論文

Gene expression of *SHARP-2* is induced by transforming growth factor beta via multiple pathways in HuH-7 hepatoma cells

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HuH-7肝癌細胞において、*SHARP-2*遺伝子の発現は、
トランスフォーミング成長因子βにより様々な経路を介して誘導される

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Summary

Transforming growth factor-β (TGF-β) inhibits cell proliferation and promotes cell differentiation. In human HuH-7 hepatocellular carcinoma cells, TGF-β reduces the expression of the *α-fetoprotein (AFP)* gene, a marker molecule for hepatocellular carcinoma cells. In this case, the amount of AT motif-binding factor-1 (ATBF1) mRNA, which encodes a transcriptional repressor of the *AFP* gene, was not changed. This suggests the involvement of factors that are induced by TGF-β and interact with ATBF1. The enhancer of split- and hairy-related protein-2 (SHARP-2) is a basic helix-loop-helix transcriptional repressor and an interacting protein of ATBF1. In this study, we examined whether TGF-β regulates the expression of the *SHARP-2* gene in HuH-7 cells. TGF-β induced the SHARP-2 mRNA level within 1 hr. Next, to explore the signaling pathway, HuH-7 cells were pretreated with various inhibitors and then treated with TGF-β. Inhibitors of TGF-β type I receptor, phosphoinositide 3-kinase, protein kinase C, Jun N-terminal kinase, mitogen-activated protein kinase, and RNA polymerase II completely or partially diminished the induction of the SHARP-2 mRNA level by TGF-β. Finally, a reporter plasmid containing from -3,700 to +265 region of the *SHARP-2* gene were transfected into HuH-7 cells and treated with TGF-β, but no change in promoter activity was observed. Thus, we concluded that TGF-β increased the level of SHARP-2 mRNA via multiple pathways at the transcription level of the *SHARP-2* gene and that SHARP-2 is a candidate protein mediating TGF-β-repression of the *AFP* gene.

Keywords

SHARP-2, TGF-β, gene regulation, signaling pathway, HuH-7 hepatoma cells

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Ⅰ. Introduction

The enhancer of split- and hairy-related protein (SHARP) family is a basic helix-loop-helix type transcriptional repressor[1]. It belongs to the clock gene family regulating the circadian rhythm[2]. The SHARP family consists of two members, SHARP-1 (also referred to as the DEC2, BHLHB3, and BHLHE41) and SHARP-2 (also referred to as the DEC1, Stra13, BHLHB2, and BHLE40). These form a homo- or a hetero-dimer each other and bind to the E box sequence (5'-CANNTG-3') locating at the transcription control region of the target gene.

Gene expression of the *SHARP family* is regulated by hormones and nutrients[3-5]. Insulin induces expression of the *SHARP-1* gene via a phosphoinositide 3-kinase (PI3-K)/atypical protein kinase C (aPKC) λ/Jun N-terminal kinase (JNK) pathway and a PI3-K/Rac/JNK pathway in H4IIE highly differentiated rat hepatoma cells[3]. Insulin also induces expression of the *SHARP-2* gene in primary cultured rat hepatocytes, H4IIE cells, mouse 3T3-L1 adipocytes, and rat L6 myotube cells[5-7]. These inductions are mediated by a PI3-K/aPKCλ pathway and a PI3-K/ mammalian target of rapamycin (mTOR) pathway in H4IIE cells, a PI3-K pathway in mouse 3T3-L1 adipocytes, and mitogen-activated protein kinase pathway in rat L6 myotube cells[5, 7]. Therefore, insulin regulates the *SHARP-2* gene expression in cell type-specific manners. Both SHARP-1 and SHARP-2 repress transcription of the rat *phosphoenolpyruvate carboxykinase (PEPCK)* gene which encodes a rate-limiting hepatic gluconeogenic enzyme[3, 8]. Thus, the SHARP family proteins are the transcription factors involved in lowering the blood glucose level by insulin.

Many transcription factors interact with other proteins to form complex transcriptional regulation networks. To explore which proteins interact with SHARP-2, a rat liver cDNA library using a yeast two-hybrid system was screened. AT motif-binding factor-1 (ATBF1) was identified as the SHARP-2-interactng protein[9]. The ATBF1 was originally cloned as a factor that represses transcription by binding to an AT-rich sequence of the *α-fetoprotein* (*AFP*) gene which encodes a hepatocellular cancer marker molecule[10, 11]. ATBF1 is a huge protein with a molecular weight of about 406 kDa, which has twenty-three zinc-finger motifs and four homeodomains. As abnormalities in the *ATBF1* gene cause cancer, it functions as an anti-oncogene[12-15].

