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Lentiviral-mediated Smad4 RNAi promotes SMMC-7721 cell migration by regulation of MMP-2, VEGF and MAPK signaling

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Abstract. Hepatocellular carcinoma (HCC) is a highly malignant cancer characterized by rapid progression, easy metastasis and frequent recurrence. Previous studies have shown that the Smad4 signaling pathway plays an important role in the cell growth and apoptosis of HCC. However, the effect of Smad4 signaling on the invasion and migration of HCC cells remains unclear. The present study aimed to examine the effects of the transforming growth factor (TGF)-β1-Smad4 signaling pathway on the migration of HCC cells. Lentiviral vectors expressing miRNA against Smad4 were constructed to block the expression of Smad4 in HCC cells, and transwell units were used to investigate the invasive potential of SMMC-7721 cells before and after TGF-β1 treatment. mRNA levels of matrix metalloproteinase (MMP)-2 and -9 were analyzed by reverse-transcription PCR, and concentrations of vascular endothelial growth factor (VEGF), p-JNK, p-p38 and p-Erk1/2 proteins were analyzed by Western blotting. The results indicate that TGF-β1 induced cellular invasion in the SMMC-7721 cells. These effects were almost completely blocked by the knockdown of Smad4. Reverse-transcription PCR and Western blot analysis revealed that MMP-2, VEGF, p-JNK and p-p38 were up-regulated by the silencing of Smad4, while the expression of MMP-9 and p-Erk1/2 was not affected by Smad4 silencing with or without TGF-β1 stimulation. These findings suggest that TGF-β1-induced SMMC-7721 cell invasion by the up-regulation of MMP-2 and VEGF is Smad4-dependent. The activation of MMP-2 and VEGF may be an important mechanism by which Smad4 is involved in metastasis. TGF-β1-Smad4 signaling may regulate SMMC-7721 cell migration through the activation of the MAPK pathway.

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Introduction

Hepatocellular carcinoma (HCC), the third leading cause of cancer-related death in the world and the second in China (1,2), is among the most prevalent and deadly cancers worldwide. The extremely poor prognosis of patients with HCC is largely due to the high rate of tumor recurrence and intrahepatic metastasis after surgical resection (3,4). The process of HCC cell metastasis has been shown to include multiple steps. In the progression of these steps, tumor cell invasion is necessary for migration to a distant organ to occur after the detachment of cells from the primary tumor. However, the mechanisms behind these steps remain unclear.

The transforming growth factor β (TGF-β) signal transduction pathway is crucial to many essential cellular processes, including cell growth, proliferation, differentiation, migration and apoptosis (5-7). It has been reported that TGF-β plays an important role in modifying the invasive and metastatic potential of malignant tumor cells (8,9). Several investigators have also reported that TGF-β may act as a strong mediator of HCC cell invasion (10).

Smad4 plays a critical role in the TGF-β signal transduction pathway (11). In models of pancreatic, breast and colon cancers, Smad4 was shown to activate the molecular mechanisms regulating tumor cell migration and invasion (12-14). However, there are only limited available data regarding whether TGF-β1-Smad4 is involved in the invasion and metastasis of HCC.

In our previous study, we reported that the expression of Smad4 was lower in HCC tissue than in adjacent tissues (15). We also validated that the down-regulation of Smad4 in HCC cells was associated with TGF-β1-induced cell growth inhibition and cellular apoptosis (16). The present study aimed to explore the effects and mechanisms of TGF-β1-Smad4 signaling on the migration of SMMC-7721 cells.

Materials and methods

Cell culture. The human HCC cell line SMMC-7721 (purchased from the Institute of Biochemistry and Cell

Research, China Life Science Academy, Shanghai, P.R. China) was cultured in DMEM supplemented with 10% fetal bovine serum (containing 100 U/ml of penicillin and streptomycin, respectively) at 37˚C in a humidified atmosphere of 5% CO_2. Exponentially growing cells were used for the experiment. The cells were treated with TGF-β1 (Sigma, MO, USA) at a concentration of 100 ng/ml (16).

