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ORIGINAL ARTICLE

ADAMTS1 is up-regulated via the SMAD dependent TGF-β signaling pathway in hepatocellular carcinoma

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Abstract

Background ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs 1), a member of the ADAMTS family, is a critical molecule due to its dual roles as a potent angiogenesis inhibitor and an aggrecanase. The dysregulation of ADAMTS1 is a common feature in many pathophysiological conditions, making the understanding of its regulation vital. The primary focus of this investigation was to explore the regulatory role of SMAD transcription factors, the central mediators of the TGF-β signaling pathway, on ADAMTS1 gene expression in Hep3B cells.

Methods and results To assess the impact of SMAD factors, changes in both transcriptional activity and mRNA levels of ADAMTS1 were quantified using co-transfection experiments. Ectopic expression of SMAD2, SMAD3, and SMAD4 was validated by preliminary Western blot and Real-time PCR steps. Co-transfection with SMAD2/SMAD4 resulted in a significant 5-fold increase in ADAMTS1 expression at 24 h, while the SMAD3/SMAD4 complex demonstrated a substantial 3.5-fold increase (with induction sustained up to 72 h). Transcriptional activity was confirmed using promoter fragment and co-transfection analyses performed via the calcium-phosphate precipitation method. Crucially, the Electrophoretic Mobility Shift Assay (EMSA) directly confirmed that SMAD transcription factors bind to the ADAMTS1 promoter region, validating a direct regulation hypothesis.

Conclusions These comprehensive findings demonstrate that ADAMTS1 gene expression is under the direct transcriptional control of SMAD factors in Hep3B cells, showing strong induction by both SMAD2/4 and SMAD3/4 complexes. This study provides the first mechanistic evidence for this direct regulation, significantly enhancing the understanding of how the TGF-β signaling axis controls ADAMTS1 expression. This insight is highly relevant to its pathological functions in angiogenesis, tissue remodeling, and cancer progression.

Keywords ADAMTS1 · TGF-beta · SMAD transcription factors · Hepatocellular carcinoma · Gene expression · Transcriptional regulation

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and a leading cause of cancer-related death globally [1]. Its occurrence varies by region, largely mirroring the distribution of its main risk factors. Chronic liver inflammation and cirrhosis are the strongest predispositions, often stemming from chronic hepatitis B or C virus infections, alcohol-related liver disease, and increasingly, non-alcoholic fatty liver disease [2]. The disease's development is complex, driven by genetic and epigenetic changes in liver cells under chronic stress, leading to uncontrolled growth and invasion [3]. The tumor microenvironment, characterized by inflammation, fibrosis, and abnormal blood vessel formation, also significantly aids tumor progression and helps it evade the immune system [4].

The extracellular matrix (ECM) is a crucial and active player in hepatocellular carcinoma (HCC) progression. Among the various proteases that regulate the ECM, ADAMTS (A Disintegrin And Metalloprotease with Thrombospondin Type-1 Motifs) represent a characterized family.

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These enzymes are hypothesized to play a significant role in both carcinogenesis and cancer dissemination. ADAMTS enzymes participate in a multitude of pathological processes, including but not limited to extracellular matrix formation, arthritis, atherosclerosis, fibrosis, organogenesis, hemostasis, and wound healing. Furthermore, specific members of this enzyme family have been implicated in the pathogenesis of both cancer and cardiovascular diseases [5].

Among the various proteases regulating the extracellular matrix, ADAMTS family members are critical players in carcinogenesis and cancer dissemination [5]. ADAMTS1, in particular, has received significant attention in oncology primarily due to its prominent anti-angiogenic activity. Studies have shown that ADAMTS1 can inhibit vascularization induced by factors like VEGF and FGF-2 [6–9], and it has been demonstrated to suppress endothelial tube formation in vitro [10]. Furthermore, ADAMTS1 is integral to several physiological processes, including organogenesis and the formation of blood and lymph vessels [11]. The multifaceted roles of ADAMTS1 highlight its potential as a key regulatory factor in HCC progression and its related microenvironment.

The dynamic interaction between cytokines and ADAMTS-1 (A Disintegrin And Metalloprotease with Thrombospondin Type-1 Motifs) is a crucial area of research, as these signaling proteins not only orchestrate vital biological processes like inflammation and immune responses but also significantly regulate the expression and activity of matrix-modifying enzymes. ADAMTS-1, a key protease involved in extracellular matrix (ECM) turnover, frequently sees its expression and activity modulated by various cytokines.

A 2023 study by Hu et al. established that ADAMTS1 positively regulates the expression and extracellular release of Transforming Growth Factor-beta TGF-β in non-small cell lung cancer (NSCLC) cells. Further investigation using siRNA targeting TGF-β demonstrated that inhibiting its expression reversed the ADAMTS1-mediated promotion of NSCLC cell migration, invasion, and epithelial-mesenchymal transition (EMT). These collective findings suggest that ADAMTS1 regulates the aggressive phenotype of NSCLC cells via the TGF-β pathway [12]. Consequently, deciphering the cytokine-ADAMTS-1 axis is crucial for understanding its involvement in diverse pathologies, including cancer progression.

Canonical TGF-β signaling is primarily mediated through the SMAD protein pathway. The cascade begins when a TGF-β ligand binds to a cell-surface complex composed of Type I and Type II TGF-β transmembrane serine/threonine kinase receptors. The activated receptor then phosphorylates receptor-regulated SMADs (R-SMADs), specifically SMAD2 and SMAD3. These phosphorylated R-SMADs subsequently complex with SMAD4 (Co-SMAD) and translocate to the nucleus [13–15]. Once in the nucleus, this SMAD complex binds to specific promoter elements, either alone or with other DNA-binding subunits, to activate target genes [16].

In our study, we studied the SMAD-mediated regulation of the ADAMTS-1 gene in the Hep3b cell line. The intracellular signaling pathways through which the TGF-β cytokine, which has been identified to have an effect on the ADAMTS1 gene, affects ADAMTS1 were studied at the mRNA, protein, and promoter levels. The activity of ADAMTS-1 promoter fragments of varying lengths was analyzed by co-transfection assays. Bioinformatically, binding motifs for the transcription factors SMAD2, SMAD3, and SMAD4 were identified in the ADAMTS1 promoter sequence. These expression plasmids, together with ADAMTS1 promoter fragments, were transiently transfected into Hep3B cells, and their contribution to ADAMTS1 promoter activity was determined by measuring luciferase activity. The presence of functional binding was determined by the non-radioactive EMSA method. ADAMTS1 mRNA expression levels were compared using a qRealTime PCR-based method after cotransfection. The effects of SMAD2, SMAD3, and SMAD4 transcription factors on ADAMTS1 mRNA levels were determined. The effect was confirmed by abolishing the effect in inhibitory experiments using a SMAD inhibitor. According to the data we obtained as a result of our study, when the response of ADAMTS1 to TGF-β cytokine is examined, it is thought that this response occurs through the SMAD-dependent pathway.

Materials and methods

Materials and vectors

The Human Liver Carcinoma (Hep3B) cell line used in this project was provided by Dr. Ramji from Cardiff University. The ADAMTS1 promoter fragments used in this study are contained in the pmetluc vector, a luciferase-based vector. The pmetluc vector system, which contains the Metridia longa (marine copepod) luciferase gene, was used as a reporter gene to determine promoter activity were purchased from ClontTech. pMET-Luc control vector and Seap control vectors were used to control the success of the transfection study. The ADAMTS1 truncated promoter constructs were kindly provided by Dr. Satoshi Hirohata (Okayama University Graduate School of Medicine, Okayama, Japan) [17, 18].