Transforming growth factor-β (TGF-β) inhibits cell proliferation and promotes cell differentiation[16]. It has been reported that some cancer cells are deficient in the TGF-β signaling pathway[17]. It has been reported that TGF-β reduces the AFP mRNA levels without affecting the ATBF1 mRNA levels in human HuH-7 hepatoma cells[18]. It suggested that protein X, which is induced by TGF-β and interacts with ATBF1, plays an important role in this reduction. We previously reported that an induction of SHARP-2 mRNA by TGF-β in renal LLC-PK1 cells was mediated by the PI3-K pathway[19]. However, the effect of TGF-β on the expression of the *SHARP-2* gene in hepatoma cells are still unknown.

In this study, we examined the regulation of the *SHARP-2* gene expressions by TGF-β and their molecular mechanisms in HuH-7 human hepatoma cells.

Ⅱ. Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and Sepasol-RNA I Super G were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from BioWest Therapeutics Inc. (Nuaille, France). Streptomycin and penicillin G were purchased from Meiji Seika Pharma Co., Ltd (Tokyo, Japan). TGF-β1 was purchased from R&D Systems (Minneapolis, U.S.A.). SB431542 was purchased from Stemgent (San Diego, U.S.A.). Wortmannin, LY294002, rapamycin,

staurosporine, PD98059, JNK inhibitor II, okadaic acid, actinomycin D (AD), and cycloheximide (CHX) were purchased from Merck Chemicals Ltd (Darmstadt, Germany). High Capacity RNA-to-cDNA Kit was purchased from Applied Biosystems Japan (Tokyo, Japan). FastStart Universal SYBR Green Master (Rox) and GenoPure Plasmid Maxi kit were purchased from Roche Diagnostics (Indianapolis, U.S.A.). Lipofectamine PLUS was purchased from Invitrogen (Groningen, The Netherlands). The phRL-CMV and pGL4.13 plasmids, and Dual-Luciferase Reporter Assay System were obtained from Promega (Madison, U.S.A.).

Cells and cell culture

HuH-7 cells, a human hepatoma cell line, were purchased from the JCRB Cell Bank (Osaka, Japan). Cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 Units/ml penicillin G at 37℃ in a 5% CO_2 incubator.

One million of cells were cultured for 24 hours in a 6 cm dishes. The medium was replaced with serum-free DMEM, then cultured for another 24 hours. The medium was replaced once more with serum-free DMEM. After 2 hours, the cells were treated with the indicated concentrations of TGF-β for 2 hours or 0.5 ng/ml TGF-β for the indicated times.

To elucidate signaling pathway(s), cells were treated for 30 minutes with each inhibitor or dimethyl sulfoxide (DS), followed by a treatment with 0.5 ng/ml TGF-β for 1 hour. Ten μM SB431542, 0.2 μM wortmannin, 50 μM LY294002, 0.1 μM rapamycin, 0.1 μM staurosporine, 25 μM PD98059, 10 μM JNK inhibitor II, 10 nM okadaic acid, 0.8 μM AD, or 10 μM CHX were used as the inhibitors.

Preparation of total RNA, reverse transcription, and real-time polymerase chain reaction (PCR) analysis

Total RNA was prepared from various cells with Sepasol-RNA I Super G. Reverse transcription was conducted using the High Capacity RNA-to-cDNA Kit as described previously[5] . Real-time PCR was performed using cDNA, primers, Fast Start Universal SYBR Green Master (ROX) (Roche) and Quant Studio 3 real-time PCR system (Applied Biosystems Japan, Co. Ltd.). Nucleotide sequences of the primers were as follows: SHARP-2 forward, 5'-GAAAGGATCGGCGCAATTAA-3' and reverse 5'-CATCATCCGAAAGCTGCATC-3'; 36B4 forward 5'-GGCGACCTGGAAGTCCAACT-3' and reverse 5'-GGATCTGCTGCATCTGCTTG-3', respectively. The expression level of SHARP-2 mRNA was normalized to the level of 36B4 mRNA.

DNA transfection and luciferase reporter assay

The pSHARP-2/Luc plasmid which contains the nucleotide sequences between -3,700 and +265 of the rat *SHARP-2* gene was previously described[5]. All plasmids used for transfection were prepared using the Genopure Plasmid Maxi Kit. HuH-7 cells were seeded on a 24-well culture plate at 5×10^4 cells per well. After 24 hours, DNA transfection into HuH-7 cells were used the Lipofectamine PLUS. 100 ng of a luciferase reporter plasmid and 5 ng of the phRLuc-CMV plasmid were used for transfection. After 3 hours, the medium was replaced with 10% FBS-DMEM. After 24 hours, the transfected cells were treated with or without 0.5 ng/ml TGF-β for 4 hours. Firefly and sea pansy luciferase assays were performed using the Dual-Luciferase assay system. A Berthold Lumat LB 9507 luminometer (Wildbad, Germany) was used for the measurement. The promoter activity was calculated by dividing the firefly luciferase activity value by the sea pansy luciferase activity value.

