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World J Gastroenterol 2003;9(4):784-787
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

Effect of transforming growth factor beta and bone morphogenetic proteins on rat hepatic stellate cell proliferation and trans-differentiation

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Received: 2002-10-04 **Accepted:** 2002-11-04

Abstract

AIM: To explore different roles of transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs) in hepatic stellate cell proliferation and trans-differentiation.

METHODS: Hepatic stellate cells were isolated from male Sprague-Dawley rats. Sub-cultured hepatic stellate cells were employed for cell proliferation assay with WST-1 reagent and Western blot analysis with antibody against smooth muscle alpha actin (SMA).

RESULTS: The results indicated that TGF-β1 significantly inhibited cell proliferation at concentration as low as 0.1 ng/ml, but both BMP-2 and BMP-4 did not affect cell proliferation at concentration as high as 10 ng/ml. The effect on hepatic stellate cell trans-differentiation was similar between TGF-β1 and BMPs. However, BMPs was more potent at trans-differentiation of hepatic stellate cells than TGF-β1. In addition, we observed that TGF-β1 transient reduced the abundance of SMA in hepatic stellate cells.

CONCLUSION: TGF-β may be more important in regulation of hepatic stellate cell proliferation while BMPs may be the major cytokines regulating hepatic stellate cell trans-differentiation.

Shen H, Huang GJ, Gong YW. Effect of transforming growth factor beta and bone morphogenetic proteins on rat hepatic stellate cell proliferation and trans-differentiation. *World J Gastroenterol* 2003; 9(4): 784-787
http://www.wjgnet.com/1007-9327/9/784.htm

INTRODUCTION

Transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs) are the members of TGF-β superfamily[7, 23]. Their biological effects are mediated through the receptors that have serine-threonine kinase activity[1, 24]. However, they bind to their respective receptors. Moreover, the molecules involved in their signal transduction are different. It is generally considered that signal transduction pathway of BMPs is mediated by Smads 1, 5, and 8, while the signaling molecules of TGF-β are Smads 2 and Smads 3[17, 29, 33]. The role of TGF-β in hepatic fibrogenesis has been investigated because of the effect of TGF-β on extracellular matrix (ECM) production and degradation[3, 27, 32]. But the effects of BMP on hepatic fibrogenesis have not been studied extensively. With recent understanding of hepatic fibrogenesis, hepatic stellate cells play an important role in the development of hepatic fibrosis[8, 14]. It is documented that hepatic stellate cells are the main cell type that synthesize and secret ECM in the liver. In addition, hepatic stellate cells migrate, proliferate and contract in response to liver injury, which contribute to the development of scar formation and portal hypertension. The effects of TGF-β1 on hepatic stellate cell proliferation and activation have been documented with some studies showed the inhibitory effect of TGF-β1[2, 6, 25, 26] while others exhibited no effect on hepatic stellate cell proliferation[20, 28]. However, there are no reports about the effects of BMPs on hepatic stellate cell proliferation and trans-differentiation. Therefore, the objective of this study is to examine the effects of TGF-β1 and BMPs on rat hepatic stellate cell proliferation and expression of smooth muscle alpha actin (SMA) - a marker of hepatic stellate cell trans-differentiation.

MATERIALS AND METHODS

Materials

Collagenase D, pronase, DNase 1, cell proliferation reagent WST-1, and monoclonal antibody against smooth muscle alpha actin were purchased from Roche Diagnostics (Laval Quebec). Rabbit antibody against mouse IgG conjugated to horseradish peroxidase, and Enhanced Chemiluminescence Detection Kit were from Amersham Pharmacia Biotech, Inc. (Baie d' Urfe, Quebec). Dulbecco' s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Nycodenz were obtained from GIBCO/BRL (Burlington, Ontario). TGF-β1, BMP-2 and BMP-4 were purchased from R&D systems (Minneapolis, MN).