Cell culture

The cells were grown in culture flasks at 37 °C in a fully humidified incubator with 5% CO_2 using DMEM Medium (Invitrogen) and 10% fetal calf serum (Sigma) solution. Trypan blue exclusion controls the viability of cells. Cells were seeded at a density of 2,000,000 cells per 25 cm^2 flask and incubated for a full night to enable cell attachment to wells for both mRNA and protein analysis [18].

MTT test

After the transfection process, cells were divided into 96-well plates with 5000 cells per well. In groups that completed the appropriate time interval, 20 µl of MTT solution was applied to each well and the cells were left at 37 °C for 4 h to metabolize MTT. The medium was removed. Formazan crystals, formed by applying isopropanol containing 0.004 M HCl, were dissolved with MTT solution by pipetting, and absorbance values of the cells were measured on a spectrophotometer at a wavelength of 550 nm [19].

Transient transfection by calcium-phosphate precipitation and cytokine application

The transient transfection of promoter constructs was performed using the calcium phosphate precipitation method, as previously detailed by Yıldırım and Koçkar [20]. For assessing basal transcriptional activity, 0.5 µg of promoter constructs were introduced into 12-well plates, each seeded with 250,000 cells. The cells were transferred to a CO_2 incubator for a 6-hour incubation. At the end of this period, the medium was removed from the wells, and all wells were filled with medium containing 0.1% BSA. One hour later, 500 U/ml of TGF-β cytokine was applied to the relevant wells. At the end of 24, 48 and 72 h of incubation, luciferase and released alkaline phosphate activities were measured by luminometer. The cells were also transfected with recombinant plasmids containing SMAD2 and SMAD4, and SMAD3 and SMAD4, along with 0.5 µg of the pMETLuc control vector to analyze transfection efficiency. In cotransfection assays, Hep3B cells received 2 µg of transcription factor expression vectors alongside their respective promoter constructs [18]. To analyze the transfection efficiency, 0.5 µg of SEAP-2 (secreted alkaline phosphatase) vector was also transfected into cells with recombinant plasmids containing the promoter fragment (pMetLuc/ADAMTS1promotor + SEAP-2) and compared with the basal activity of the SMAD transfected groups (pMetLuc/ADAMTS1promotor + SEAP-2) (pMetLuc/ADAMTS1promotor + SEAP-2 + SMAD2-4 or SMAD3-4). The Clontech Ready-To-Glow™ Secreted Luciferase System was used to determine luciferase enzyme activity. Measurements were taken with a Luminoskan Ascent luminometer. The results were normalized by the ratio of luciferase activity to SEAP activity in Excel. Transfection efficiency was evaluated by co-transfection of the pMetLuc control vector and the SEAP control vector, and separately.

RNA preparation and semi quantitative RT-PCR

To ensure optimal cell density for transfection, Hep3B cells were seeded into 25 cm^2 flasks 24 h prior, aiming for an approximate count of 2×10^6 cells. For the transfection procedure, 10 µg of each DNA construct (SMAD2, SMAD3, and SMAD4) was prepared in separate tubes. A 2 M $CaCl_2$ solution was then added to each DNA preparation. As a control for the transfection efficiency and reporter activity, the pMETLuc Control vector was also included. Subsequently, 2X HEPES buffer was introduced, and the mixtures were incubated at room temperature for 45 min to facilitate DNA precipitation [18]. Following a six-hour incubation period after transfection, the transfection solution was replaced with fresh medium. The subsequent day, all flasks received medium containing 0.1% Bovine Serum Albumin (BSA). One hour later, relevant flasks were treated with TGF-β cytokine at a concentration of 500 U/ml. At designated harvest time points, cells were detached using trypsin and collected as pellets for subsequent RNA isolation. RNA isolation was performed from the collected cell pellets, encompassing both control and experimental groups. RNA was extracted by diligently following the steps outlined in a commercial kit (Fermentas). The isolated RNA samples were then stored long-term at −80 °C in a freezer.

RT-PCR was performed using primers designed using information published in GenBank on ADAMTS1, Hβ2, SMAD2, SMAD3, and SMAD4 mRNA sequences. All primer specifications and accession numbers for each gene are shown in Table 1.

Real time (Quantitative) PCR

For each experimental set, real-time PCR was performed in triplicate using the 'LightCycler 480 SSYBR Green I Master' real-time PCR kit under the specified conditions. The specified amounts of the indicated solutions were added to 96-well plates. The plate was covered with film and placed in the Roche Real-Time PCR device. The Ct values obtained were first averaged for each experimental group, as three replicates were used. Then, since a single control was intended, the average of the control groups for each time period was calculated. The Ct values obtained with the Human-β−2 microglobulin gene, our control gene, were evaluated using the livac method. The PCR was performed

Table 1 Primers and product lengths used in gene expression analyses

Gene name	Expression primers	Product lenght	Accession number	Annealing temperature
ADAMTS1	FP- CAGCCCAAGGTTGTAGATGGTA RP- TTCACTTCGATGTTGGTGGCTC	241 bp	NM_006988	50 °C
hβ2	FP-TTTCTGGCCTGGAGGCTATC RP-CATGTCTCCATCCCACTTAACT	314 bp	NM_004048	60 °C
SMAD2	FP- CGCCAGTTGTGAAGAGACTGCTGG RP- GGGATACCTGGAGACGACCATCAA	329 bp	XM_006722451.1	62 °C
SMAD3	FP- GTGAAGCGCCTGCTGGGCTGGAA RP- ATTGAAGGCGAACTCACACAGCTCC	135 bp	NM_005902.3	63 °C
SMAD4	FP- TGAAAGTTTGGTAAAGAAGCTGAAG RP- TCACCATCATACTTGATGGAGCAT	355 bp	NM_005359.5	55 °C

using a protocol that was previously optimized [18]. Following amplification, product specificity was confirmed by melting curve analysis, and the data were quantified using the ΔCt method.

Cell treatment, protein isolation and western blot

Hep3B cells were seeded to achieve optimal confluency at the time of transfection. The cells were then transfected with SMAD2, SMAD3, and SMAD4 DNA constructs using the calcium phosphate precipitation method. Following a 6-hour incubation, the transfection medium was replaced with fresh medium. The next day, cells were serum-starved with medium containing 0.1% Bovine Serum Albumin (BSA) for one hour, after which they were stimulated with TGF-β cytokine (500 U/ml). At the designated time points, cells were harvested via trypsinization, collected as pellets, and stored at − 80 °C for subsequent protein isolation. Protein was extracted from frozen cell pellets using RIPA buffer. The Sigma Lowry method was used to calculate the protein concentration of the cell extracts. Western blot analysis was performed as described in our previous publication [18]. The membrane was incubated with a primary antibody (Smad2/3 Sc-133098 lot#j0112 mouse monoclonal IgG2) followed by a HRP-conjugated secondary antibody (Goat anti-mouse IgG-HRP). Following the antibody incubations, the membrane was subjected to imaging.

In Silico analysis and electromobility shift assay (EMSA)

Transcription factors likely to bind to the human ADAMTS1 promoter were identified through bioinformatic analyses (Supplemantary Fig. 1). EMSA studies were conducted to determine whether SMAD transcription factors bind to the relevant region in the ADAMTS1 promoter. Furthermore, unlabeled probes for Sp1, Usf, C/EBP, and Ap1, transcription factors identified by bioinformatics analysis on the ADAMTS1 promoter region, were included in EMSA competition experiments. For this purpose, nuclear extract was first prepared and binding reactions were set up with pre-biotinylated and double-stranded oligonucleotides. For competition reactions, approximately 1000-fold excess of unlabeled oligonucleotides was added to the reaction tube. Nuclear extracts from Hep3B SMAD2/4, SMAD3/4, and untransfected control Hep3B cells were used in binding reactions. After incubation for 10 min on ice, the cells were incubated for 20 min at room temperature. Binding reactions were loaded onto a non-denaturing gel and run at 90 V, 400 mA. The proteins were then transferred to a nylon membrane. After cross-linking, the membrane was blocked and labeled with HRP-conjugated streptavidin. To visualize the complexes, the membrane was treated with ECL and visualized on a Fusion FX VILBER LOURMAT instrument [21] (Table 2).