Design and cloning of lentiviral shRNA vectors. The target siRNAs against human Smad4 (GenBank, gi: 34147555) for RNAi were designed as follows: siRNA1, 5'-GCACAAGGT TGGTTGCTAA-3'; siRNA2, 5'-CCAGCTACTTACCATC ATA-3'; siRNA3, 5'-GTACTTCATACCATGCCGA-3'; and the control sequence of RNAi, 5'-TTCTCCGAACGTGTCACGT-3'. siRNA3 was the published sequence designed by Jazag *et al* (17). In our previous study, we confirmed that siRNA3 was the optimal target siRNA of the above three sequences (18). The lentiviral vector system, purchased from Tronolab (http://tronolab.com/lentivectors.php), was also used. This vector system includes three plasmids: the pLVTHM vector, pCMV-dR8.74 and pMD2G. The targeting sequence was subcloned into the pLVTHM vector, which contained H1 promoter and green fluorescent protein (GFP). The resulting lentiviral vector containing Smad4 shRNA was named RNAi-Smad4, and its sequence was confirmed by PCR and sequencing. The lentiviral vector containing the negative control sequence of Smad4 shRNA (RNAi-NC), containing the non-silencing sequence, was used as the infection control for lentiviral vector production and cell infection. Lentiviral vectors were produced by transient transfection of HEK293 cells according to standard protocol as previously reported (16). The HEK293 cell line was supplemented with 10% fetal calf serum and, when subconfluent, transfected with 1,800 μl DNA solution: 20 μg LV-shSmad4, 15 μg pCMV-dR8.74 and 7.5 μg pMD2G. All virus stocks were produced by calcium phosphate-mediated transfection. After 48 h of post-transfection, cell supernatants containing viral particles were filtered using the 0.45-μm Steriflip vacuum filtration system (Millipore) and concentrated by ultracentrifugation at 25,000 rpm at 4˚C. The titer of the virus was tested according to the expression level of GFP. The day before infection, the SMMC-7721 cells were seeded on dishes with a conflluence of 30-40%. On the day of infection, the SMMC-721 cells were infected by packaged lentiviral production. Parallel non-infected SMMC-721 cells were observed simultaneously. Cells were cultured in normal growth medium for 72 h after infection. RNAi-NC as infection control was performed as described above.

In vitro invasion Matrigel assays. The invasive ability of the SMMC-7721 cells was determined using a transwell unit (Corning, USA) with polycarbonate filters (Neuro Probe, USA). The upper part of the filter was coated with Matrigel (Boyden chamber), and the lower compartments were filled with SMMC-7721 cell culture supernatants (control, RNAi-NC and RNAi-Smad4, respectively). The cells were incubated at 37˚C in 5% CO_2 for 36 h, fixed with paraformaldehyde and stained with hexamethylpararosaniline. The invasive phenotype was assessed by counting the cells that had migrated to the lower part of the filter using light microscopy at a magnification of x200. Five fields were counted for each filter, and each sample was assayed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. For RT-PCR analysis of MMP-2 and MMP-9 mRNA levels, total RNA was isolated from cells using TRIzol (Invitrogen, USA), and cDNA was synthesized using an RT-PCR kit (Santa Cruz, USA) according to the manufacturer's instructions. The sequences of the primers were as follows: forward primer 5'-TACAACTTCTTCCCTCGCAAGC-3' and reverse primer 5'-GTCATCAAAATGGGAGTCTCCC-3' for MMP-2; forward primer 5'-CGCCCATTTCGACGATGACGAG-3' and reverse primer 5'-GCGGAGTAGGATTGGCCTTGGA-3' for MMP-9;forwardprimer5'-TGGGGAAGGTGAAGGTCGGA-3' and reverse primer 5'-GGGATCTCGCTGCTCGAAGA-3' for GAPDH. PCR analysis was performed under the following conditions: denaturation at 94˚C for 10 min, and then 30 cycles of denaturation for 1 min at 94˚C, annealing for 1 min at 60˚C (for MMP-2) or 58˚C (for MMP-9), and polymerization for 1 min at 72˚C followed by 7 min at 72˚C. The amplified products were analyzed by 1.0% agarose gel electrophoresis, followed by ethidium bromide staining.

