

Effect of TGF- β_1 Stimulation on the Smad Signal Transduction Pathway of Human Peritoneal Mesothelial Cells

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ABSTRACT

Objective: To prove whether the SMAD signal transduction pathway in human peritoneal mesothelial cells (HPMCs) influenced the process of human peritoneal fibrosis stimulated by TGF- β_1 .

Methods: HPMC were isolated from normal human omentum and the third generation cells were stimulated by 5 ng/ml TGF- β_1 . Immunohistochemistry, Western blotting, ELISA and RT-PCR were employed to investigate the protein expression of p-Smad2/3 and the protein and mRNA expressions of SMAD 7, fibronectin (FN) and collenge-I collagen-I (COL1)

Results: The protein expression of p-Smad2/3 in HPMC was remarkably increased 15 min (29% p-Smad2/3-positive cells) after TGF- β_1 stimulation, peaking from 30 min (81%) to 1 h (84%) and dropping after 2 h (37%); Meanwhile, p-Smad2/3 mainly distributed in cytoplasm at 15 min, concentrated in cell nucleus and peri-nucleus from 30 min to 1 h, and distributed in cytoplasm again at 2 h. The protein expression of SMAD7 in cells was obviously increased 24 h after TGF- β_1 stimulation, peaking at 48 h. The mRNA expression of SMAD7 was time-dependently increased. The expressions of extracellular FN protein, intracellular FN mRNA, as well as intracellular COL1 protein and mRNA were significantly increased and all of them displayed time dependency.

Conclusions: The SMAD signal transduction pathway of HPMC can be specifically activated by TGF- β_1 and influence the process of human peritoneal fibrosis. The protein and mRNA expression of SMAD 7 (an inhibitor of SMAD pathway) are significantly increased as a result of feedback.

Keywords: Mesothelial cells; TGF- β_1 ; Smad2/3; Smad7; Extracellular matrix

Ultrafiltration failure caused by peritoneal fibrosis is the major cause for patients with continuous ambulatory peritoneal dialysis (CAPD) to give up peritoneal dialysis therapy. The increase in endogenous TGF- β_1 induced by a variety of intrinsic or extrinsic factors results in excessive accumulation of extracellular matrix around HPMC, which contributes to the formation of peritoneal fibrosis^{1,2}. Smad signaling pathway was found recently to be an important TGF- β_1 downstream signaling pathway, which was closely related to fibrosis in many tissues such as cardiac muscle, liver, lung and kidney^{3,4}. Does TGF- β_1 dependent Smad signaling pathway exert its effect in the process of peritoneal

fibrosis? However, no definite answers are available as yet. Therefore, it is necessary for us to elucidate the underlying mechanisms for the development of peritoneal fibrosis, and further to find safe and effective methods to elongate dialysis time and improve life quality of patients with peritoneal dialysis.

MATERIALS AND METHODS

Reagents

Goat anti-human phosphorylated Smad2/3 (P-Smad2/3) polyclonal antibody and goat anti-human Smad7 polyclonal antibody were purchased from Santa Cruz Company, USA; mouse anti-

human CTGF(connective tissue growth factor) monoclonal antibody was purchased from R&D Company, USA; mouse anti-human α -SMA monoclonal antibody, mouse anti-human COL1 (human collagen type 1) and pure TGF- β 1 were purchased from Sigma Company,USA. ELISA kits for Plasminogen Activator Inhibitor type 1 (PAI-1) and fibronectin (FN) were purchased from Shanghai Sun Biotechnology Company. Immunohistochemistry staining kits were purchased from Beijing Zhongshan Biotechnology Co. Ltd.

