human hepatoma cell line and it characterizes all the phenotypic hallmarks of cancer including that of HCC; therefore, a good choice for investigating signaling pathways implicated in HCC. Equally, MAPK-specific inhibitors are a class of anti-cancer drugs, which are under various stages of clinical investigations. Specifically, the MAPK-specific inhibitors as a class of anti-cancer drugs target the MAPK pathway as well as the TGF- β /Smad signaling pathway, so they were used in the present study to explore other targets relevant for therapy against HCC.

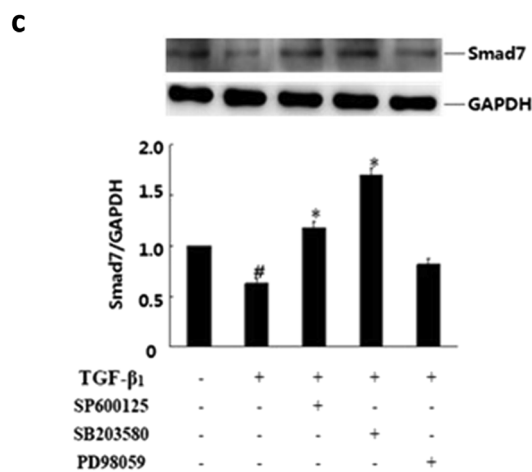
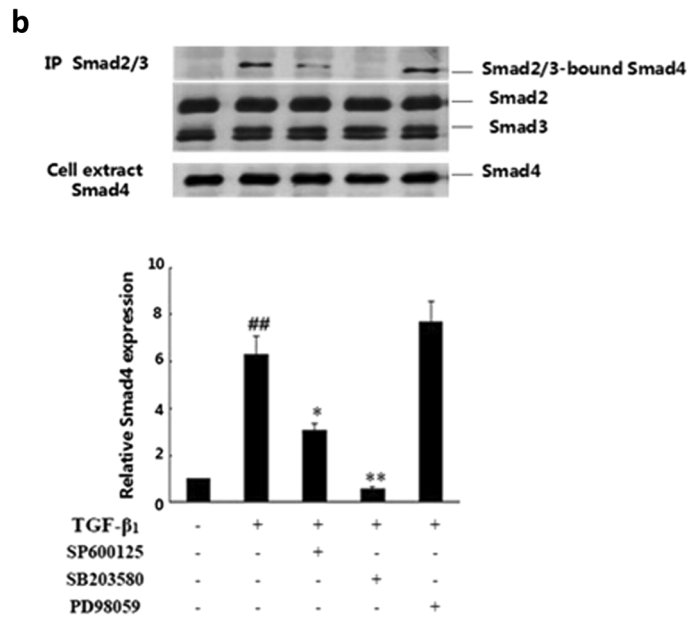
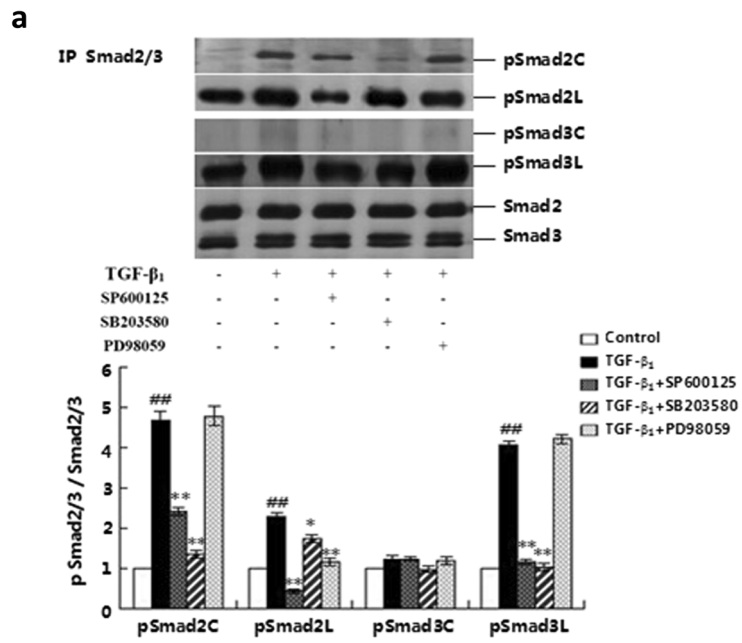
Previously, in an *in vivo* study [18] have shown the expression of only phosphorylated pJNK in both tumor and non-tumor liver tissues in DEN-induced HCC in rats over a period of 84 days but not pp38 and pERK. However, we report *in vivo* expression of not only phosphorylated pJNK but also pp38 and pERK1/2 in DEN-induced HCC in rats over a period of 16 weeks (Fig. 1a). Before the present study, we have shown 100 % incidence rate of HCC in DEN-induced HCC in rats over a period of 16 weeks [11] and we attributed that observation to prolonged exposure (16 weeks) of rats to DEN, but our present results seem to emphasize the crucial role of the MAPK pathway in HCC progression corroborating earlier reports [4, 19].