Isolation of rat hepatic stellate cells

Male Sprague-Dawley rats (450-550 gram body weight) were purchased from Central Animal Care of the University of Manitoba and maintained under 12-hour light/dark cycles with food and water ad libitum. In conducting the research described in this report, all animals received humane care in compliance with the Institution' s guidelines (Animal Protocol No. 98-053), which is in accordance with the Canadian Council on Animal Care' s criteria. Hepatic stellate cells were isolated from rat liver by two steps of collagenase and pronase methods as previously described[10]. The liver was perfused via the portal vein first with Ca^{2+} free Hanks' balanced salt solution (HBSS), pH7.4, for 10 minutes at 37 ℃ and then with Ca^{2+} HBSS containing 0.125 mg/ml collagenase D, 0.5 mg/ml pronase and 15 μg/ml DNase 1 for 20 minutes. After being dispersed gently, the cells were incubated with 0.125 mg/ml collagenase D, 0.5 mg/ml pronase and 15 μg/ml DNase 1 for another 12 minutes with constant low speed stirring at 37 ℃. Cell suspension was filtered through a 100 μm mesh. After removing hepatocytes

by centrifugation at 500 rpm on a bench-top centrifuge, Hepatic stellate cells were separated from other non-parenchymal cells by density gradient centrifugation on 11.3 % Nycodenz with sodium chloride at 1 400 g for 17 minutes at 8 ℃. Hepatic stellate cells were harvested from the interface between suspension buffer on the top and 11.3 % Nycodenz solution, washed and plated on uncoated plastic tissue culture dishes (Costar) at a density of 25 000 cells/cm^2. Hepatic stellate cells were identified by the typical star-like configuration under light microscopic appearance. The purity was always higher than 95 %. Hepatic stellate cells were incubated in DMEM supplemented with 10 % FBS, antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) and 2 mM L-glutamine at 37 ℃ in a humidified atmosphere of 5 % CO_2. The first change of culture medium was made 24 hours after seeding and then change the medium every 48 hours. Sub-cultured Hepatic stellate cells were obtained from 9-day-old primary culture hepatic stellate cells by detaching from the dishes with trypsin-EDTA.

Cell proliferation assay

Cell proliferation was measured by cell proliferation reagents WST-1[12]. Sub-cultured hepatic stellate cells (2×10^3) in 200 μl culture medium were seeded into 96 well plates. After incubation for 24 hours, the medium was changed with 100 μl of fresh culture medium containing different concentrations of recombinant TGF-β1, BMP-2 and BMP-4. The media and reagents were changed every other day. Cell proliferation was documented after 3, 6, 9 days of treatment. At the end of treatment, 10 μl of WST-1 reagent were added into wells and incubated for 2 hours. The absorbence of the treated samples against a blank control was measured using a THERMOmax microplate reader (ELISA) (molecular Devices Co., Menlo Park, CA) with 420nm as detection wavelength and 650nm as reference wavelength for WST-1 assay.

Western blot analyses of SMA

Sub-cultured hepatic stellate cells were lysed in 100 μl protein extract solution (1 mM Tris-HCl pH7.5, 1 mM EDTA pH 8.0, 10 mM NaCl, 1 % sodium dodecyl sulfate (SDS), 1 mM PMSF and 0.25 M sucrose)[11]. Cell membrane was broken by sonicating the cells for 1 minutes with Sonicator (Vibra Cell, Sonics and Material Inc. Danbury, CT) and cell debris was pelleted by centrifugation at 14 000 rpm at 4 ℃ for 5 minutes. Protein concentration was determined by Lowry method[9]. 20 g of protein from each sample was mixed with gel loading buffer (2×: 125 mM Tris-HCl, pH6.8, 4 % SDS, 20 % glycerol, 0.1 % bromophenol blue and 2.5 % β-mercaptoethanol), boiled for 5 minutes, separated on 12 % SDS-polyacrylamide gel under reducing conditions, and transferred to Nitroplus-2000 membrane (Micron Separations Inc. Westborough, MA). Nonspecific antibody binding was blocked by pre-incubation of the membranes in 1×Tris-buffered-saline (TBS) containing 5 % skim milk for 1 hour at room temperature. Membranes were then incubated overnight at 4 ℃ with primary antibody against SMA at dilution of 1:1 000 in 1×TBS containing 2 % skim milk. After washing, they were incubated for 1 hour at room temperature with sheep anti-mouse IgG at 1:1 000 dilutions. Bands were visualized by employing the enhanced chemiluminescence kit per the manufacturer' s instruction.