Isolation, culture and identification of HPMCs⁵

Dissection of greater omentum from healthy male adults with removal of blood vessel and lipid tissue was performed in sterile room where tissue was dissected into 1 \times 1cm² pieces, washed twice with Phosphate Buffered Saline (PBS) and F12 respectively. **After the tissue pieces were transferred** into sterile centrifuge tube containing 20ml 0.125% pancreatin and 0.01% EDTA, and the cultured cells were incubated on rocking bed at 250 rpm at 37°C for 15min. The digestion was stopped by addition of 3ml 15% Fetal Calf Serum (FCS)-F12 and the samples centrifugated at 1000rpm at 40C for 10min. The pellet was blown once with 5ml F12, centrifugated at 1000rpm at 4°C for 5min and after removal of supernatant, 5ml of complete medium (15%FCS-F12, 0.5 μ g/ml insulin, 1 μ g/ml hydrocortisone, 5 μ g/ml transferrin, 2 μ mol/ml glutamine) was added to the cells. The cell suspension was transferred into 25cm² culture flask coated with 0.1% gelatin and containing culture media? And supplemented with 100U/ml penicillin and 100U/ml streptomycin. The culture flasks were placed into incubator at 37°C, 5% CO₂ for cell culturing. Cell culture media was refreshed every three days. When the cultured cells were grown into confluence, the cells were detached with 0.125% pancreatin-0.01% EDTA and plated. The third passage of the cells was used in experiments. 95% of the cells of the third passage were found to possess characteristics of mesothelial cells by inverted microscopy, transmission electron microscopy and scanning electron microscopy.

Cytokeratin and VIII factor immunohistochemistry staining.

Immunohistochemistry analysis of intracellular Smad2/3 phosphorylation

The confluent cells of the second passage were adjusted to the concentration of 1 \times 10⁵/ml, and plated on cover glass, which was placed in 24-well culture plate. After cells reached confluence, 0.1% FCS-F12 was added, synchronized for 24h, and after they entered the resting stage the cells were divided into 0, 15min, 30min, 1h, 2h and incubated with 5ng/ml TGF- β 1. After specified periods of time the supernatant was removed and the cover glass was taken out and fixed with pure acetone. SP method was used in p-Smad2/3 immunohistochemical staining (1:100). In control samples PBS was added instead of primary antibody. The translocation of p-Smad2/3 from membrane to nucleus was observed. Positive rate was calculated by counting the positive cells in 1000 cells selected randomly in each slice, and four slices were counted for each stage to get the mean value.

Western blot assay of intracellular Smad7, CTGF, α -SMA, COL1 protein expression

The confluent cells of the second passage were adjusted to the concentration of 1 \times 10⁵/ml, plated into 25cm² culture flask. After reaching confluence, the cells were cultured in 0.1% FCS-F12 and synchronized for 24h. After reaching the resting stage, the cells were divided into 0, 15min, 30min, 1h and 2h group and incubated with 5ng/ml TGF- β 1 for specified time, for p-Smad2/3 measurements. After removal of supernatant, The cells were washed twice with PBS, and lysed with protein lysis buffer at 40C for 60min. centrifugated at 12,000rpm for 15min and the resulting supernatant preserved at -70°C. 50ug total protein was electrophoresed on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked with blocking buffer containing 3% bovine serum albumin (BSA) and 5% skim milk powder at room temperature, incubated

with anti p-Smad2/3 phosphorylated polyclonal antibody, Smad7 antibody and COL1 antibody, and the result of immune reaction was detected with ECL agent.

ELISA assay of FN level

The confluent cells of the second passage were adjusted to the concentration of 1×10^5 /ml, and plated into 24-well culture plate for cell culturing as described above. After reaching confluence, 0.1% FCS-F12 was added, the cells were synchronized for 24h and after entering resting stage divided into 0, 24h, 48h, 72h groups and incubated with 5ng/ml TGF- β 1 for specified periods of time. The supernatants were collected and preserved at -20°C . FN in supernatant content was determined according to the manufacturers' instruction (Shanghai Sun Biotechnology Inc.).