In the progression of HCC, the two most important defining phenotypic hallmarks of the tumor cells are cell proliferation and invasion. Indeed, after tumor cells have evaded the tumor suppressive effects of hepatocytic TGF- β_1 to their advantage, they usurp proliferative and invasive capacities with which they metastasize into distant tissue sites. Before, we have shown induction of cell migratory capacity by TGF- β_1 (9 pM) in HepG2 cells but no effect on cell proliferation [10]. Nonetheless, in the present study by increasing the concentration of TGF- β_1 (40 pM for 12 h) led to induction of HepG2 cell proliferation and invasion, perhaps indicating that

Fig. 3 Effects of SP600125, SB203580, and PD98059 on TGF- β_1 -induced phosphorylation of Smad2/3, Smad2/3/4 complex formation, and down-regulation of Smad7 expression in HepG2 cells. **a** HepG2 cells were starved overnight in serum-free medium in the presence or absence of SP600125 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 5 h in each case; subsequently, they were each treated of exogenous TGF- β_1 (9 pM) for 1 h. Following IP of HepG2 cell lysates with anti-Smad2/3 antibody (α Smad2/3), phosphorylation (P) of Smad2 and Smad3 were analyzed by IB using each anti-pSmad2/3 antibody. Expressions of endogenous Smad2, Smad3 in HepG2 cell lysate were monitored by IB using anti-Smad2/3 antibody. Intensities of Smad2/3 bands were normalized to those of total Smad2/3 of the corresponding treatment groups. The ratio of the phosphorylated protein: total protein without exogenous TGF- β_1 was assigned a value of 1. **b** HepG2 cells were starved overnight in serum-free medium in the presence or absence of SP600125 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 5 h in each case; subsequently, they were each treated with exogenous TGF- β_1 (9 pM) for 1 h. Following IP of HepG2 cell lysates with anti-Smad2/3 antibody (α Smad2/3), Smad4 bound to Smad2/3 was analyzed by IB using anti-Smad4 antibody. Expressions of endogenous Smad2, Smad3, and Smad4 in HepG2 cell lysate were monitored by IB using anti-Smad2/3 antibody and anti-Smad4 antibody. Intensities of Smad2/3/4 complex bands were normalized to those of total Smad2/3 and Smad4 of the corresponding treatment groups. The ratio of the Smad2/3/4 complex: total protein without exogenous TGF- β_1 was assigned a value of 1. **c** HepG2 cells were starved overnight in serum-free medium in the presence or absence of SP600125 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 5 h in each case; subsequently, they were each treated with exogenous TGF- β_1 (9 pM) for 1 h. Expression of Smad7 was monitored by IB by using anti-Smad7 antibody. Intensities of Smad7 bands were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the corresponding treatment groups. The ratio of Smad7: GAPDH without exogenous TGF- β_1 was assigned a value of 1. All the data presented is based on at least three independent experiments ($^{\#}P < 0.05$ compared with control group; $^*P < 0.05$ compared with TGF- β_1 group) ($n = 3$). **c** (C-terminal), L (Linker region)