Inhibition studies of SMAD transcription factors

To address the observed effect, inhibition studies were undertaken targeting SMAD transcription factors. These factors were previously identified, through Electrophoretic Mobility Shift Assay (EMSA) studies, to exhibit specific binding to fragments of the ADAMTS1 promoter. The aim of these inhibition studies was to abrogate or reduce the aforementioned effect mediated by SMAD binding.

To evaluate the impact of the Sis3 inhibitor on cell proliferation, an MTT assay was performed. For this experiment, cells were seeded into 96-well plates 24 h prior to transfection at a density of 50.000 cells per well. The following day, all wells received medium containing 0.1% Bovine Serum Albumin (BSA). One hour later, Sis3 inhibitor was applied at a final concentration of 3 µM. After an additional hour, TGF-β cytokine was administered at 500 U/ml. Following a 24-hour incubation period, MTT solution was applied as described in MTT section, and absorbance values were measured.

To assess the effects on both mRNA and protein levels, cells were equally seeded into 25 cm^2 flasks 24 h prior to the experimental procedures. The following day, all cells were pre-incubated in medium containing 0.1% Bovine Serum

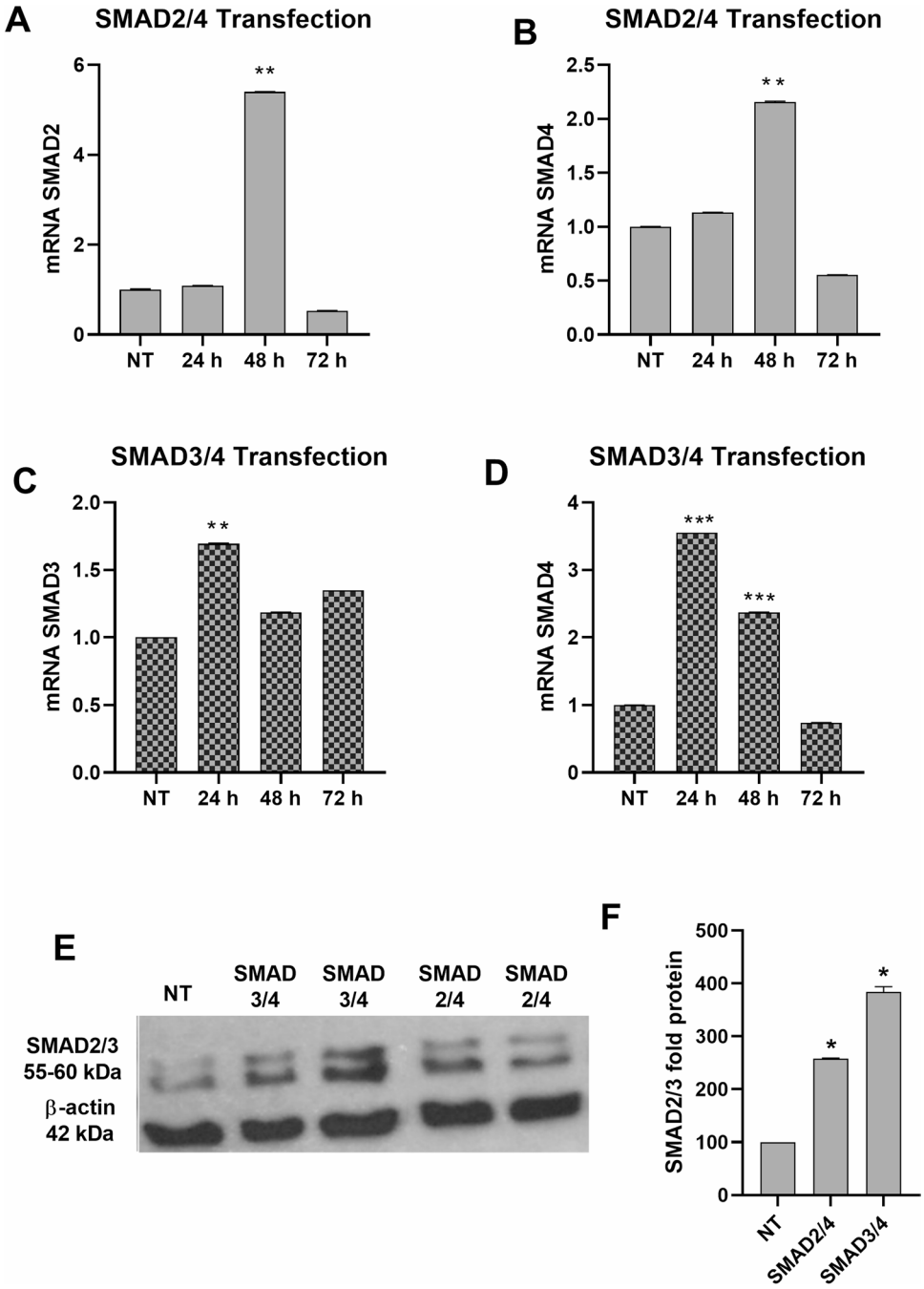

Fig. 1 Control of ectopic expression of SMAD factors transfected in Hep3B cells at the mRNA level and Analysis of SMAD2/3 and β-actin proteins in SMAD 2/4 and SMAD 3/4 transfected Hep3B cells by Western Blot (**A**) SMAD2 mRNA level in SMAD2/4 transfection, (**B**) SMAD4 mRNA level in SMAD2/4 transfection, (**C**) SMAD3 mRNA level in SMAD3/4 transfection, (**D**) SMAD4 mRNA level in SMAD3/4 transfection, (**E**) Membrane image obtained in Western blot, (F) Densitometric analysis of SMAD2/3 and β-actin proteins. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$

Table 2 EMSA primers used in the study

ADAMTS1 (+106/+166)	FP-GGCGGAGGCCGAAGAGGG GCGCCAGGCACCAATCTCCGC GTTGCCTCAGCCCCGGAGGCG
	RP- CCGCCTCCGGCTTCTCCCC GCGGTCCGTGGTTAGTGGCGC AACGGAGTCGGGGCCTCCGC
ADAMTS1 (−1365/−1305)	FP-GGGGAAGATCTGGGGAGG AGCGAGGAAAGGACCCAGATC TACTTGGAGCCAACCAAGAGA
	RP- CCCCTTCTAGACCCCTCCT CGCTCCTTTCCTGGGTCTAGAT GAACCACGGTTGGTTCTCT

Albumin (BSA). One hour later, 3 µM Sis3 (a SMAD inhibitor) was applied. Following an additional one-hour incubation, 500 U/ml of TGF-β cytokine was added to the cells.

Statistical analysis

Real-Time PCR was used to evaluate the cT values of the control and experimental groups using the Livak method, along with their standard deviations. Numerical values of the bands were determined using Image-j for Western Blotting. Results were analyzed in Microsoft Excel. Statistical

analyses of the experimental results were performed using one-way ANOVA (Minitab15 version). A p value of ≤0.05, ≤ 0.01 and ≤0.001 was considered significant.