Statistical analyses

Statistical significance of differences was performed by employing the ANOVA and Fisher' s PLSD test as Post hoc test with StatView software (version 5.0, SAS Institute Inc. Cary, NC). Differences were considered to be significant when *P* was less than 0.05.

RESULTS

Effect of TGF-b1, BMP2 and BMP4 on rat hepatic stellate cell proliferation

Effect of TGF-β1, BMP2 and BMP4 on rat hepatic stellate cell proliferation was shown in Figure 1. TGF-β1 significantly inhibited hepatic stellate cell proliferation after 6 days of incubation at the concentrations of 0.1, 0.5 and 1 ng/ml, respectively, $P<0.05$ (Figure 1A). The inhibition was more dramatic at 9-days treatment of TGF-β1, $P<0.01$. However, both BMP2 and BMP4 did not affect hepatic stellate cell proliferation at the concentration as high as 10 ng/ml (Figure 1B and 1C).

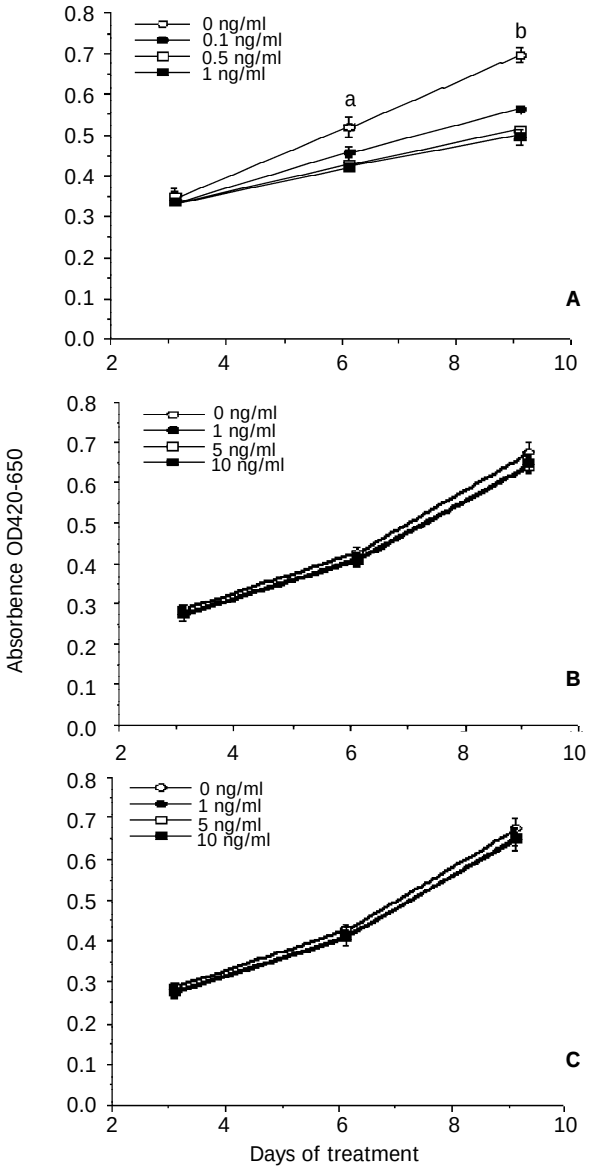

Figure 1 Regulating effect of TGF-β1, BMP-2 and BMP-4 on the proliferation of rat hepatic stellate cells. Hepatic stellate cells were incubated with TGF-β1 (A), BMP-2 (B) and BMP-4 (C) as described in Materials and methods. The data represent mean ± SE from ten wells. The experiments were repeated two times. Denotes: a represents $^aP<0.05$ *vs* the concentrations of 0.1, 0.5 and 1 ng/ml, b means $^bP<0.01$*vs* the concentrations of 0.1, 0.5 and 1 ng/ml.