Statistical analysis

All experiments were performed at least three times. Data were represented as the mean and

standard error and analyzed by one-way ANOVA, followed by Fisher's protected LSD multiple comparison test.

Ⅲ. Results

Effect of TGF-β on expressions of the *SHARP family* genes

First, we examined whether TGF-β increase the SHARP-2 mRNA level in HuH-7 cells. When HuH-7 cells were treated with various concentrations of TGF-β, the SHARP-2 mRNA level was increased (Fig. 1A). Next, when HuH-7 cells were treated with 0.5 ng/ml TGF-β at various times, the expression of SHARP-2 mRNA increased from 1 hour (Fig. 1B).

These results indicated that the level of SHARP-2 mRNA was upregulated by TGF-β in HuH-7 cells.

Analysis of the regulatory mechanism of the *SHARP-2* gene expression by TGF-β

Next, we analyzed the signaling pathway involved in the induction of SHARP-2 mRNA by TGF-β. TGF-β binds to TGF-β type II receptor (TRII) on the plasma membrane of target cells and this complex then binds to the type I receptor (TRI)[20]. TRII has serine/threonine kinase activity, which phosphorylates and activates ALK5 of the TRI. The ALK5 is a serine/threonine kinase domain of the TRI. The active form of ALK5 phosphorylates receptor-regulated Smad, Smad 2, 3. The phosphorylated Smad2, 3 binds to common-mediator Smad, Smad4, and is translocated from the cytoplasm to the nucleus. In the nucleus, the Smad2, 3, 4 complex binds to the Smad-binding element (SBE, 5'-GTCT-3') in the transcriptional regulatory region of the target gene. SB431542 is an inhibitor that selectively inhibits the kinase activity of ALK5 of TRI, and inhibits the phosphorylation of Smad2, 3. First, HuH-7 cells were treated with SB431542, then treated with TGF-β. The induction of SHARP-2 mRNA by TGF-β was completely inhibited (Fig. 2A). This result indicated that the induction of SHARP-2 mRNA by TGF-β is mediated by the TGF-β receptor.

Next, we used the PI3-K inhibitor wortmannin and LY294002, and the mTOR inhibitor rapamycin.

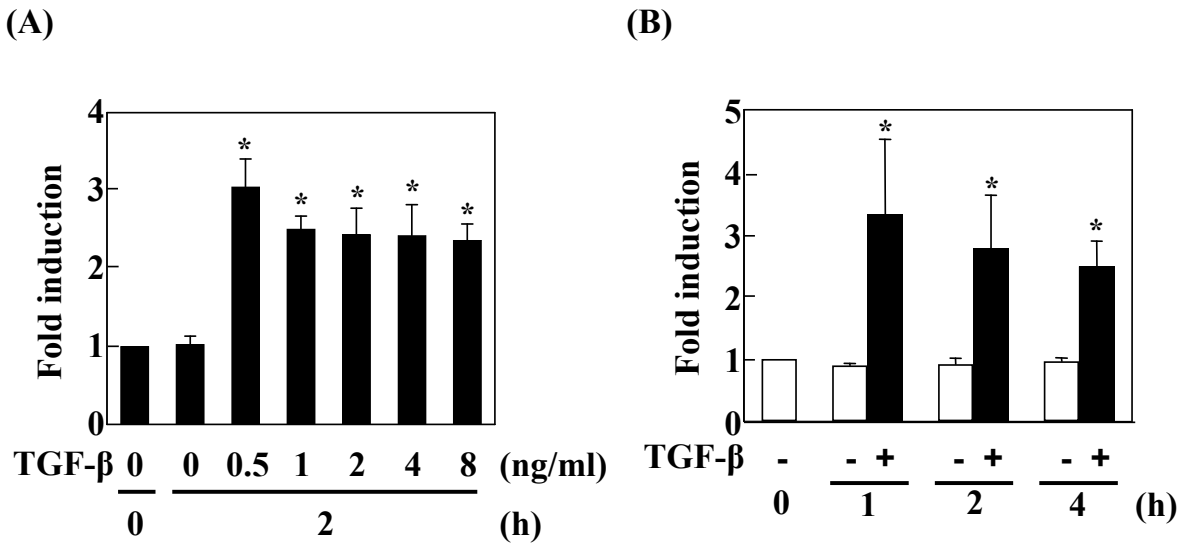

Fig. 1. Effects of TGF-β on the expression of the *SHARP-2* gene.