increasing TGF- β_1 concentration may increase the risk of HCC, an observation that reinforces our previous report [9–11] that the extent of fibrosis and cirrhosis is directly associated with the progression of HCC because TGF- β_1 is a key factor in fibrosis. In this study, all of the MAPK-specific inhibitors except PD98059 suppressed TGF- β_1 -induced HepG2 cell proliferation (Fig. 2a), while all the three MAPK-specific inhibitors blocked cell invasion (Fig. 2b). In as much as the MAPK-specific inhibitors suppressed cell proliferation and invasion in HepG2 cells induced by TGF- β_1 coupled with the fact that proliferation and invasion are crucial for HCC progression and metastasis emphasizes the role of the MAPK pathway in HCC and in effect the therapeutic significance of the inhibition of MAPK-dependent linker phosphorylation of Smad3 by SB203580 and SP600125. To ascertain the underlying mechanism by which the MAPK-specific inhibitors suppressed HepG2 cell proliferation and invasion, we investigated the TGF- β /Smad signaling at the protein and mRNA levels.

Mechanistically, pSmad2/3 and Smad2/3/4 complex formation are indispensable for canonical TGF- β /Smad signaling in HCC. Importantly, TGF- β requires Smad2/3, Smad2/3/



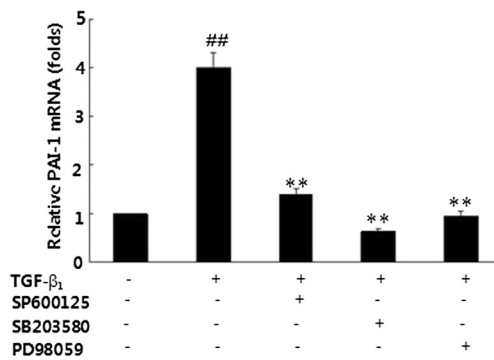


Fig. 4 Effects of SP600125, SB203580, and PD98059 on TGF- β_1 -induced repression of PAI-1 mRNA transcription in HepG2 cells. HepG2 cells were starved overnight in serum-free medium in the presence or absence of SP600125 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 5 h in each case; subsequently, they were each treated with TGF- β_1 (9 pM) for 3 h. Cellular RNA was collected to determine the changes in PAI-1 mRNA levels by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Results were expressed as fold increases of PAI-1 mRNA after normalization to β -actin. Each column represents mean \pm SD; $n=3$; ### $P<0.01$ compared with control group; ** $P<0.01$ compared with TGF- β_1 group

4 complex, and their nuclear translocation for the transcription of its target genes including PAI-1 mRNA, which mediate oncogenic roles of TGF- β in cancer. Quite noteworthy, SP600125, SB203580, and PD98059 in a selective but varied manner blocked TGF- β_1 -induced phosphorylation of pSmad2/3 at both C- and linker phospho-domains, thereby increasing the unlikely chance of pSmad2/3/4 complex formation in HepG2 cells. Our previous results showed increased positive staining of pSmad2L and oncogenic pSmad3L in hepatoma nodule areas than in adjacent normal liver tissues in rats treated with DEN, while positive staining of pSmad2C and tumor suppressor pSmad3C were increased in normal liver tissues than in adjacent hepatoma tissues areas [9–11]. In this study, SB203580 (a p38 MAPK-specific inhibitor) and SP600125 (a JNK-specific inhibitor) both blocked the phosphorylation of oncogenic pSmad3L but not tumor suppressor pSmad3C (Fig. 3a); perhaps, this may be peculiar to HepG2 cells, since they are hepatoma cell lines. Also, the above observation agrees with [18] who have shown that inhibition of c-jun NH₂-terminal kinase switches Smad3 signaling from tumor promotion to tumor suppression in DEN-induced HCC in rats. The consistency of these two independent observations emphasizes the possible therapeutic importance of Smad3L as a target for future investigations. Still with canonical TGF- β /Smad signaling, Smad7 through a negative feedback mechanism disrupts TGF- β_1 -receptor-specific phosphorylation of Smad2/3 through competitive blockade of transphosphorylation of Smad2/3 by TGF- β -type 1 receptor (T β R1) and also disrupts Smad2/3/4 complex formation and their nuclear translocation by recruiting ubiquitin ligases that induce proteasomal degradation [20]. This action of Smad7 opposes TGF- β /Smad signaling in cancer to reduce cancer