Effect of TGF-b1, BMP-2 and BMP-4 on SMA expression

TGF-β1, BMP-2 and BMP-4 increased the expression of SMA, which is well recognized by the antibody as a 42-kDa protein. BMP-2 and BMP-4 seemed to have more potent effect on the expression of SMA than TGF-β1. After incubation with TGF-

β1 (concentrations from 0.1 to 1 ng/ml) for 3 days, SMA level was only elevated about 50 % as compared with control group (Figure 2). While after BMP-2 or BMP-4 treatment, SMA level was two to four times higher than that of the untreated group (Figure 3). Moreover, we observed that the abundance of SMA in hepatic stellate cells was reduced after 3 hours of TGF-β1 treatment. The abundance of SMA was reduced to about 20 % of original level and the level of SMA was elevated (about 60 % higher than original level) after 18 hours' treatment (Figure 4).

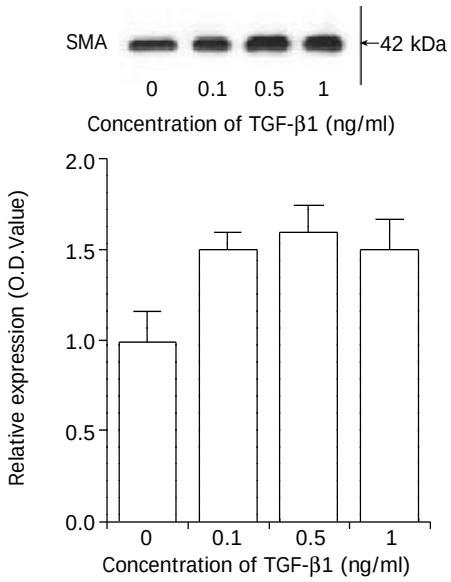

Figure 2 Regulating effect of TGF-β1 on rat hepatic stellate cell trans-differentiation. Hepatic stellate cells were incubated with different concentrations of TGF-β1 for three days. The media and TGF-β1 were changed every other day. Western blot was performed as described in Materials and methods. The top panel represents typical Western blot of SMA. The lower panel represents histogram of densitometric data from four-separated Western blot (mean ± SE).

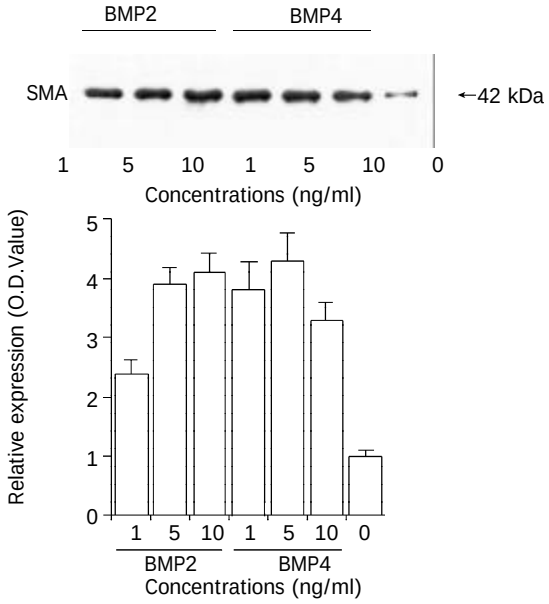

Figure 3 Regulating effect of BMP-2 and BMP-4 on hepatic stellate cell trans-differentiation. Hepatic stellate cells were incubated with different concentrations of BMP-2 or BMP-4 for three days. The media and BMPs were changed every other day. Western blot was performed as described in Materials and methods. The top panel represents typical Western blot of SMA. The lower panel represents histogram of densitometric data from four-separated Western blot (mean ± SE).