Detection of Smad7, FN and COL1 intracellular gene expression by RT-PCR

The confluent cells of the second passage were adjusted to the concentration of 1×10^5 /ml, divided into 25cm² culture flask for cell culture. After reaching confluence, 0.1% FCS-F12 was added and the cells were synchronized for 24h. After entering the resting stage the cells were divided into 0, 24h, 48h, 72h groups and incubated with 5ng/ml TGF-

β 1 for specified periods of time. The total RNA was extracted according to the method described in instruction for Trizol reagent (GibcoBRL Inc., USA). RNA OD values were determined by ultraviolet spectrophotometer and wavelength pair of 260/280 nm. OD values ranged from 1.7 to 2.0. Three ribosome RNA strips of 28s, 18s and 5s were clearly recognized on 1.2% agarose gel electrophoresis, suggesting that no pollution and degradation took place in total RNA isolation. The first cDNA was synthesized by reverse transcription according to the instruction for reverse transcription kit (Promega Inc.), which was then used as a template for PCR reaction. Primer sequences (synthesized by Shanghai Boya Biotechnology Co. Ltd.) are shown in Table 1. The reaction condition: pre-denaturation at 95 $^\circ\text{C}$ for 5min, denaturation at 95 $^\circ\text{C}$ for 30s, annealing at 56 $^\circ\text{C}$ for 30s, elongation at 72 $^\circ\text{C}$ for 50s, followed by 21 cycles for FN and COL1, and 27 cycles for smad7, elongation at 72 $^\circ\text{C}$ for 10min, terminated 40 $^\circ\text{C}$. The experiments using the cycles did not yet reach the plateau phase of PCR. PCR products were isolated by 6% polyacrylamide gel electrophoresis. Gel scanning and analysis was performed on Gene Genius image analyzer (Syngene Inc). The ratio of optical density of objective fragment to that of GAPDH fragment acting as control was used in semi-quantification comparison.

Table 1 primer sequence

Primer	Sequence	Length of PCR products(bp)
Smad7-F	5'-AGC AGG CCA CAC TTC AAA CT-3'	375
-R	5'-CAC GTT GTC TCC CCA TCT G-3'	
FN -F	5'-AGC CGC CAC GTG CCA GGA TTA C-3'	439
-R	5'-CTT ATG GGG GTG GCC GTT GTG G-3'	
COL1-F	5'-AGG CTG GTG TGA TGG GAT T-3'	544
-R	5'-GGA GAG CCA TCA GCA CCT TT-3'	
GAPDH -F	5'-CAT GGG TGT GAA CCA TGA GA-3'	695
-R	5'-ACT GAG TGT GGC AGG GAC TC-3'	

Statistical Analysis

Experimental results were expressed as mean \pm standard deviation ($\bar{x} \pm SD$). One-way ANOVA or T test were used for statistical analysis using SPSS11.0 statistical software package and least significant difference method to analyze the means further.

RESULTS

Effect of TGF- β_1 on intracellular

The results of immunochemical (Fig.1) were accord with Western blotting's (Fig.2). The HPMCs hardly expressed p-Smad2/3 protein in

the control group and the cells stained lightly (the positive rate: $3\% \pm 1.7\%$). After TGF- β_1 stimulation of HPMCs Smad2/3 was significantly activated at 15min (the positive rate: $29\% \pm 5.8\%$), and staining was distributed in cytoplasm; its activity peaked between 30min and 1h (the positive rate: $81\% \pm 5.0\%$ and $84\% \pm 3.7\%$ respectively). At that time the stain was deepened and distributed in nucleus or peri-nucleus. The phosphorylation activity was obviously reduced at 2h (the positive rate: $37\% \pm 5.7\%$), and staining was lightened and distributed in cytoplasm again. Compared with the control, $P < 0.01$