Results

Ectopic SMAD transcription factor expression and cell viability in Hep3B cells

This study was conducted in two main phases. The first phase focused on confirming the mRNA and protein expression of the ectopically produced SMAD transcription factors. The second phase evaluated potential cytotoxic effects of the transfection and cytokine treatment on Hep3B cells using an MTT assay. All transfection experiments were performed in triplicate and repeated in three independent trials. The activities of the experimental groups were compared to their respective controls, with relative expression levels normalized to the housekeeping gene B2M (β−2 microglobulin). Results demonstrated a significant increase in both SMAD2 and SMAD4 mRNA expression in transfected cells at 48 h compared to the control group (Fig. 1A, B). Similarly, in SMAD3/SMAD4 co-transfected cells, a notable increase in SMAD3 mRNA expression was observed at 24 h (Fig. 1C), while SMAD4 expression also contributed to this effect at both 24 and 48 h (Fig. 1D).

To confirm the successful transfection and expression of the transcription factors central to our study, we performed Western blot analysis to assess the protein levels of SMAD2, SMAD3, and SMAD4 following ectopic expression. Following ectopic transfection, Western blot analysis confirmed a significant increase in SMAD2, SMAD3, and SMAD4 protein levels compared to the control group (Fig. 1E). Densitometric analysis, with values normalized to β-Actin as a loading control, revealed elevated protein expression in the transfected groups, as depicted in Fig. 1F.

As a critical methodological control, the potential cytotoxic effects of both the SMAD plasmid transfection and the TGF-β cytokine treatment on Hep3B cell viability were assessed using the MTT assay at the 24, 48, and 72-hour time points. Overall, neither the transfection nor the cytokine application had a statistically significant impact on cell viability compared to the control group. Specifically, at the 24-hour time point, a non-significant decrease in viability was observed in both the SMAD2/4 and SMAD3/4 transfected groups (Supplementary File 1). At 48 h, the SMAD2/4 group showed no change in viability, while the SMAD3/4 group experienced a non-significant decrease. This trend continued at the 72-hour mark, where no effect was seen in the SMAD2/4 group, but a persistent non-significant decrease was observed in the SMAD3/4 group (Supplementary File 1).

Co-transfection experiments and SMAD-acting activities of ADAMTS1 promoter fragments

A schematic diagram illustrating the ADAMTS1 promoters is provided in Fig. 2A. To assess their activity, transient transfections of the promoter fragments were initially performed in Hep3B cells. The transcriptional activities were subsequently determined at 48 and 72 h using both luciferase and Secreted Alkaline Phosphatase (SEAP) assays. Since the promoter fragments were cloned into a luciferase-based vector, a SEAP-containing vector was co-transfected into Hep3B cells to account for and normalize potential variations in transfection efficiency. Consequently, the luciferase values were normalized to the SEAP activity values (Fig. 2B).

To determine the localization of SMAD binding sites and some transcription factors on the ADAMTS1 promoter, the ADAMTS1 promoter sequence was analyzed using the TF-Binding MATINSPECTOR data program. This analysis identified potential SMAD binding sites (Fig. 2A). These binding sites were observed in the P1:1834 bp (−1415/+419) and P7:548 bp (−129/+419) promoter fragments (Fig. 2A). For this purpose, the P1 and P7 promoter fragments containing the SMAD binding site were used in co-transfection experiments, rather than the entire promoter fragments. In a series of co-transfection and cytokine stimulation experiments, cells were transfected with reporter vectors containing ADAMTS1 promoter fragments along with SMAD expression plasmids. TGF-β was also applied to assess its influence.

Analysis of the P1 promoter fragment revealed no significant transcriptional response to the applied SMAD transcription factors or TGF-β treatment. Although a time-dependent decrease in transcriptional activity was observed at 48 and 72 h compared to basal activity, this effect was not statistically significant (Fig. 2C). In contrast, the P7 promoter fragment showed a clear response. A statistically significant increase in transcriptional activity was observed, particularly following the application of SMAD2/4 and TGF-β together. SMAD3/4 application also increased the transcriptional activity of the P7 promoter fragment; however, this effect was more pronounced in the absence of TGF-β than in its presence (Fig. 2C).

The differential transcriptional activity of the P1 and P7 promoter fragments provides insight into the complex regulation of ADAMTS1. The observation that P7 responds to SMAD2/4, whereas the larger P1 fragment does not, suggests the presence of a repressive regulatory element or a repressor binding site within the P1 sequence that is absent