phenotypic hallmarks including cell proliferation and invasion. From our study, TGF- β_1 -induced down-regulation of Smad7 expression in HepG2 cells was restored by all the MAPK-specific inhibitors except in the case of PD98059 where it was comparatively minimal (Fig. 3c). Since the oncogenic role of TGF- β_1 in HCC is partly mediated through repression of Smad7 expression, it is quite noteworthy that SP600125 and SB203580 ameliorated HCC progression by enhancing the expression and inhibitory roles of Smad7 in HepG2 cells. Canonical TGF- β /Smad signaling is incomplete without expression of TGF- β -specific target genes; key among them is PAI-1 mRNA.

PAI-1 mRNA is one of the important target genes of TGF- β signaling, and severally [15, 18, 21] oncogenic pSmad3L-dependent signaling have been shown to up-regulate PAI-1 mRNA transcription, which has become a prerequisite for shifting hepatocytic TGF- β signaling from tumor suppression (pSmad3C/p21^{WAF1}) to tumor promotion (pSmad3L/c-myc) [22]. This action of oncogenic pSmad3L-dependent pathway eventually leads to up-regulation of PAI-1 mRNA transcription to promote HCC progression. In the present study, exogenous TGF- β_1 -stimulated HepG2 cells showed increased transcription of PAI-1 mRNA, but interestingly, the three MAPK-specific inhibitors repressed the TGF- β_1 -induced PAI-1 mRNA transcription (Fig. 4). Strangely, PD98059 unlike SP600125 and SB203580 failed to inhibit HepG2 cell proliferation, failed to block Smad2/3/4 complex and minimally restored Smad7 expression; nonetheless, it produced a significant repression of PAI-1 mRNA. It remains to be explained how PD98059 represses PAI-1 mRNA without affecting Smad2/3/4 complex formation. Indeed, this observation certainly needs further investigations. The MAPK-specific inhibitors may not only target PAI-1 mRNA but also other TGF- β target genes; therefore, further studies are needed to investigate other TGF- β -specific targets of the MAPK-specific inhibitors. For example, SP600125, SB203580, and PD98059 have been shown to disrupt TGF- β -dependent effects in cancer cells including abolishing beta1 integrin-induced resistance of HepG2, Huh7, and HepG3 cells to TGF- β -induced apoptosis [23, 24] and specifically, SB203580 was shown to have suppressed TGF- β -induced down-regulation of phosphatase tensin homologue (PTEN) in HCC [25]. These reports together with our present results provide a convincing rationale for further studies on the MAPK pathway.

Finally, we report that oncogenic Smad3L, Smad2/3/4 complex formation, and PAI-1 mRNA may be possible targets of the MAPK-specific inhibitors, specifically SB203580 and SP600125, while PD98059 represses PAI-1 mRNA possibly through an unknown mechanism. Moving forward, the mechanism underlying how PD98059 represses PAI-1 mRNA without necessarily affecting mediators of TGF- β /Smad signaling needs to be investigated and clarified.

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Conflicts of interest None

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