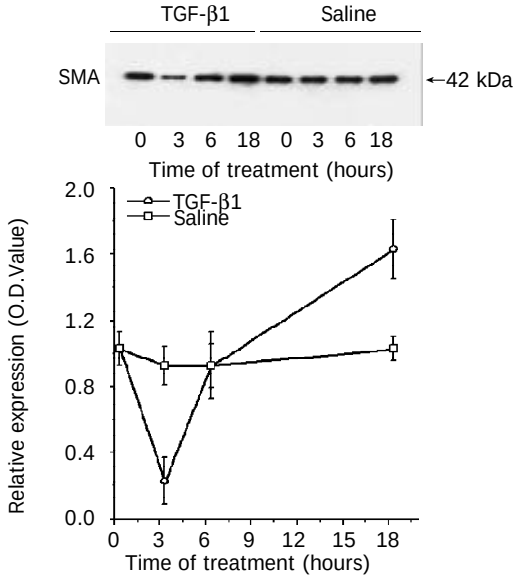

Figure 4 Regulating effect of TGF-β1 on SMA protein expression. Hepatic stellate cells were cultured for three days and the media were removed. Media with 1 ng/ml TGF-β1 or saline were added into culture dishes and cells were collected at different times as indicated. The data represent mean ± SE from four experiments.

DISCUSSION

Hepatic stellate cells are non-parenchymal liver cells located at the perisinusoidal space. Recent studies have demonstrated that these cells proliferate and activate in response to liver injury. It is now established that hepatic stellate cells are the cell type involved in hepatic fibrogenesis[4, 5]. It is known that the proliferation of these cells is one response during hepatic fibrogenetic process. The other feature of these cells is their trans-differentiation, which is indicated by increased expression of SMA[19]. During the trans-differentiation process of hepatic stellate cells, these cells are trans-differentiated into myofibroblast-like phenotype. To understand hepatic fibrogenesis, it is important to know the mechanism of proliferation and trans-differentiation of hepatic stellate cells. In this report, we employed TGF-β1 and BMPs to examine their effects on hepatic stellate cell proliferation and trans-differentiation. Our study indicated that both TGF-β1 and BMPs were important in the trans-differentiation of hepatic stellate cells. However, BMP-2 and BMP-4 had more potent effect on trans-differentiation of these cells than TGF-β1 did. The difference can be due to the different signaling proteins involved in TGF-β1 and BMPs signal transduction. It is known that Smads are the intracellular molecules for both TGF-β and BMP signal transduction pathways[17, 29, 33]. However, Smad-2 and Smad-3 are believed to mediate TGF-β signal transduction while Smad-1, Smad-5 and Smad-8 mediate BMP signaling[13, 16, 18, 21]. It has been demonstrated that Smad-3 is not necessary to the trans-differentiation of hepatic stellate cells but is required for TGF-β1 mediated cell proliferation[31]. Although TGF-β1 had some effect on the trans-differentiation of hepatic stellate cells in this study, it might not be related to Smad-2 and Smad-3. It has been documented that TGF-β1 can phosphorylate Smad-1 in human breast cancer cells[22]. However, it still remained be tested what is the mechanism mediating the transient reduction of SMA by TGF-β1 in hepatic stellate cells. The direct TGF-β1 regulation of SMA gene expression could not be excluded.