Total RNA was prepared from HuH-7 cells treated with or without TGF-β. The levels of SHARP-2 and 36B4 mRNAs were determined by reverse-transcription and quantitative real-time PCR. Each column and bar represent the mean and standard error of the ratio of the level of SHARP-2 mRNA and 36B4 mRNA of at least three independent experiments. The expression level ratio shown as "0 h" was normalized to 1. *P < 0.05. (A) HuH-7 cells were treated with or without the indicated concentrations of TGF-β for 2 hours. (B) HuH-7 cells were treated with or without 0.5 ng/ml TGF-β for the indicated times.

As shown in Fig. 2B, the induction of the SHARP-2 mRNA level by TGF-β was completely diminished by treatments with both wortmannin and LY294002. In contrast, rapamycin does not affect the induction of the level of SHARP-2 mRNA by TGF-β (Fig. 2C). In addition, protein kinase C (PKC) inhibitor staurosporine, mitogen-activated protein kinase (MAPK) inhibitor PD98059, Jun N-terminal kinase (JNK) inhibitor JNK inhibitor II, and protein phosphatase inhibitor okadaic acid were used as the inhibitors. The induction of SHARP-2 mRNA level by TGF-β was completely inhibited by staurosporine and JNK inhibitor II and partially inhibited by PD98059 (Fig. 2D).

These results suggested that the PI3-K, the PKC, the JNK and the MAPK pathways are involved in the induction of SHARP-2 mRNA by TGF-β.

Furthermore, we examined whether the induction of SHARP-2 mRNA level by TGF-β depends on the transcription level of the *SHARP-2* gene or whether its induction requires the synthesis of a novel protein. When HuH-7 cells were treated with the transcription inhibitor AD or the translation inhibitor CHX, TGF-β-induced SHARP-2 mRNA level was inhibited by AD but not CHX (Fig. 3).

These results suggested that the induction of *SHARP-2* mRNA by TGF-β occurred at the transcription level of the *SHARP-2* gene, and that *de novo* protein synthesis was not required for this induction.

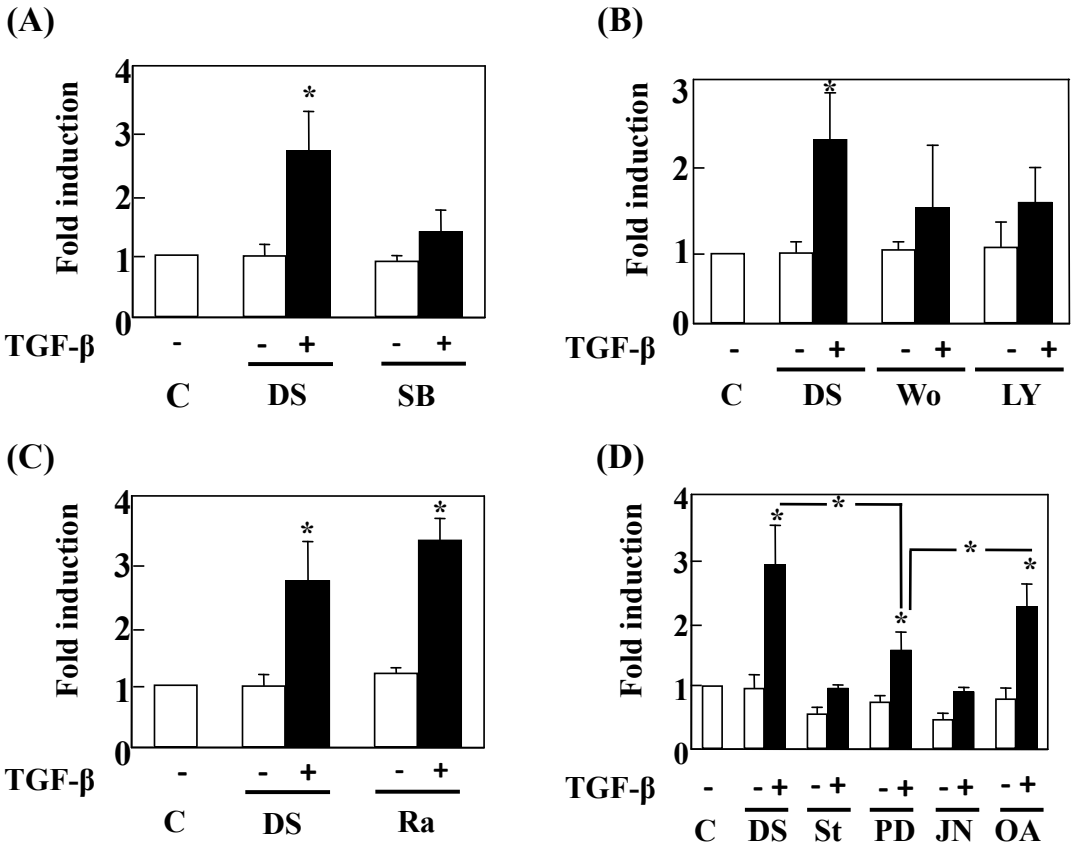

Fig. 2. Analysis of a signaling pathway(s) involved in the induction of the level of SHARP-2 mRNA by TGF-β.