Western blot analysis. Samples of cell extracts were homogenized in SDS-PAGE sample buffer containing 2% SDS (19). After being heated at 80˚C for 5 min and clarified by spinning in a microcentrifuge at 12,000 rpm at room temperature for 5 min, the total protein extracts were resolved on polyacrylamide gel using the Laemmli buffer system. The resulting gels were stained with Coomassie Blue R-250 to reveal the resolved protein bands. Gels were electrically transferred to nitrocellulose membranes. After blocking in TBS containing 5% non-fat milk, the membranes were incubated with rabbit polyclonal antibodies against Smad4 (Upstate) (1:1000 in TBS containing 5% non-fat milk), p38 (Cell Signaling) (1:1000), Erk1/2 (Cell Signaling) (1:1000), JNK (Abcam) (1:1000), vascular endothelial growth factor (VEGF) (Abcam) (1:1000) and β-actin (Sigma) (1:2000). The membranes were then washed with TBS containing 0.05% Tween-20 and incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma) (1:2000) and washed again. The blots were developed in ECL substrate solution (Sigma) to reveal the immunoreactive bands. Protein expression was analyzed by Bandscan software and normalized by the quantity of β-actin in the same membrane.

Statistical analysis. Statistical analysis was performed using SPSS 11.0. Data were calculated as the mean ± SD. One-way analysis of variance (ANOVA) followed by the LSD-test was used to assess significant differences among groups. The two independent samples test was used to assess the differences between two groups. Each assay was performed at least three times. P-values <0.05 were considered statistically significant.

Results

Inhibition of Smad4 expression by RNA interference. As in our previous study (18), in the present study siRNAs against human Smad4 were identified and effective siRNA target sites were validated. Smad4 in SMMC-7721 cells was knocked

Table I. Results of the transwell assay.

Group	Migrated cells	
	TGF-β1(-)	TGF-β1(+)
Control	19.2±2.34	23.4±4.31[a]
RNAi-NC	18.1±3.14	22.6±2.42[a]
RNAi-Smad4	23.8±2.01[b]	23.2±2.35

SMMC-7721 cells were infected with RNAi-NC or RNAi-Smad4 for 24 h, and then incubated in the presence or absence of 100 ng/ml TGF-β1 for 36 h. Cellular invasion was assayed by transwell units. Significance between groups with or without TGF-β1 stimulation: 23.4±4.31 vs. 19.2±2.34 ([a]P<0.01); 22.6±2.42 vs. 18.1±3.14 ([a]P<0.01, t-test). Significance among TGF-β1(-) groups, [b]P<0.05 (LSD-test). The results represent the average of three individual experiments.

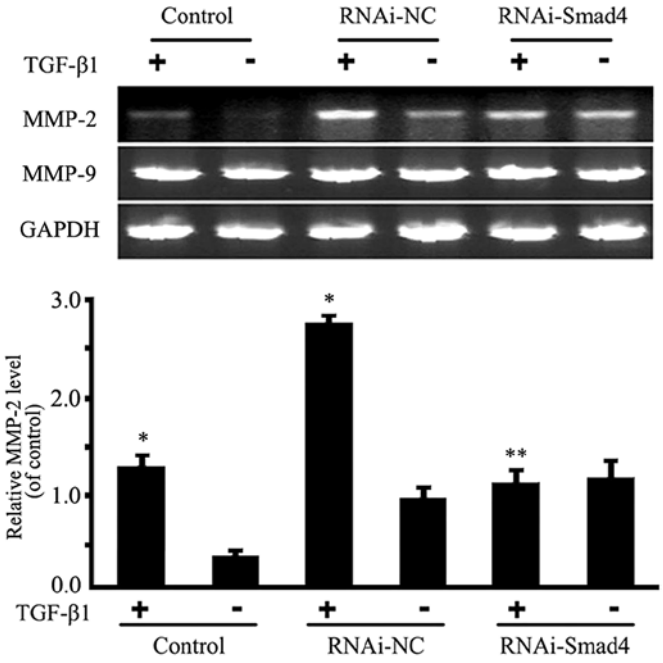

Figure 1. Regulation of MMP-2 and MMP-9 by the TGF-β1-Smad4 signaling pathway in SMMC-7721 cells. SMMC-7721 cells were infected with RNAi-NC or RNAi-Smad4 for 24 h. The cells were then treated or not with 100 ng/ml TGF-β1 for 36 h. Expression of MMP-2 and -9 was measured by RT-PCR analysis. The level of MMP-2 was related to that of the corresponding control cells without TGF-β1 stimulation. Data are the average of three independent experiments. *P<0.05 vs. the related group untreated by TGF-β1. **P<0.05 vs. RNAi-NC-infected cells with TGF-β1(+) or TGF-β1(-) stimulation.