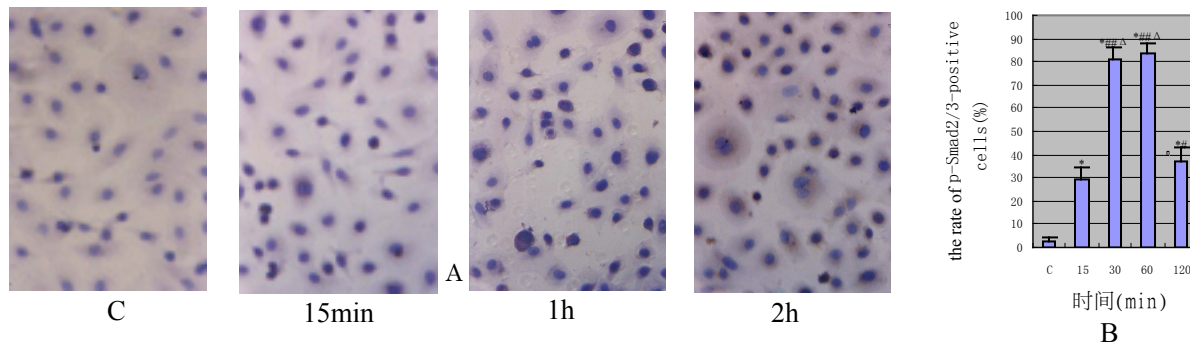


Fig.1 A: the protein expression of p-Smad2/3 displayed by immunohistochemistry (400); B: TGF- β_1 -induced the rate of p-Smad2/3-positive cells (* $P < 0.01$ vs C group; # $P < 0.05$, ## $P < 0.01$ vs 15 min group; $\Delta P < 0.01$ vs 120 min)

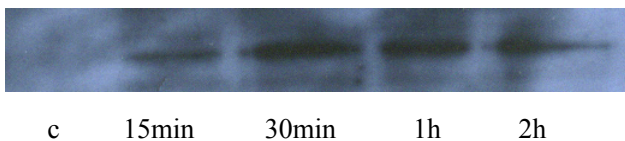


Fig.2 the protein expression of p-Smad2/3 detected by western blotting

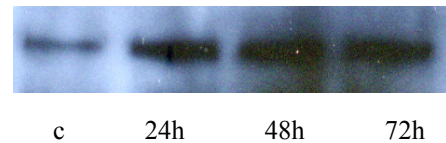


Fig.3 Western blot Aanalysis of Smad7

TGF- β_1 effect on Smad7 protein and gene expression in HPMC

The results of Western blot analysis (Fig.3): the expression of Smad7 in HPMCs was expressed at a low level in control group, and increased at 24h, peaked at 48h after TGF- β_1 stimulation of HPMC, and then decreased at 72h.

The results of semi-quantification RT-PCR (Fig.4) of TGF- β_1 stimulated HPMC have shown that

mRNA expression of Smad7 was increased compared with the control (p value < 0.05 at 24h, < 0.01 at 48h and 72h) in a time-dependent manner.

TGF- β_1 effect on FN, COL1 protein and gene expression in HPMC

The results of Western blot analysis (Fig.5) have shown that COL1 showed very limited expression in the control, increased at 24h in a

time-dependent manner after TGF- β_1 stimulation of the cells. ELISA assay showed FN protein expression of supernatants at 24h, 48h, 72h were 2.79 ± 0.44 mg/L, 3.11 ± 0.48 mg/L, 3.55 ± 0.52 mg/L respectively and the results were in a time-dependent manner compared with the control

(0.67 ± 0.07 mg/L and $p < 0.01$). The results of semi-quantification RT-PCR of TGF- β_1 stimulated HPMC (Fig.6,7) have shown that mRNA expression of FN and COL1 was increased at 24 h (p value < 0.01) compared with the control, also in a time-dependent manner.

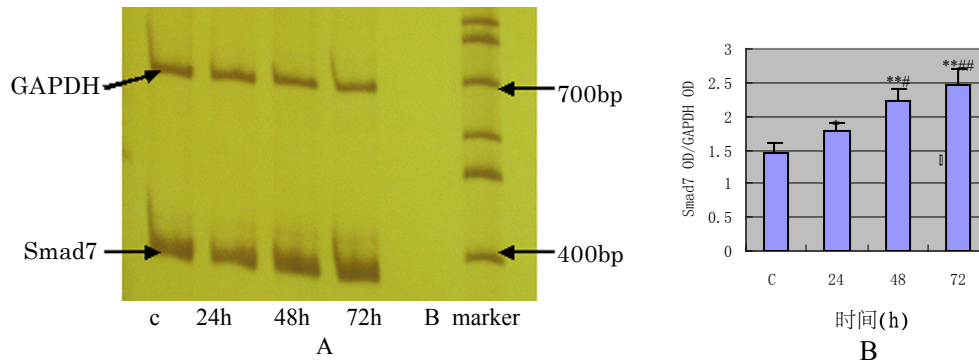


Fig.4 A: the mRNA expression of Smad7 detected by RT-PCR, B: the semi-quantification result of Smad7 OD/GAPDH OD (* $P < 0.05$, ** $P < 0.01$ vs C group; # $P < 0.05$, ## $P < 0.01$ vs 24 h group)