HuH-7 cells were treated with dimethyl sulfoxide (DS), (A) 10 μM SB431542 (SB), (B) 0.2 μM wortmannin (Wo), 50 μM LY294002 (LY), (C) 0.1 μM rapamycin (Ra), (D) 0.1 μM staurosporine (St), 25 μM PD98059 (PD), 10 μM JNK inhibitor II (JN), or 10 nM okadaic acid (OA) for 30 minutes, then treated with (+) or without (-) 0.5 ng/ml TGF-β for 1 hour. Details for determination of the levels of various mRNAs are described in the legend of Fig. 1.

Effect of TGF-β on the *SHARP-2* gene transcription

Finally, we examined whether TGF-β stimulates transcription of the *SHARP-2* gene using the luciferase reporter system. The SHARP-2/Luc reporter plasmid which contains the region from -3,700 to +265 of the *SHARP-2* gene or the pGL4.13 plasmid which contains simian virus 40 enhancer/promoter were used as the reporter plasmid. HuH-7 cells were transfected with a luciferase reporter plasmid, then treated with or without TGF-β. However, promoter activities from both plasmids were not affected by TGF-β (Fig. 4). These results indicated that nucleotide sequences between -3,700 and +265 of the *SHARP-2* gene does not contain a TGF-β-responsive element.

Ⅳ. Discussion

TGF-β inhibits cell proliferation and promotes cell differentiation[16] . It has been reported that TGF-β decreased the AFP mRNA level without

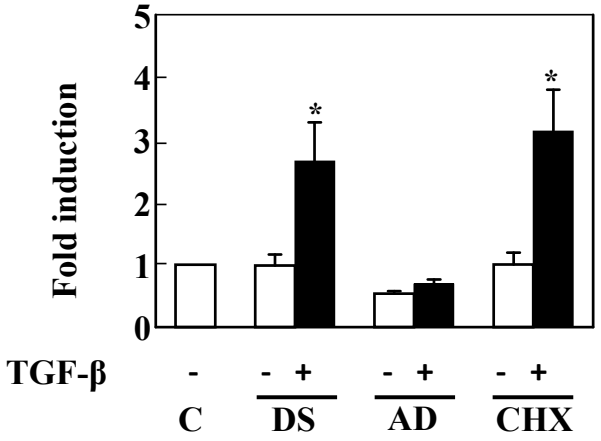

Fig. 3. Effects of actinomycin D or cycloheximide on the induction of the SHARP-2 mRNA level by TGF-β.

HuH-7 cells were treated with dimethyl sulfoxide (DS), 0.8 μM actinomycin D (AD) or 10 μM cycloheximide (CHX) for 30 minutes, then treated with (+) or without (-) 0.5 ng/ml TGF-β for 1 hour. Details for determination of the levels of various mRNAs are described in the legend of Fig. 1.

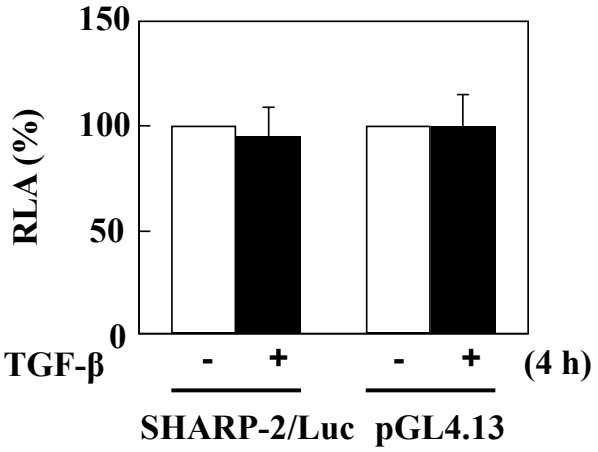

Fig. 4. An effect of TGF-β on transcriptional activity of the *SHARP-2* gene.