down by lentiviral-mediated RNAi. The infection efficiency of the lentiviral RNAi vectors of Smad4 (RNAi-Smad4) and the negative control (RNAi-NC) in SMMC-7721 cells was >85%. Smad4 was significantly knocked down by infection with RNAi-Smad4.

Lentiviral-mediated Smad4 RNAi-induced invasion of SMMC-7721 cells. Invasive activity was assessed using Matrigel-coated transwell chambers. The cell invasion of SMMC-7721 cells was measured and displayed with a 36-h TGF-β1 stimulation.

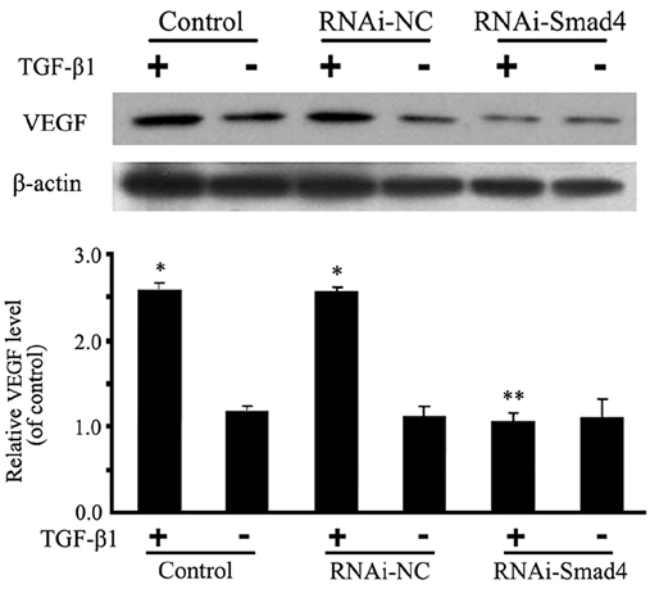

Figure 2. Regulation of VEGF by the TGF-β1-Smad4 signaling pathway in SMMC-7721 cells. SMMC-7721 cells were infected with RNAi-NC or RNAi-Smad4 for 24 h. The cells were then treated or not with 100 ng/ml TGF-β1 for 36 h. Expression of VEGF was measured by Western blot analysis. The level of VEGF was related to that of the corresponding control cells without TGF-β1 stimulation. Data are the average of three independent experiments. *P<0.05 vs. the related group untreated by TGF-β1. **P<0.05 vs. RNAi-NC-infected cells with TGF-β1(+) or TGF-β1(-) stimulation.

As shown in Table I, TGF-β1 had a significant promotive effect on SMMC-7721 cells (without transfection) and on cells transfected with RNAi-NC. However, no significant difference was found between RNAi-Smad4-transfected cells with (23.2±2.35) and without (23.8±2.01) TGF-β1 stimulation (P<0.05). The migrated cell numbers were higher in SMMC-7721 cells transfected with lentiviral-mediated Smad4 RNAi (23.8±2.01) than in the control or RNAi-NC groups (P<0.05)

Effect of lentiviral-mediated Smad4 RNAi on the expression of MMP-2 and MMP-9. To explore the possible mechanisms behind Smad4 regulation of the TGF-β1-induced invasion of SMMC-7721 cells, the expression levels of MMP-2 and -9 were investigated in SMMC-7721 cells transfected with miRNA against Smad4. Notably, compared with the controls, a significantly increased expression of MMP-2 was detected in SMMC-7721 cells treated with TGF-β1 (which served as a positive control) (P<0.05) (Fig. 1). However, no significant alteration in MMP-2 levels was observed between RNAi-NC and RNAi-Smad4, suggesting that the effects of the down-regulation of TGF-β1 on the invasion of SMMC-7721 cells were not related to the activation of Smad4 response genes. As shown in Fig. 1, unlike MMP-2, the expression of MMP-9 in each group was not regulated by the silencing of Smad4 or TGF-β1 stimulation. These results indicate that MMP-9 is not associated with TGF-β1-induced invasion in SMMC-7721 cells.