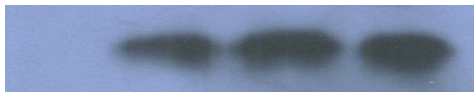


Fig.5 the protein expression of COL1 detected by western blotting

DISCUSSION

TGF- β_1 has been well known as one of the most important trigger factor in tissue fibrosis. Does TGF- β_1 have the same effect on the peritoneal fibrosis? In this study, when the third passage of HPMCs cells from its primary cultures were stimulated with TGF- β_1 , the subsequently obtained Western blot assay showed that the production of FN and COL1 proteins significantly increased in a time-dependent manner comparing to the control. The results of ELISA indicated that FN and COL1 protein levels in supernatants obtained from TGF- β_1 -stimulated cells were significantly elevated after 24 h of stimulation. This effect was time-dependent

and compared to the control group significantly elevated at the $p < 0.01$. This data are similar to the amplification data of RT-PCR for FN and COL1 mRNA. The up-regulation of FN and COL1, the major components of extracellular matrix, demonstrates that ECM components deposit and because of excessive accumulation result in peritoneal fibrosis. Therefore, our experiments have demonstrated that TGF- β_1 plays an important trigger role in peritoneal fibrosis.

There have been many researches that have attempted to block the expression or activity of TGF- β_1 from different aspects, in an effort to reduce the accumulation of extracellular matrix and prevent tissue fibrosis. However, TGF- β_1 which is one of the growth factors with the most complicated function that have been found so far has a wide range of biological activity on development, proliferation, differentiation, apoptosis and immune function of a variety of cells, TGF- β_1 has also many side effects such as loss of cell growth control, immune disturbance, severe inflammatory and even death that

will unavoidably occur if expression or activity of TGF- β_1 is to be inhibited as in treatment of tissue fibrosis. As a result, its application in clinical practice is limited^[6]. Recently, with the advances in TGF- β_1 downstream signaling pathway research,

the regulatory mechanism of TGF- β_1 in tissue fibrosis is gradually being recognized, and inhibition or blockade in TGF- β_1 downstream signaling pathway and its effective mediators have become the focus of research in prevention of tissue fibrosis.

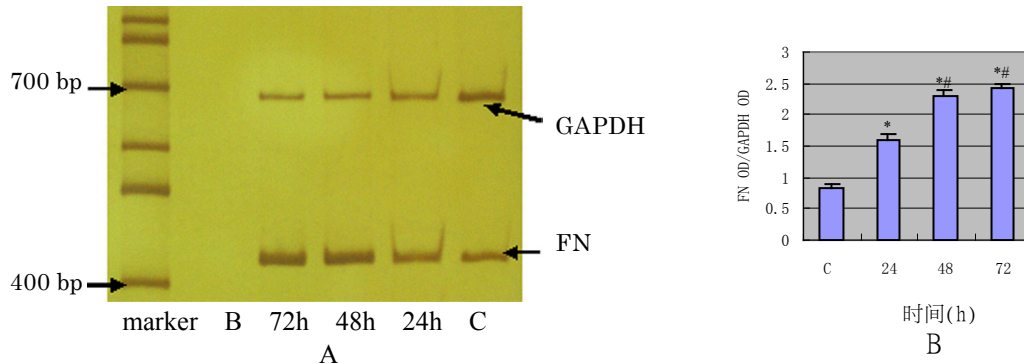


Fig.6 A: the mRNA expression of FN detected by RT-PCR; B: the semi-quantification result of FN OD/GAPDH OD (* P <0.01 vs C group; # P <0.01 vs 24 h group)