Either the pSHARP2/Luc or pGL4.13 plasmid shown on the *bottom* was used as the reporter plasmid. These transfected cells were cultured for 24h in the absence (-) or presence (+) of 0.5 ng/ml TGF-β. A value of 100 was assigned to the promoter activity from each reporter plasmid in the absence of TGF-β. Each column and bar represent the mean and standard error of four independent experiments.

affecting the ATBF1 mRNA level in HuH-7 cells[18]. It suggested that TGF-β could induce expression of transcriptional repressors or corepressors that interact with ATBF1. SHARP-2 is a possible candidate because it is a transcriptional repressor interacting with ATBF1[9].

In this study, we examined whether TGF-β increased the level of SHARP-2 mRNA. When HuH-7 cells were treated with TGF-β, SHARP-2 mRNA expression was induced at 1 hour (Fig. 1). Since expression of the *SHARP-2* gene has been reported to be involved in differentiation of some cell types, it is possible that SHARP-2 mRNA expression mediates the anti-proliferation activity of TGF-β[21-23] . This induction was completely inhibited by the TRI inhibitor, indicating that the induction of SHARP-2 mRNA by TGF-β was mediated by the TGF-β receptor (Fig. 2A).

We previously reported that an induction of SHARP-2 mRNA by TGF-β in renal LLC-PK1 cells was mediated by the PI3-K pathway[19]. It has been reported that TGF-β stimulates the PI3-K pathway in other systems[24] . In HuH-7 cells, inhibitors of the PI3-K, JNK, MAPK, and PKC were diminished the induction of SHARP-2 mRNA level by TGF-β (Fig. 2). In addition, among these, only an inhibitor of the MAPK partially inhibited the induction of SHARP-2 mRNA expression by TGF-β (Fig. 2). These findings suggested that TGF-β regulated the *SHARP-2* gene expression in cell type-specific manners and that the PI 3-K/PKC/JNK pathway and the MAPK pathway were independent each other for TGF-β regulation in HuH-7 cells. PKC has more than 10 isozymes[25, 26] . These are classified into classical PKC, novel PKC, and aPKC. It has been reported that aPKCλ exists downstream of the PI3-K[27] . We previously reported that insulin induced the expression of the *SHARP-2* gene via the PI 3-K/aPKCλ pathway and that it induced that of the *SHARP-1* gene via the PI 3-K/aPKCλ /JNK pathway[3, 5]. Further studies will be required for identification of the PKC isozyme in the case of TGF-β.

In addition, it was shown that the induction of SHARP-2 mRNA by TGF-β occurred at the transcription level of the *SHARP-2* gene, and that its induction did not require *de novo* protein synthesis (Fig. 3). Therefore, this induction is a direct effect of TGF-β signaling pathway. However, the transcription control region from -3,700 to +265 of the *SHARP-2* gene did not respond by TGF-β, even though two putative SBEs were existed in this region (Fig. 4). To elucidate the mechanism of TGF-β-induced transcriptional stimulation of the *SHARP-2* gene, it is necessary to search for other regulatory regions.

Thus, we concluded that TGF-β induced the SHARP-2 mRNA expression in the PI3-K/PKC/JNK and PI 3-K/MAPK pathways and it stimulates transcription of the *SHARP-2* gene (Fig. 5). These results indicate that SHARP-2 is a candidate molecule that interacts with ATBF1 to repress transcription of the *AFP* gene in a TGF-β-dependent manner in HuH-7 cells. To investigate whether SHARP-2 is involved in the transcriptional repression of the *AFP* gene, further studies will be required.