Effect of lentiviral-mediated Smad4 RNAi on the expression of VEGF. As shown in Fig. 2, there was no significant difference in the expression of VEGF among the three groups not stimulated by TGF-β1. The VEGF protein was highly expressed in TGF-β1-treated control and RNAi-NC cells. As for the cells

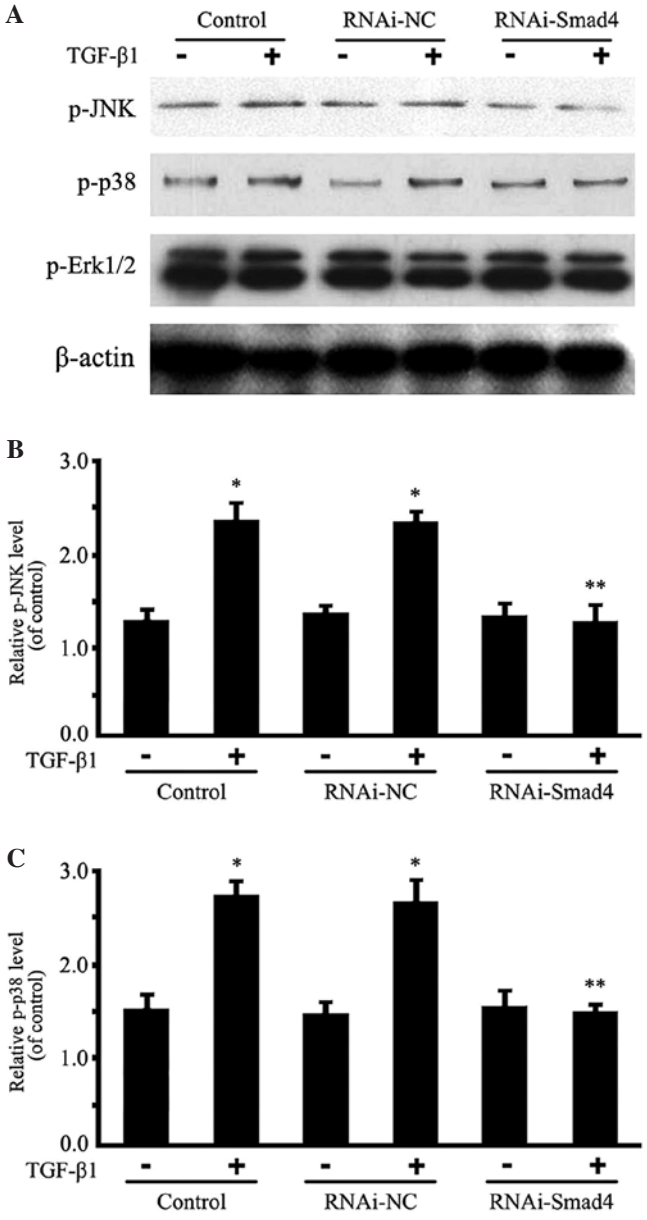

Figure 3. Regulation of p-JNK, p-p38 and p-Erk1/2 by the TGF-β1-Smad4 signaling pathway in SMMC-7721 cells. SMMC-7721 cells were infected with RNAi-NC or RNAi-Smad4 for 24 h. The cells were then treated or not with 100 ng/ml TGF-β1 for 36 h. Expression of p-JNK, p-p38 and p-Erk1/2 was measured by Western blot analysis. The levels of p-JNK and p-p38 were related to those of the control cells without TGF-β1 stimulation. The expression of p-Erk1/2 was not regulated by Smad4 silencing in cells with or without TGF-β1 stimulation. Data are the average of three individual experiments. *P<0.05 vs. the related group untreated by TGF-β1. **P<0.05 vs. RNAi-NC-infected cells with TGF-β1(+) or TGF-β1(-) stimulation.

transfected with RNAi-Smad4, VEGF expression was not affected significantly by TGF-β1 stimulation, indicating that the up-regulating effect of TGF-β1 on VEGF expression was blocked by Smad4 silencing.