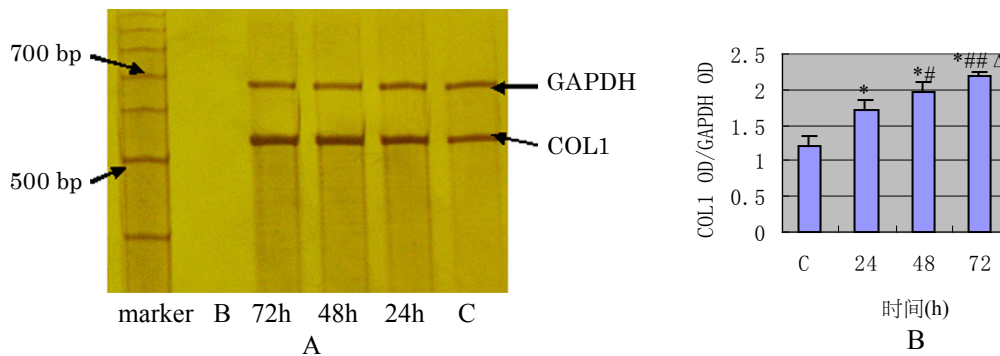


Fig.7 A: the mRNA expression of FN detected by RT-PCR; B: the semi-quantification result of COL1 OD/GAPDH OD (* P <0.01 vs C group; # P <0.05, ## P <0.01 vs 24 h group; Δ P <0.05 vs 48 h group)

Smad signaling pathway plays a very important role in TGF- β -induced tissue fibrosis: (1) SB-431542, a specific blocker for Smad signaling pathway, is able to suppress the TGF- β_1 induced synthesis of extracellular matrix FN and COL1A1⁷, (2) Smad heterogenous oligomer binding site (SBE) exists in promoters of many genes such as FN, COL1, COL3, COL6, COL7 and COL13, TGF- β can induce extracellular matrix synthesis through Smad signaling pathway^{3, 4}. Moreover, SBE structure is also found in promoter of other genes including CTGF

⁸, TIMP-1³, α -SMA⁹, PAI-1¹⁰ and Smad7¹⁰ which are closely related to tissue fibrosis, (3) During the course of interaction with other signaling pathway, Smad pathway is of great importance because other signaling pathways as it either intervenes with the transmission of Smad pathway or cooperate with Smad heterogenous oligomer to regulate target gene expression through its specifically activated transcriptional factor¹¹, (4) although little is known about whether specific blockade of Smad signaling pathway can reduce the side effects caused by

direct blockade of TGF- β . Recent studies suggest that selective blockade of Smad signaling pathway and p38MAPK pathway by small protein SB-505124 can not only inhibit the synthesis of extracellular matrix but also prevent the cell death induced by direct blockade of TGF- β .¹²

Does TGF- β 1 specially suppress Smad signaling pathway in HPMCs? Smad2/3 is the receptor-regulated Smads and its activation by phosphorylation is the most important step in the Smad signaling pathway. Therefore, the expression of p-Smad2/3 implicates the extent to which Smad signaling pathway is activated.¹³ In our study, it was proven by both immunohistochemical staining and Western blot assay that the expression of p-Smad2/3 was increased at 15min after TGF- β 1 stimulation, peaking at 1h and simultaneously, the increased p-Smad2/3 translocated from membrane to cytoplasm then bounded to Smad4 and in such form entered into nucleus where it exerted the effect of inducing target genes transcription. Our results confirmed that TGF- β 1 could specifically activate Smad signaling pathway in HPMCs. It was also found in our experiment that the activation of Smad signaling pathway by TGF- β 1 in HPMCs could persist for long time (at least 2h) and peak at a later timethen the time observed in renal tubular epithelial cell¹³ Smad7, an inhibitory Smads, negatively regulate Smad signaling pathway.^{3,4} Based on the inhibitory effect of Smad7 on Smad signaling pathway, studies have been carried out to prevent the tissue fibrosis –induced by TGF- β 1 using Smad7 gene transfection. For example, transfected Smad 7 gene into normal rat kidney TEC line (NRK52E cells) and found that overexpression of Smad7 resulted in marked inhibition of TGF- 1 induced Smad2 activation with the prevention of collagen synthesis.¹³ Similarly, the data of Torada et al indicated that Smad7 gene transfer via adenovirus electroporation prevents unilateral ureteral obstruction(UUO)-induced renal fibrosis.¹⁴ In this study, the Western blot analysis showed that Smad7 protein remained at a low level in the absence of TGF- β 1 but that it was up-regulated remarkably 24 h after the stimulation with TGF- β 1, peaking at 48 h