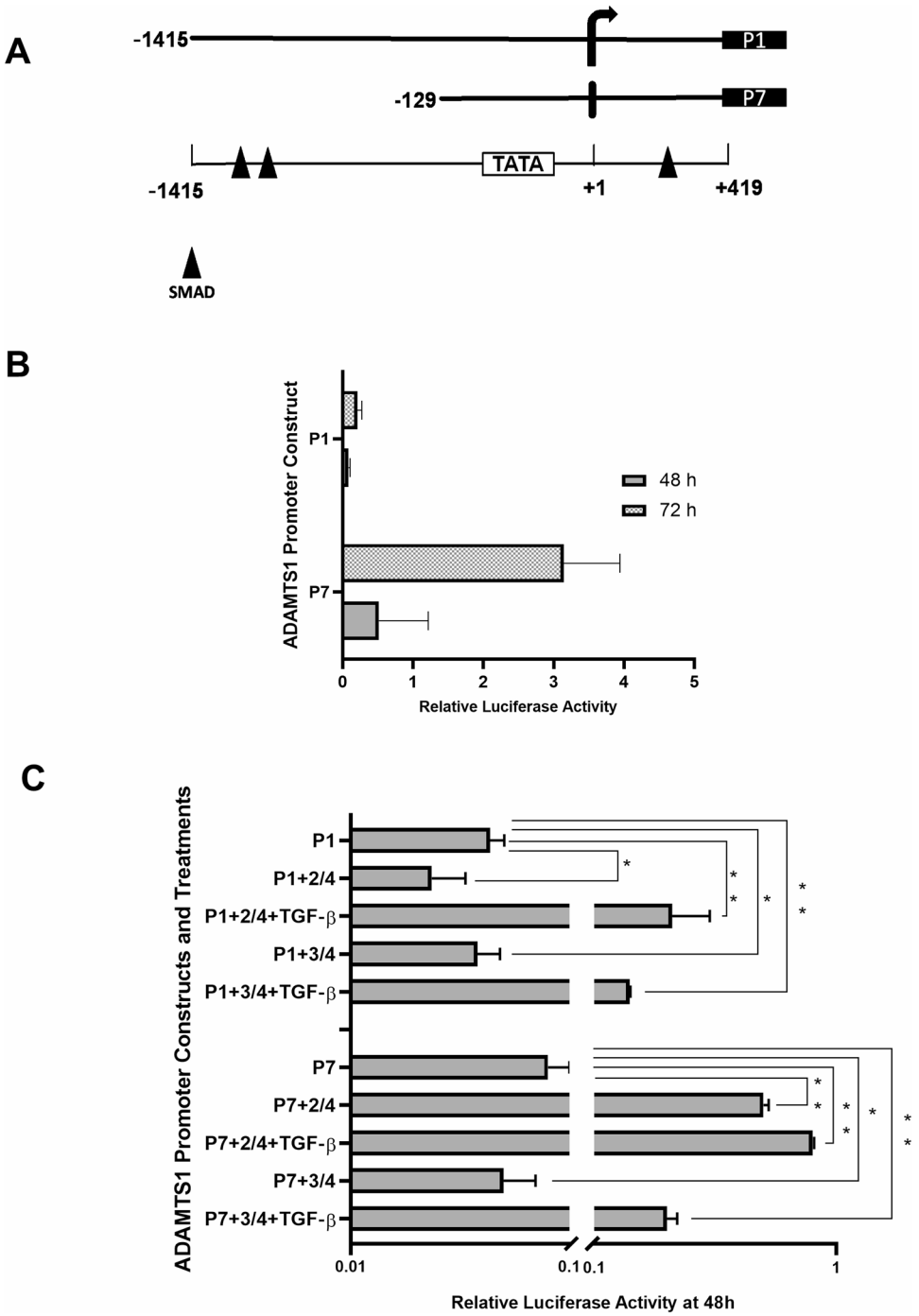

Fig. 2 Transcriptional Activity and Structural Analyses of ADAMTS1 Promoter Fragments in Hep3B Cells. (**A**) ADAMTS1 Promoter Structural Schematic and Bioinformatic Analysis: Schematic representation of the ADAMTS1 promoter fragments (P1 and P7) utilized in the transient transfection experiments, indicating the corresponding base pair positions relative to the transcription start site (+1). This panel also incorporates the results of the bioinformatic analysis which illustrates the predicted localization of potential SMAD binding sites (▲) within the ADAMTS1 promoter sequence, as identified by the TF-Binding MATINSPECTOR program. (**B**) Basal Transcriptional Activity: Basal activity results of the ADAMTS1 promoter fragments (P1 and P7) transfected in Hep3B cells. (**C**) Regulation by TGF-β and SMAD Overexpression: ADAMTS1 promoter activities in Hep3B cells following SMAD co-transfection or treatment with 500 U/mL TGF-β for 48 h

in the shorter P7 construct. This indicates that the positive regulatory effect of the SMAD pathway on the P7 fragment is likely masked in the full-length promoter by an inhibitory mechanism.

Furthermore, the responsiveness of the P7 fragment to TGF-β treatment implies that the SMAD pathway is not the sole mediator of TGF-β signaling at this promoter. The data suggest that multiple regulatory pathways, beyond the canonical SMAD pathway, may converge to control ADAMTS1 expression, highlighting a more intricate regulatory network (Fig. 2C).

Effects of SMAD and TGF-β on ADAMTS1 mRNA expression levels

To determine the functional consequences of the SMAD complexes on the gene expression level, we employed qRT-PCR to quantify ADAMTS1 mRNA levels following the ectopic expression of SMAD2/4 and SMAD3/4 vectors,

both with and without TGF-β stimulation, across a 72-hour time course.

The ectopic expression of SMAD2/4 transcription factors led to a significant increase in ADAMTS1 mRNA levels. Specifically, a 5-fold increase was observed at 24 h compared to the control group, and this elevated expression was sustained at both the 48 and 72-hour time points (Fig. 3A). Conversely, when this group was treated with TGF-β, ADAMTS1 expression was reduced at all time points (Fig. 3B).

Similarly, the ectopic expression of SMAD3/4 also resulted in an increase in ADAMTS1 mRNA levels, with a 3.5-fold increase recorded at 24 h, which persisted at 48 and 72 h (Fig. 3C). However, the effect of TGF-β on the SMAD3/4 group was more complex; ADAMTS1 expression decreased at 24 and 72 h but increased at 48 h (Fig. 3D).

Detection of SMAD binding to the ADAMTS1 promoter by EMSA

An electrophoretic mobility shift assay (EMSA) was performed to investigate whether SMAD transcription factors directly bind to the ADAMTS1 promoter at the −1365/−1305 and +106/+166 regions (Fig. 4A). Nuclear extracts from Hep3B cells transfected with SMAD2/4 and SMAD3/4 revealed specific DNA-protein complexes when incubated with a probe designed for the +106/+166 region. Two distinct complexes, designated C1 and C2, were formed. The intensity of these complexes was notably increased in the SMAD-transfected groups compared to the untransfected control (Fig. 4B, lanes 2, 3, and 4). To confirm the binding specificity, a competition assay was performed. The addition of a 1000-fold molar excess of an unlabeled probe successfully eliminated complex formation, particularly C2, at the +106/+166 site (Fig. 4B, lane 5). These results demonstrate that the SMAD transcription factors ectopically expressed in Hep3B cells specifically bind to the +106/+166 region of the ADAMTS1 promoter.

An electrophoretic mobility shift assay (EMSA) was performed to investigate whether SMAD transcription factors also bind to a second potential site on the ADAMTS1 promoter at the −1365/−1305 region. Nuclear extracts from Hep3B cells ectopically expressing SMAD2/4 and SMAD3/4 were incubated with a probe for this region, revealing the formation of a single distinct DNA-protein complex (C1). A densitometric comparison showed that the level of this complex was increased in the SMAD2/4 transfected group compared to the control Hep3B nuclear extract (Fig. 4C). To confirm binding specificity, a competition assay was conducted. The addition of a 1000-fold molar excess of unlabeled probe led to a noticeable decrease in complex formation (Fig. 4C, lane 5), providing evidence that this region is indeed a SMAD binding site.

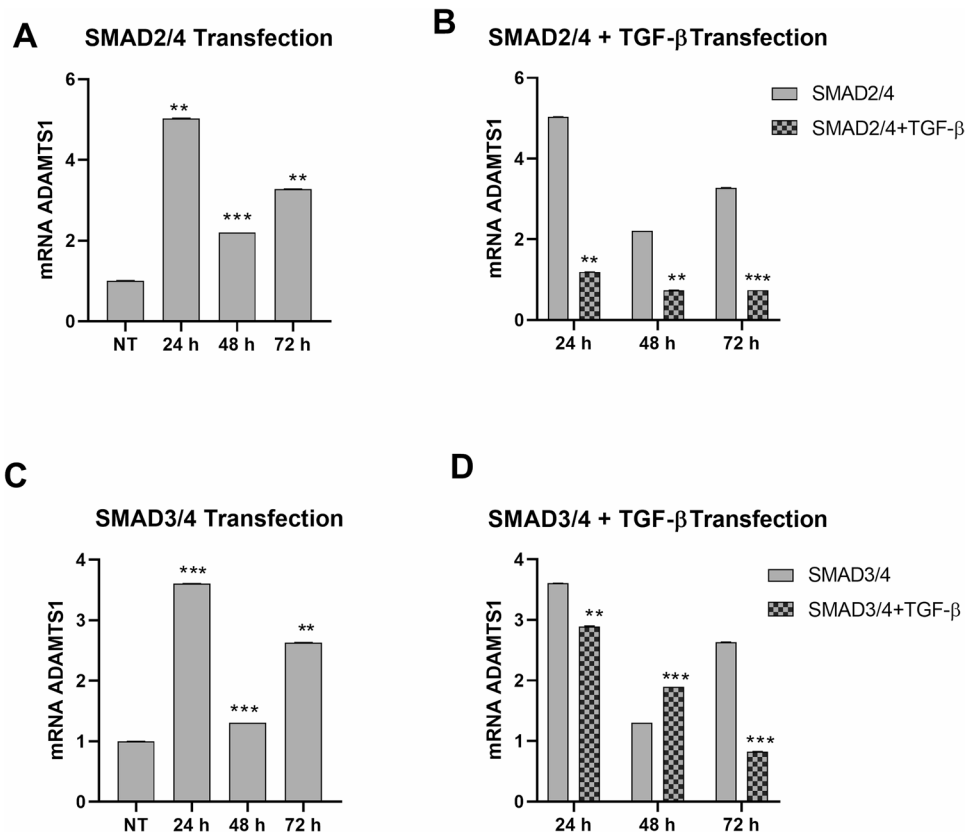

Fig. 3 ADAMTS1 mRNA expression analaysis in SMAD transfected Hep3B cells (**A**) ADAMTS1 mRNA level in SMAD2/4 transfection, (**B**) ADAMTS1 mRNA level in 500 U/mL TGF-β treated SMAD2/4 transfection, (**C**) ADAMTS1 mRNA level in SMAD3/4 transfection, (D) ADAMTS1 mRNA level in 500 U/mL TGF-β treated SMAD3/4 transfection. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$