Effect of lentiviral-mediated Smad4 RNAi on the expression of p-JNK, p-p38 and p-Erk1/2. As shown in Fig. 3, in TGF-β1-stimulated cells, a clearly enhanced expression of p-JNK was detected using Western blot analysis including control cells and RNAi-NC-transfected cells. Neither p-JNK nor p-p38 were regulated by TGF-β1-induced RNAi-Smad4. Unlike p-JNK and p-p38, the expression of p-Erk1/2 was not regulated by Smad4 silencing in cells with or without TGF-β1 stimulation.

Discussion

The data indicate that the down-regulation of Smad4 induced by RNAi leads to an obvious increase in the migration ability of SMMC-7721 cells. This implies that Smad4 expression is critical for the tumor invasion and metastasis of human HCC, in addition to playing an important role in proliferation and growth. This is consistent with previous reports (20,21), and provides further evidence to suggest that Smad4 plays a key role in HCC. The present study investigated the effects of Smad4 signaling on the migration of HCC cells, and the mechanisms behind these effects.

Tumor cells and their neighbor interstitial cells may produce a great quantity of proteinase for the degradation of the basement membrane and the stromal extracellular matrix (ECM), and aid in tumor cell migration (22,23). Among these proteinases, the MMPs are important. MMPs can degrade the stroma collage, which favors the action of tumor cells, leading them to shake off the yoke of ECM and migrate (24,25). It is known that MMP-2 and/or -9 levels are associated with the invasive phenotype of tumor cells (26). To further explore the possible mechanisms involved in the TGF-β1-Smad4-induced invasion and metastasis of HCC, we examined the effects of Smad4 on the expression and activity of MMP-2 and -9 in SMMC-7721 cells. MMP-2 was found to be significantly enhanced in RNAi-NC and control HCC cells, and the level of MMP-2 was increased after treatment with TGF-β1. No significant alteration in the level of MMP-2 was observed after Smad4 silencing. However, there was no significant change in MMP-9 in any of the groups. These data suggest that MMP-2 rather than MMP-9 is one of the key molecules involved in the effect of TGF-β1 on invasion and metastasis in HCC, and that these effects are not related to Smad4.

It is known that the formation of new blood vessels plays an important role in the growth and metastasis of solid tumors, and that various growth factors secreted from tumor cells determine the pace of the progression (27). VEGF is found up-regulated in most solid tumors, whereas there is little or no VEGF in normal tissues. Its expression level is positively related to the microvessel density, invasion and metastasis of tumors, and to patient prognosis (28,29). The present results show that VEGF was activated in SMMC-7721 cells by TGF-β1 stimulation, and that this activation was inhibited by lentiviral-mediated Smad4 silencing. These findings suggest that VEGF regulated by Smad4 may mediate the invasion of SMMC-7721 cells induced by TGF-β1.

The mitogen-activated protein kinase (MAPK) pathway plays an integral role in coordinating growth, survival and signaling, which may in turn play an important role in tumor development and progression. Increasing evidence suggests that this pathway is abnormally regulated in HCC and plays a central role in tumorigenesis and the maintenance of tumor growth (30-32). In addition, targeted inhibition of this pathway has been regarded as an alternative approach for the treatment of HCC (33,34). Our results indicate that TGF-β1 significantly enhances the activation of the MAPK pathway, as evidenced

by the phosphorylation of downstream p38 and JNK. Thus, TGF-β1 may affect the development and progression of SMMC-7721 cells by regulating the MAPK pathway.

Taken together, our data suggest that TGF-β1-Smad4 signaling plays an important role in HCC tumor invasion and growth. The activation of MMP-2 and VEGF may be an important mechanism by which Smad4 is involved in SMMC-7721 cell metastasis, and TGF-β1-Smad4 signaling may regulate SMMC-7721 cells by activating the MAPK pathway. In conclusion, the present study sheds light on the role of Smad4 in HCC metastasis, and suggests that Smad4 has an anti-metastatic effect *in vitro*.

Acknowledgements

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