and persisted for 72h. The result of RT-PCR showed that the level of Smad7 mRNA was increased in a time-dependent manner with p value less than 0.05 at 24 h and less than 0.01 at both 48 and 72 h. These data demonstrate that a definite level of Smad7 protein is produced by normal HPMCs and that it act mainly by keeping the Smad signaling pathway from activation. Once TGF- β 1 signaling is initiated, the expression of Smad7 gene is rapidly up regulated in a feedback manner. However, the feedback is so weak that the up-regulation of Smad7 cannot offset the TGF- β 1-incuded synthesis of FN and COL1. In our studies, the difference between Smad7 protein production level and mRNA expression level may be associated with the degradation of Smad7 protein by obiquitination of activated molecules in Smad signaling pathway.¹⁵

REFERENCE

1. LIU Yinghong , LIU Fuyou , DUAN Shaobing , et al. Influence of huangqi on TGF2beta 1 inducing extracellular matrix secretion of human peritoneal mesothelial cell. BULL HUNAN MED UNIV. 2003, 28 (2):141-144
2. Ha H, Lee HB. Effect of high glucose on peritoneal mesothelial cell biology[J]. Perit Dial Int, 2000, 20(Suppl 2): S15-18.
3. Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation[J]. J Invest Dermatol, 2002, 118(2):211-215.
4. Chen W, Fu X, Sheng Z. Review of current progress in the structure and function of Smad Proteins[J]. Chin Med J (Engl), 2002, 115(3): 446-450.
5. LIU Fuyou , DUAN Shaobin , LONG Zhigao , et al. Culture and characterization of human peritoneal mesothelial cells. BULL HUNAN MED UNIV: 2001, 26(4):321-324.
6. Huang Haichang, Li Jingzi, Wang Haiyan. Transformation of renal fibroblast to myofibroblast introduced by CTGF [J]. Science Bulletin, 2002, 47(1):37-40.
7. Laping NJ, Grygielko E, Mathur A, et al. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542 [J]. Mol Pharmacol, 2002,62(1):58-64.
8. Chen Y, Blom IE, Sa S et al. CTGF expression in mesangial cells: involvement of SMADs, MAP kinase, and

- PKC [J]. *Kidney Int*, 2002, 62(4): 1149-1159.
9. Hu B, Wu Z, Phan SH. Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression [J]. *Am J Respir Cell Mol Biol*, 2003,29(3 Pt 1): 397-404.
 10. Hua X, Miller ZA, et al. Benchabane H, Synergism between transcriptions factors TFE3 and Smad3 in transforming growth factor-beta-induced transcription of the Smad7 gene [J]. *J Biol Chem*, 2000, 275 (43): 33205-33208.
 11. Lo RS, Wotton D, Massague J. Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIP. *EMBO J*, 2001, 20(12): 128-136.
 12. DaCosta Byfield S, Major C, et al. SB-505124 Is a Selective Inhibitor of Transforming Growth Factor- β Type I Receptors ALK4, ALK5, and ALK7 [J]. *Mol Pharmacol*, 2004, 65(3): 744-752.
 13. Li JH, Zhu HJ, Huang XR, et al. Smad7 inhibits fibrotic effect of TGF-beta on renal tubular epithelial cells by blocking smad2 activation. *J Am Soc Nephrol*, 2002, 13(6): 1464-1472.
 14. Torada Y, Hanada S, Nakao A, et al. Gene transfer of Smad7 using electroporation of adenovirus prevents renal fibrosis in post-obstructed kidney. *Kidney Int*, 2002, 61(suppl 1): 94-98.
 15. Lin Haiyan, Wang Hongmei, Zhu Cheng, et al. The smad signal transmitted by TGF-. *Science in China (series C)*. 2003 vol.13 (2):97-109.