Ⅴ. Acknowledgements

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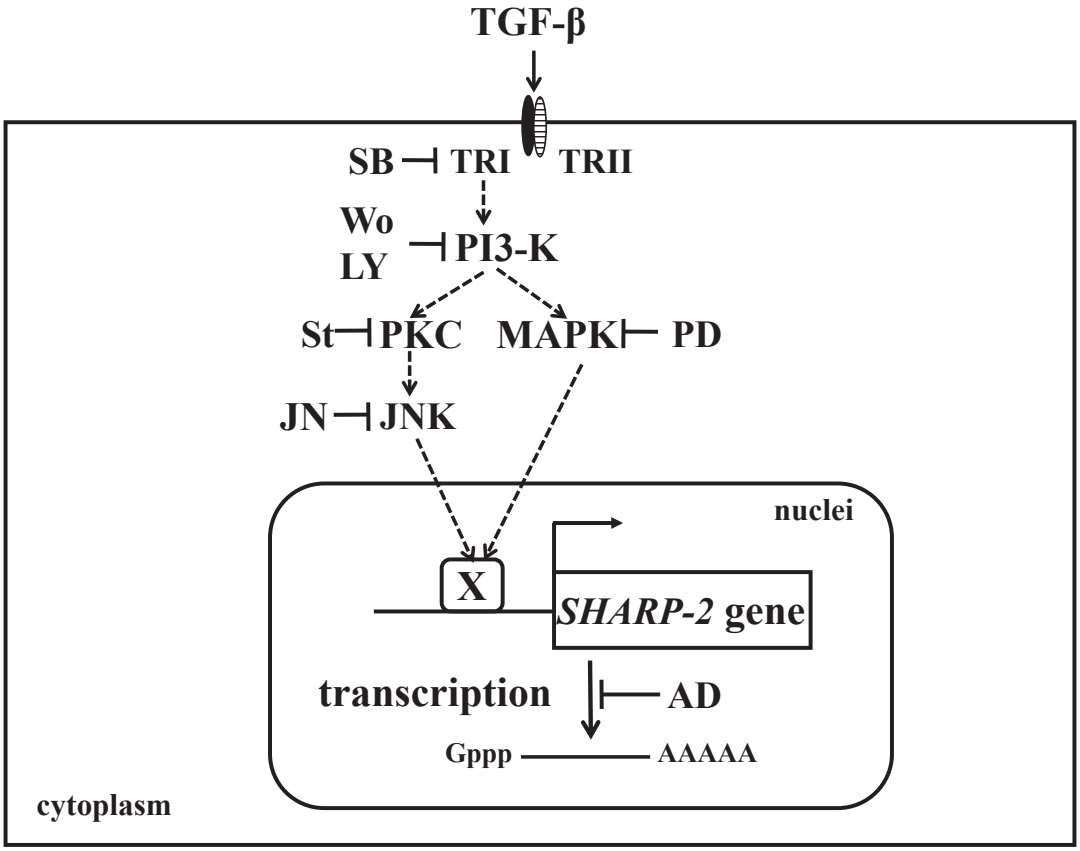

Fig. 5. Schematic drawing of regulatory expression of the *SHARP-2* gene by TGF-β.

TGF-β activates transcription factor X via the PI 3-K/PKC/JNK and the PI 3-K/MAPK pathways. The X enhances transcription of the *SHARP-2* gene.