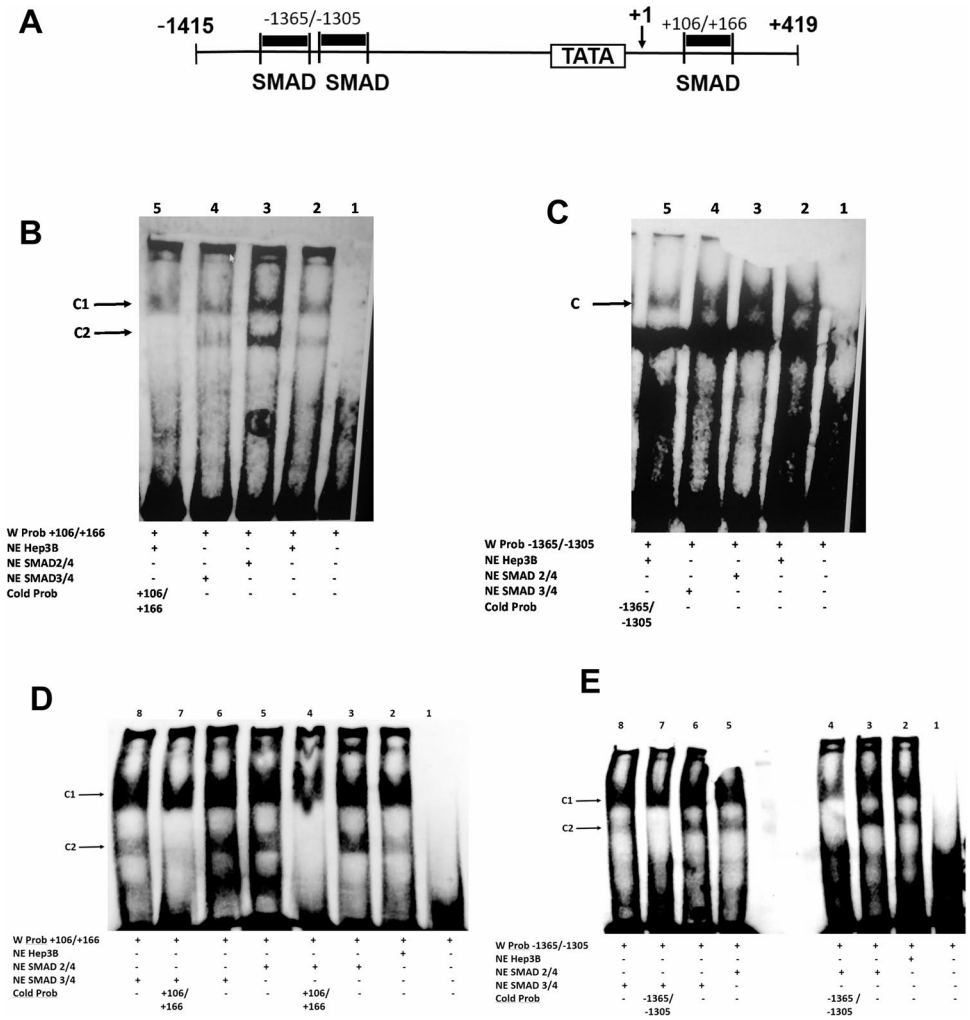

Fig. 4 (**A**) Schematic diagram of the possible SMAD binding sites (−1365/−1305; +106/+166) selected for EMSA located on the −1415/+419 human ADAMTS-1 promoter 1. (**B**) EMSA results of the '+106/+166' ADAMTS1 promoter region based on the ADAMTS1 Smad binding site (Competition experiments) (**C**) EMSA results of the −1365/−1305 ADAMTS1 promoter region based on the ADAMTS1 Smad binding site (Competition experiments). (**D**) Supershift EMSA result of the '+106/+166' ADAMTS1 promoter region according to the ADAMTS1 Smad binding site (**E**) Supershift EMSA result of the −1365/−1305 ADAMTS1 promoter region according to the ADAMTS1 Smad binding site

To confirm the presence of SMAD proteins within the DNA-protein complexes, supershift experiments were performed using the +106/+166 probe. Consistent with the previous findings, increased binding complexes (C1 and C2) were observed in the nuclear extracts from SMAD2/4 and SMAD3/4 transfected cells compared to the control (Fig. 4D). Upon the addition of a SMAD2/3 specific antibody to the SMAD2/4 transfected nuclear extracts, a notable reduction was observed in Complex C2. Similarly, the addition of the antibody to the SMAD3/4 transfected nuclear extracts also resulted in a decrease in Complex C2. While the complexes were clearly reduced by the antibody, a distinct supershift band was not apparent. This suggests that the antibody-bound complexes may have been too large to migrate effectively into the gel matrix.

Supershift analysis was also conducted to confirm the presence of SMAD proteins in the DNA-protein complexes formed at the −1365/−1305 region. The C2 complex observed in nuclear extracts from SMAD2/4 transfected cells was notably stronger than in the control. When a SMAD2/3-specific antibody was added, the C2 complex was substantially diminished, indicating that SMAD proteins are indeed part of the binding complex. However, a distinct supershift band was not apparent, likely due to the size and stability of the antibody-bound complex, which may have inhibited its migration into the gel matrix (Fig. 4E).

Effect of Sis3 inhibitor on SMAD and ADAMTS1 expression levels

To definitively confirm that the observed ADAMTS1 regulation is SMAD-dependent, we utilized the Sis3 inhibitor to specifically block SMAD transcription factors. We then assessed the resulting effects on SMAD mRNA and protein levels, as well as ADAMTS1 mRNA expression and promoter activity across various time points and experimental conditions.

The results show that the cis inhibitor successfully inhibited SMAD transcription factors compared to the control group (A, B, C in Fig. 5). When looking at ADAMTS1 levels, inhibitor application at 6 h statistically significantly reduced ADAMTS1 mRNA levels compared to the control