The inhibitory effect of TGF-β1 on hepatic stellate cell proliferation was consistent with effect of TGF-β1 on most mammalian cells. It inhibited the proliferation of hepatic stellate

cells. Studies relating the effect of TGF-β1 on hepatic stellate cell proliferation from other groups have revealed some conflicting results. Most of the studies indicated that TGF-β1 inhibited the proliferation of hepatic stellate cells[2, 6, 25, 26, 28] while few studies had found no significant effect on these cell proliferation[14, 20]. The difference of TGF-β1 regulation of hepatic stellate cells may be related to stage of cell differentiation or the period of TGF-β1 treatment. One of the interesting findings in this study is that both BMP-2 and BMP-4 did not affect the proliferation of hepatic stellate cells. It is well known that the main role of BMPs is bone morphogenesis, which is a sequential cascade with three key phases: chemotaxis and mitosis of mesenchymal cells, differentiation of the mesenchymal cells initially into cartilage, and replacement of the cartilage by bone. Its function is more related to differentiation then proliferation[15, 30]. Therefore, no effect of BMPs on hepatic stellate cell proliferation is well consistent with their functions.

CONCLUSION

Our study documented that both TGF-β and BMPs play important roles in hepatic stellate cell proliferation and trans-differentiation. TGF-β is more important in the regulation of hepatic stellate cell proliferation while BMPs is more potent in the regulation of trans-differentiation of hepatic stellate cells. However, the *in vitro* observations of TGF-β and/or BMPs on hepatic stellate cell proliferation and trans-differentiation may represent one aspect of the complex interaction among varieties of factors in human body.

ACKNOWLEDGEMENTS

This work was supported by grants from Canadian Institute of Health Research and Manitoba Medical Service Foundation.