References

1) Yamada K, and Miyamoto K, "Basic helix-loop-helix transcription factors, BHLHB2 and BHLHB3; their gene expressions are regulated by multiple extracellular stimuli", Front Biosci 10, pp.3151-3171, (2005).
2) Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, and Honma K, "Dec1 and Dec2 are regulators of the mammalian molecular clock", Nature 419, pp.841-844, (2002).
3) Takagi K, Asano K, Haneishi A, Ono M, Komatsu Y, Yamamoto T, Tanaka T, Ueno H, Ogawa W, Tomita K, Noguchi T, and Yamada K, "Insulin stimulates the expression of the SHARP-1 gene via multiple signaling pathways", Horm Metab Res 46, pp.397-403, (2014).
4) Hirano S, Yamada K, Kawata H, Shou Z, Mizutani T, Shigematsu Y, Mayumi M, and Miyamoto K, "The rat enhancer of split- and hairy-related protein-2 gene: hepatic expression, genomic structure, and promoter analysis", Arch Biochem Biophys 422, pp.81-90, (2004).
5) Kanai Y, Asano K, Komatsu Y, Takagi K, Ono M, Tanaka T, Tomita K, Haneishi A, Tsukada A, and Yamada K, "Induction of the SHARP-2 mRNA level by insulin is mediated by multiple signaling pathways", Biosci Biotechnol Biochem 81, pp.256-261, (2017).
6) Yamada K, Kawata H, Shou Z, Mizutani T, Noguchi T, and Miyamoto K, "Insulin induces the expression of the SHARP-2/Stra13/DEC1 gene via a phosphoinositide 3-kinase pathway", J Biol Chem 278, pp.30719-30724, (2003).
7) Kawata H, Yamada K, Matsuura K, Shou Z, and Miyamoto K, "Insulin regulates the expression of the enhancer of split- and hairy-related protein-2 gene via different pathways in 3T3-L1 adipocytes and L6 myotubes", Horm Metab Res 36, pp.526-530, (2004).
8) Yamada K, Ogata-Kawata H, Matsuura K, and Miyamoto K, "SHARP-2/Stra13/DEC1 as a potential repressor of phosphoenolpyruvate carboxykinase gene expression", FEBS Lett 579, pp.1509-1514, (2005).
9) Yamada K, Mishima A, Hanaoka Y, Misaki T, Kamijo A, Sakamoto N, Chino Y, Horiuchi S, Kondo H, Nagai A, Maruyama Y, Yamane Y, Tanokuchi A, Tsukada A, and Takagi K, "Identification and characterization of the SHARP-2-interacting proteins.", J. Matsumoto Univ. 20, pp.105-115, (2022).
10) Morinaga T, Yasuda H, Hashimoto T, Higashio K, and Tamaoki T, "A human alpha-fetoprotein enhancer-binding protein, ATBF1, contains four homeodomains and seventeen zinc fingers", Mol Cell Biol 11, pp.6041-6049, (1991).
11) Yasuda H, Mizuno A, Tamaoki T, and Morinaga T, "ATBF1, a multiple-homeodomain zinc finger protein, selectively down-regulates AT-rich elements of the human alpha-fetoprotein gene", Mol Cell Biol 14, pp.1395-1401, (1994).
12) Sun X, Frierson HF, Chen C, Li C, Ran Q, Otto KB, Cantarel BL, Vessella RL, Gao AC, Petros J, Miura Y, Simons JW, and Dong JT, "Frequent somatic mutations of the transcription factor ATBF1 in human prostate cancer", Nat Genet 37, pp.407-412, (2005).
13) Sun X, Zhou Y, Otto KB, Wang M, Chen C, Zhou W, Subramanian K, Vertino PM, and Dong JT, "Infrequent mutation of ATBF1 in human breast cancer", J Cancer Res Clin Oncol 133, pp.103-105, (2007).
14) Cho YG, Song JH, Kim CJ, Lee YS, Kim SY, Nam SW, Lee JY, and Park WS, "Genetic alterations of the ATBF1 gene in gastric cancer", Clin Cancer Res 13, pp.4355-4359, (2007).
15) Kai K, Zhang Z, Yamashita H, Yamamoto Y, Miura Y, and Iwase H, "Loss of heterozygosity at the ATBF1-A locus located in the 16q22 minimal region in breast cancer", BMC Cancer 8, pp.262, (2008).
16) Morikawa M, Derynck R, and Miyazono K, "TGF-β and the TGF-β Family: context-dependent roles in cell and tissue physiology", Cold Spring Harb Perspect Biol 8, (2016).
17) Vander Ark A, Cao J, and Li X, "TGF-β receptors: In and beyond TGF-β signaling", Cell Signal 52, pp.112-120, (2018).
18) Sakata N, Kaneko S, Ikeno S, Miura Y, Nakabayashi H, Dong XY, Dong JT, Tamaoki T, Nakano N, and Itoh S, "TGF- β signaling cooperates with AT motif-binding factor-1 for repression of the α -fetoprotein promoter", J Signal Transduct 2014, pp.970346, (2014).
19) Shou ZF, Zhou Q, Cai JR, Chen JH, Yamada K, and Miyamoto K, "PI-3 kinase pathway can mediate the effect of TGF-beta1 in inducing the expression of SHARP-2 in LLC-PK1 cells", J Zhejiang Univ Sci B 10, pp.702-706, (2009).
20) Hata A, and Chen YG, "TGF-β signaling from receptors to Smads", Cold Spring Harb Perspect Biol 8, (2016).
21) Hughes M, Dobric N, Scott IC, Su L, Starovic M, St-Pierre B, Egan SE, Kingdom JC, and Cross JC, "The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling

to promote terminal differentiation of trophoblast stem cells", Dev Biol 271, pp.26-37, (2004).

22) Boudjelal M, Taneja R, Matsubara S, Bouillet P, Dolle P, and Chambon P, "Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells", Genes Dev 11, pp.2052-2065, (1997).

23) Shen M, Yoshida E, Yan W, Kawamoto T, Suardita K, Koyano Y, Fujimoto K, Noshiro M, and Kato Y, "Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages", J Biol Chem 277, pp.50112-50120, (2002).

24) Zhang L, Zhou F, and ten Dijke P, "Signaling interplay between transforming growth factor-β receptor and PI3K/AKT pathways in cancer", Trends Biochem Sci 38, pp.612-620, (2013).

25) Martelli AM, Faenza I, Billi AM, Falà F, Cocco L, and Manzoli L, "Nuclear protein kinase C isoforms: key players in multiple cell functions?", Histol Histopathol 18, pp.1301-1312, (2003).

26) Farese RV, Sajan MP, and Standaert ML, "Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): actions and defects in obesity and type II diabetes", Exp Biol Med (Maywood) 230, pp.593-605, (2005).

27) Matsumoto M, Ogawa W, Akimoto K, Inoue H, Miyake K, Furukawa K, Hayashi Y, Iguchi H, Matsuki Y, Hiramatsu R, Shimano H, Yamada N, Ohno S, Kasuga M, and Noda T, "PKClambda in liver mediates insulin-induced SREBP-1c expression and determines both hepatic lipid content and overall insulin sensitivity", J Clin Invest 112, pp.935-944, (2003).