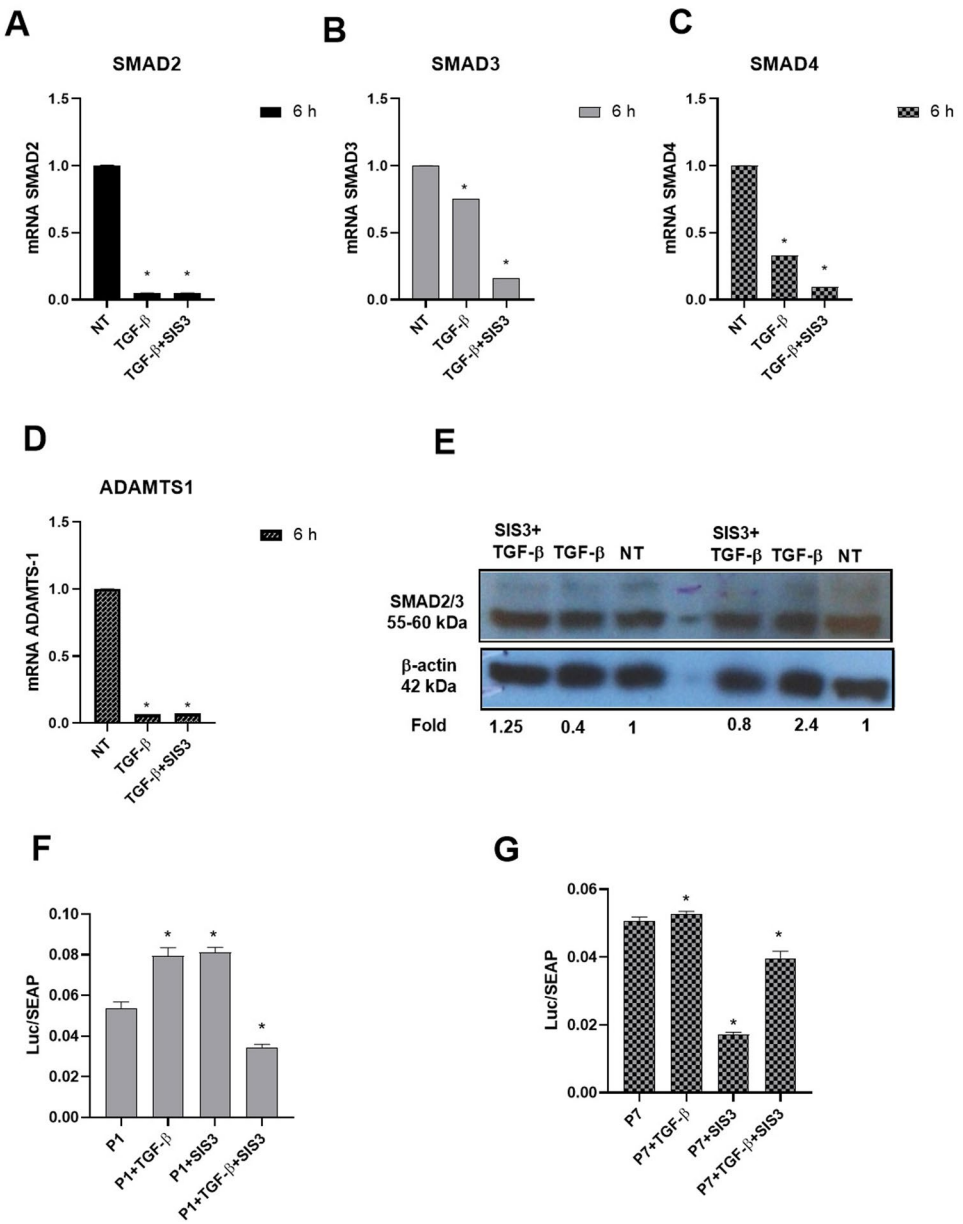

Fig. 5 Effect of SIS3 and TGF-β on (**A**) SMAD2, (**B**) SMAD3, (**C**) SMAD4, and (**D**) ADAMTS1 mRNA Levels in Hep3B cells (**E**) Western blot image and densitometric analysis of Smad2/3 and Human Beta Actin protein expression in cells treated with SIS3 and TGF-β cytokine (**F**) Effect of SIS3 inhibitor and TGF-β cytokine on ADAMTS1 promoter P1 (−1415/+419) in Smad-treated HEP3B cell line 48 h (**G**) Effect of SIS3 inhibitor and TGF-β cytokine on ADAMTS1 promoter P7 (−129/+419) in Smad-treated HEP3B cell line 48 h, $*p \leq 0.05$

group (Fig. 5D). In this experiment, the 6-hour period is particularly important because the inhibitor's effect is observed early at the mRNA level.

As shown in the analysis results in Fig. 5E, no decrease in SMAD protein levels was detected after Sis application in the 6-hour group. The values were almost identical to the control group (1 and 1.25). When the 24-hour results were examined, it was seen that Sis application reduced SMAD factors (1 and 0.8).

When the results are examined, it is seen that the Sis3 inhibitor reduced the activity of the ADAMTS1 P1 promoter fragment at 48 h in the TGF-β + Sis3 inhibitor group (Fig. 5F). Figure 5 shows that the Sis3 inhibitor reduced the transcriptional activity of the P7 promoter fragment at 48 h compared to basal activity. Co-administration of TGF-β and Sis3 re-increased activity. This suggests that even if SMADs were inhibited, TGF-β treatment re-increased activity. This suggests the existence of both SMAD and a different mechanism.

Discussion

Alterations within the tumor microenvironment are fundamental for driving cancer progression and metastasis. Traditionally, the activities of secreted or membrane-associated metalloproteases have been causally linked to an augmented tumorigenic potential in cancer cells. However, a growing

body of contemporary research now substantiates a tumor-suppressive role for various distinct metalloproteases [22, 23].

With specific regard to ADAMTSs (A Disintegrin And Metalloprotease with Thrombospondin Motifs), these enzymes are secreted by either tumor or stromal cells and possess the capacity to modulate the primary tumor microenvironment through both proteolytic-dependent and independent mechanisms. Consequently, it can be postulated that the pro-tumoral functions or anti-oncogenic properties instigated by ADAMTSs may be contingent upon the specific substrates or interacting partners present within the cellular microenvironment.

A prospective challenge of considerable importance involves the precise differentiation of ADAMTS-mediated interactions that potentiate tumor growth and metastasis from those that effectively suppress them. This distinction is critical for understanding the dual role of these enzymes in cancer progression. A deeper understanding of the molecular mechanisms orchestrated by ADAMTSs would significantly advance our comprehension of the true impact these metalloproteases have on tumorigenesis. The clarification of these intricate processes is expected to ultimately facilitate the introduction of more highly specific and less toxic personalized anti-tumor therapeutic strategies.

ADAMTS-1 plays a role in physiological events such as organogenesis, blood/lymphatic vessel formation, ovarian folliculogenesis, and ovulation [11]. ADAMTS1 inhibits VEGF-induced angiogenesis. It also suppresses fibroblast growth factor-2-induced vascularization [9]. ADAMTS1 also has aggrecanase activity in the degradation of aggrecan, a major cartilage protein. It has also been found to degrade versican, which is found in blood vessels and brevican, which is heavily expressed in the central nervous system. Aggrecan is the primary proteoglycan of cartilage and is responsible for the tissue's resistance to compression by hydrating and swelling against the type II collagen spiral. Numerous studies investigating the roles of ADAMTS1 in cancer have revealed changes in ADAMTS1 mRNA and protein levels during the progression of prostate, liver, and mammary gland tumors [11]. These studies have also demonstrated the anti-angiogenic effects and role of ADAMTS1. Angiogenesis plays a crucial role in tumor progression. Several reports indicate that ADAMTS1 inhibits angiogenesis through multiple mechanisms.

The role of the protease ADAMTS1 in cancer progression is highly complex and remains context-dependent, exhibiting both pro- and anti-tumor characteristics across different malignancies. While some studies suggest a tumor-suppressive role, such as the finding that ADAMTS1 deficiency reduced survival and tumor burden in the MMTV-PyMT breast cancer model [24], other evidence points towards an oncogenic function. Specifically, ADAMTS1 is frequently observed to be overexpressed in metastatic carcinomas and has been linked to increased lymph node metastasis and retroperitoneal invasion in pancreatic carcinoma patients [25]. This variability is further highlighted by conflicting expression patterns across tumor types, showing up-regulation in cervical carcinoma [26] but down-regulation in some breast [5], colorectal [27], and prostate carcinomas [28]. Furthermore, its expression appears to be regulated epigenetically, as demonstrated by the high frequency of ADAMTS1 methylation in gastric tumor tissues [29]. This broad functional inconsistency necessitates further investigation into the specific signaling pathways, such as the Smad cascade, that may modulate ADAMTS1 expression and activity within the HCC microenvironment.