REFERENCES

1 **Atfi A**, Lepage K, Allard P, Chapdelaine A, Chevalier S. Activation of a serine/threonine kinase signaling pathway by transforming growth factor type beta. *Proc Natl Acad Sci USA* 1995; **92**: 12110-12114
2 **Bachem MG**, Riess U, Gressner AM. Liver fat storing cell proliferation is stimulated by epidermal growth factor/transforming growth factor alpha and inhibited by transforming growth factor beta. *Biochem Biophys Res Commun* 1989; **162**: 708-714
3 **Baricos WH**, Cortez SL, Deboisblanc M, Xin S. Transforming growth factor-beta is a potent inhibitor of extracellular matrix degradation by cultured human mesangial cells. *J Am Soc Nephrol* 1999; **10**: 790-795
4 **Brenner DA**, Waterboer T, Choi SK, Lindquist JN, Stefanovic B, Burchardt E, Yamauchi M, Gillan A, Rippe RA. New aspects of hepatic fibrosis. *J Hepatol* 2000; **32**: 32-38
5 **Davis BH**, Kresina TF. Hepatic fibrogenesis. *Clin Lab Med* 1996; **16**: 361-375
6 **Davis BH**, Rapp UR, Davidson NO. Retinoic acid and transforming growth factor beta differentially inhibits platelet-derived-growth-factor-induced Ito-cell activation. *Biochem J* 1991; **278**: 43-47
7 **Derynck R**, Chen RH, Ebner R, Filvaroff EH, Lawler S. An emerging complexity of receptors for transforming growth factor-beta. *Princess Takamatsu Symp* 1994; **24**: 264-275
8 **Friedman SL**. Hepatic stellate cells. *Prog Liver Dis* 1996; **14**: 101-130
9 **Fryer HJ**, Davis GE, Manthorpe M, Varon S. Lowry protein assay using an automatic microtiter plate spectrophotometer. *Anal Biochem* 1986; **153**: 262-266
10 **Gong W**, Roth S, Michel K, Gressner AM. Isoforms and splice variant of transforming growth factor beta-binding protein in rat hepatic stellate cells. *Gastroenterology* 1998; **114**: 352-363
11 **Gong Y**, Blok LJ, Perry JE, Lindzey JK, Tindall DJ. Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* 1995; **136**: 2172-2178
12 **Gong Y**, Zhang M, Minuk GY. Regulation of transforming growth factor-beta1 gene expression and cell proliferation in human hepatocellular carcinoma cells (PLC/PRF/5) by tamoxifen. *J Lab Clin Med* 1999; **134**: 90-95
13 **Goumans MJ,** Mummery C. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol* 2000; **44**: 253-265
14 **Gressner AM**. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. *Kidney Int Suppl* 1996; **54**: S39-S45
15 **Hogan BL**. Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 1996; **6**: 432-438
16 **Hullinger TG**, Pan Q, Viswanathan HL, Somerman MJ. TGFbeta and BMP-2 activation of the OPN promoter: roles of smad- and hox-binding elements. *Exp Cell Res* 2001; **262**: 69-74
17 **Itoh S**, Itoh F, Goumans MJ, Ten Dijke P. Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem* 2000; **267**: 6954-6967
18 **Kawabata M**, Imamura T, Miyazono K. Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev* 1998; **9**: 49-61
19 **Khan MA**, Poulos JE, Brunt EM, Li L, Solomon H, Britton RS, Bacon BR, Di Bisceglie AM. Hepatic alpha-smooth muscle actin expression in hepatitis C patients before and after interferon therapy. *Hepatogastroenterology* 2001; **48**: 212-215
20 **Knittel T**, Janneck T, Muller L, Fellmer P, Ramadori G. Transforming growth factor beta 1-regulated gene expression of Ito cells. *Hepatology* 1996; **24**: 352-360
21 **Lagna G,** Hemmati-Brivanlou A. A molecular basis for Smad specificity. *Dev Dyn* 1999; **214**: 269-277
22 **Liu X**, Yue J, Frey RS, Zhu Q, Mulder KM. Transforming growth factor beta signaling through Smad1 in human breast cancer cells. *Cancer Res* 1998; **58**: 4752-4757
23 **Massague J**. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990; **6**: 597-641
24 **Massague J**, Weis-Garcia F. Serine/threonine kinase receptors: mediators of transforming growth factor beta family signals. *Cancer Surv* 1996; **27**: 41-64
25 **Matsuoka M**, Pham NT, Tsukamoto H. Differential effects of interleukin-1 alpha, tumor necrosis factor alpha, and transforming growth factor beta 1 on cell proliferation and collagen formation by cultured fat-storing cells. *Liver* 1989; **9**: 71-78
26 **Meyer DH**, Bachem MG, Gressner AM. Modulation of hepatic lipocyte proteoglycan synthesis and proliferation by Kupffer cell-derived transforming growth factors type beta 1 and type alpha. *Biochem Biophys Res Commun* 1990; **171**: 1122-1129
27 **Paulus W**, Baur I, Huettner C, Schmausser B, Roggendorf W, Schlingensiepen KH, Brysch W. Effects of transforming growth factor-beta 1 on collagen synthesis, integrin expression, adhesion and invasion of glioma cells. *J Neuropathol Exp Neurol* 1995; **54**: 236-244
28 **Pinzani M**, Gesualdo L, Sabbah GM, Abboud HE. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. *J Clin Invest* 1989; **84**: 1786-1793
29 **Schiffer M**, von Gersdorff G, Bitzer M, Susztak K, Bottinger EP. Smad proteins and transforming growth factor-beta signaling. *Kidney Int Suppl* 2000; **77**: S45-S52
30 **Schmitt JM**, Hwang K, Winn SR, Hollinger JO. Bone morphogenetic proteins: an update on basic biology and clinical relevance. *J Orthop Res* 1999; **17**: 269-278
31 **Schnabl B**, Kweon YO, Frederick JP, Wang XF, Rippe RA, Brenner DA. The role of Smad3 in mediating mouse hepatic stellate cell activation. *Hepatology* 2001; **34**: 89-100
32 **Tipton DA**, Dabbous MK. Autocrine transforming growth factor beta stimulation of extracellular matrix production by fibroblasts from fibrotic human gingiva. *J Periodontol* 1998; **69**: 609-619
33 **Visser JA**, Themmen AP. Downstream factors in transforming growth factor-beta family signaling. *Mol Cell Endocrinol* 1998; **146**: 7-17

Edited by Xu XQ