ADAMTS1 mRNA and protein expression levels have been shown to increase rapidly in endothelial cells under hypoxia. They also demonstrated that ADAMTS1 is a target gene of HIF1α [17]. Another study investigated its regulation by SP1, USF, and c/Ebp transcription factors in HEP3B cells under normal and hypoxic conditions (Turkoglu and Kockar, 2016). The transforming growth factor (TGF-β) family comprises a large group of extracellular growth factors that control multiple aspects of development. TGF-β members, which include numerous similar polypeptide growth factors, are capable of regulating cellular processes such as cell proliferation, differentiation, motility, adhesion, and death. TGF-β and its related factors play crucial roles in the development, homeostasis, and repair of all tissues in the body. All of these factors play a crucial role in the regulation of intracellular signals [30].

The rise in hepatocellular carcinoma (HCC) is a significant public health problem due to delayed diagnosis and limited treatment options. TGF-β is known to provide cytostatic signals in the early stages of liver injury and regeneration, but it also exerts a potentiating effect on tumor initiation and liver cancer progression [31]. The biochemical backbone of the TGF-β signaling pathway and how it translates cellular responses to these signals have been thoroughly elucidated. SMAD transcription factors are at the core of this pathway [32].

Our study aimed to elucidate the regulatory role of SMAD transcription factors on the ADAMTS1 promoter. The ectopic expression of SMAD2, SMAD3, and SMAD4 in Hep3B cells was successfully validated at both the mRNA and protein levels. Importantly, neither the transfection procedure nor the subsequent TGF-β treatment yielded any statistically significant cytotoxic effects, thus validating the integrity of our experimental model.

Based on bioinformatic analysis, we identified potential SMAD binding motifs within the ADAMTS1 promoter sequence. To test their functional relevance, we performed

a series of co-transfection experiments using promoter fragments of varying lengths. Our analysis of the full-length P1 promoter fragment, however, did not show a statistically significant change in transcriptional activity in response to SMAD transfection or TGF-β treatment over a 72-hour period. This initial finding, despite the presence of predicted binding sites, suggests that the regulation of the full-length promoter is more complex, potentially involving other regulatory elements that mask the direct effect of SMADs.

Our findings demonstrate a key difference in the transcriptional regulation of the ADAMTS1 promoter. Unlike the full-length P1 promoter, which showed no significant response to SMAD transcription factors, the shorter P7 promoter segment exhibited a statistically significant increase in transcriptional activity. This differential response suggests that the larger P1 fragment likely contains a repressive element or binding site that is absent in the smaller P7 segment, effectively masking the stimulatory effect of SMADs. The responsiveness of the P7 promoter to SMAD application provides further insight into the regulatory mechanisms. Specifically, the combined application of SMAD2/4 and TGF-β resulted in a notable increase in transcriptional activity. Interestingly, the effect of SMAD3/4 was more complex; while it increased activity on its own, its effect was diminished in the presence of TGF-β, suggesting a potential antagonistic interaction. The responsiveness of P7 to both SMADs and TGF-β collectively indicates that ADAMTS1 regulation involves the canonical SMAD pathway along with other distinct signaling pathways that work together to modulate expression.

Electrophoretic mobility shift assays (EMSA) confirmed that SMAD transcription factors ectopically expressed in Hep3B cells specifically bind to two distinct sites on the ADAMTS1 promoter, located at +106/+166 and −1365/−1305. The specificity of this interaction was demonstrated by competition assays, where a molar excess of unlabeled probe successfully eliminated complex formation. Additionally, supershift analysis further indicated that SMAD proteins are a component of these complexes, although a distinct supershift band was not observed.

Despite the definitive evidence of transcriptional induction, a functional paradox remains when comparing the initial activation signal with the final steady-state mRNA levels. The Luciferase data confirms that the TGF-β signal has the capacity to directly activate the ADAMTS1 promoter via the SMAD pathway [33, 34]. However, qRT-PCR measures the net mRNA accumulation, which is a balance between transcription and degradation. We propose that TGF-β simultaneously activates non-SMAD signaling pathways (MAPK, ERK) and post-transcriptional mechanisms that promote the rapid degradation of the nascent ADAMTS1 transcript [35, 36]. This simultaneous counter-regulation leads to the observed net decrease in mRNA despite the initial promoter activation. The dual regulatory mechanism of TGF-β in cancer is characterized by its ability to act as both an inhibitor and a promoter, entirely depending on the cellular context [37–39].

The incorporation of Electrophoretic Mobility Shift Assay (EMSA) results, which confirm the direct physical binding of the SMAD complex to the ADAMTS1 consensus sequence in the promoter, definitively validates the conclusion from the Luciferase Reporter Assay that ADAMTS1 is a direct transcriptional target of the TGF-β/SMAD pathway. This evidence establishes the initial signal as one of transcriptional induction. However, the functional paradox remains: despite this confirmed positive transcriptional signal, the endogenous mRNA expression analysis shows a net reduction in mature ADAMTS1 mRNA levels. This strongly suggests that the transcriptional activation is rapidly and dominantly countered by a subsequent, overriding mechanism. This potent inhibitory action is most likely attributed to post-transcriptional gene silencing (e.g., accelerated mRNA decay or targeted microRNA-mediated degradation induced concurrently by the SMAD pathway), or an antagonistic regulatory loop (a repressive cofactor recruited to the native chromatin locus) that effectively dictates the final steady-state mRNA level [40, 41], ultimately resulting in a functional suppression of ADAMTS1 expression. This complex finding suggests a critical regulatory loop in Hep3B cells that rapidly curtails the ADAMTS1 transcript, potentially influencing the tumor microenvironment [42].

Our findings provide new insights into the complex regulation of ADAMTS1 by the TGF-β/SMAD signaling pathway in hepatocellular carcinoma (HCC). We first confirmed the efficacy of the SIS inhibitor, observing a significant reduction in SMAD transcriptional activity and a corresponding decrease in ADAMTS1 mRNA levels as early as 6 h after treatment. This rapid transcriptional effect, preceding any detectable decrease in SMAD protein levels, highlights the immediate and potent action of the inhibitor on its target pathway.

The differential regulation of the P1 and P7 promoter fragments by TGF-β and SIS3 is particularly revealing. While the SIS3 inhibitor alone reduced the activity of both promoter fragments, suggesting a SMAD-dependent regulation, the co-administration of TGF-β and SIS3 restored transcriptional activity in the P7 promoter. This intriguing finding indicates that TGF-β can regulate the ADAMTS1 promoter through both a SMAD-dependent pathway and an alternative, SMAD-independent mechanism. This observation is consistent with the current understanding that TGF-β signaling is highly complex and context-dependent, particularly in HCC, where its role in tumor progression is well-documented but not yet fully understood.

Our results align with recent studies suggesting that the TGF-β/SMAD signaling pathway is associated with poor prognosis in HCC, even though genetic alterations in this pathway are relatively rare in this cancer type [43, 44]. The presence of a SMAD-independent regulatory mechanism for ADAMTS1 suggests that other signaling members, such as p38/MAPK, JAK/STAT, and PI3K/mTOR, may contribute to TGF-β-mediated gene regulation through protein-protein interactions or by influencing the transcriptional activity of other downstream factors [45]. These findings underscore the intricate nature of ADAMTS1 regulation and the broader TGF-β signaling network in the context of the tumor microenvironment. Further research is warranted to fully elucidate the roles of these different signaling pathways.

Conclusion

This study provides significant mechanistic insights into the complex and multi-layered role of the TGF-β/SMAD signaling pathway in the regulation of the ADAMTS1 promoter in Hep3B cells. In conclusion, our results demonstrate that ADAMTS1 is controlled in a precise and context-sensitive manner within the TGF-β signaling network, which is critical in cancer progression. Deciphering this complex regulatory network provides a vital foundation for developing new therapeutic strategies targeting ADAMTS1 in the context of HCC treatment and tumor microenvironment management.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Not applicable.

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