

# TGF- $\beta$ 1 Transactivates ADAMTS-2 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 2) in Saos-2 Cells through Canonical and Non-Canonical Pathways

Meltem Alper\*, \*\* (ORCID: 0000-0001-6359-9979)

Department of Translational Oncology, Institute of Oncology, Dokuz Eylül University, Balçova, İzmir, 35330 Turkey

\*e-mail: biologmeltem@hotmail.com

\*\*e-mail: meltem.alper@deu.edu.tr

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**Abstract**—Osteosarcoma is a malignant bone tumor that is common in children and adolescents. The tumor microenvironment is highly effective in the development and progression of osteosarcoma. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is one of the most abundant cytokines in the tumor microenvironment, and can regulate tumor initiation, progression, and metastasis promoting extracellular matrix (ECM) remodeling and epithelial-mesenchymal transition (EMT). ADAMTS (ADAM Metallopeptidase with Thrombospondin Motifs) proteases have critical functions in normal and tumor microenvironments by processing individual proteins in the ECM. ADAMTSs contribute to tissue remodeling, inflammation, cell migration and, angiogenesis. Among the family members, ADAMTS-2 is a well-known example for ECM remodeling which cleaves the N-terminal propeptide of procollagen and promotes correct collagen fibrillogenesis. Cytokines can regulate normal and tumor microenvironments by affecting ECM proteins. In this study, the effect of TGF- $\beta$ 1, on the transcriptional regulation of the ADAMTS-2, which is an essential enzyme for ECM remodeling was investigated in Saos-2 cells. TGF- $\beta$ 1 upregulated ADAMTS-2 expression both at mRNA and protein levels. Transient transfection assays revealed that TGF- $\beta$ 1 was also induced ADAMTS-2 promoter activity. According to the pathway inhibition studies, both canonical and non-canonical signaling pathways and post-translational mechanisms were responsible for the induction. These studies will contribute to future research on ADAMTS-2 mediated ECM remodeling in osteosarcoma.

**Keywords:** ADAMTS-2, TGF- $\beta$ 1, osteosarcoma, Saos-2, transcriptional regulation

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## 1. INTRODUCTION

Osteosarcoma is a malignant primary bone tumor that predominantly occurs in children and adolescents. The tumor microenvironment (TME), which contains many different kinds of cytokines, is highly effective in the development and progression of osteosarcoma (Li et al., 2014). Cytokines are signaling proteins that are secreted by various cell types to control cellular stress factors such as carcinogen-induced injury, infection, and inflammation. In the tumor microenvironment, cytokines regulate the host response to stress factors, preventing consistent cytokine production and thus minimizing tissue destruction. Host responses to cellular stress can affect cancer formation and progression at different levels (Xiao et al., 2014; Lopes-Júnior et al., 2016). Transforming growth factor- $\beta$  (TGF- $\beta$ ) is one of the most abundant cytokines in the tumor microenvironment, and can regulate tumor initiation, progression, and metastasis. While TGF- $\beta$ 1 acts as a tumor suppressor by suppressing cell proliferation and triggering apoptosis in nor-

mal cells, it acts as an oncogenic factor by inducing metastasis and tumor progression in advanced cancer. TGF- $\beta$  contributes to metastasis by promoting extracellular matrix remodeling and epithelial-mesenchymal transition (EMT). Regarding osteosarcoma, increased TGF- $\beta$ 1 expression has been determined in high-grade osteosarcomas (Li et al., 2014; Lopes-Júnior et al., 2016; Zhang et al., 2017). ADAMTS proteases are involved in the processing of various individual proteins and protein families in the extracellular matrix (Rose et al., 2021). They regulate cell-cell and cell-matrix communication and therefore they play key roles in numerous biological processes such as tissue remodeling, inflammation, and cell migration. In addition to the physiological functions, ADAMTS family members also contribute to the tumor microenvironment, metastasis, and angiogenesis. In that context, transcriptional regulation of the ADAMTSs is an important issue to be investigated. Due to their connection with some pathological cases, structural and evolutionary aspects and substrate specificity of

ADAMTSs have widely been studied (Kelwick et al., 2015). Knowledge of the transcriptional regulators of the ADAMTS family members is rather limited. Among the family members, ADAMTS-2 is a well-known example for ECM remodeling which cleaves the N-terminal propeptide of procollagen and promotes correct collagen fibrillogenesis. The promoter region of the ADAMTS-2 was cloned and functional analyses were performed in our previous studies (Alper and Kockar, 2014). We have also elucidated upregulation of ADAMTS-2 expression and promoter activity in the presence of some inflammatory cytokines such as IL-6 and IL1- $\alpha$  in Saos-2 cells (Alper et al., 2015). Here we aimed to determine the regulatory effect of TGF- $\beta$ 1 on ADAMTS-2 transcriptional regulation in Saos-2 cells. We also performed pathway inhibition studies in Saos-2 cells to find out intracellular signaling pathways that are effective in this regulation. These studies will contribute to future research on ADAMTS-2 mediated ECM remodeling in osteosarcoma.

## 2. MATERIALS AND METHODS

### 2.1. Cell Culture and Measurement of Cell Proliferation

Human osteosarcoma cell line Saos-2 was kindly gifted by Prof. Kenneth Wann from Cardiff University, UK. Cells were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM, Sigma) containing 10% fetal calf serum (FCS; Sigma) and 1% L-Glutamine (Sigma) at 37°C incubators with 5% CO<sub>2</sub> in the air. Cell proliferation was measured by MTT Assay. Saos-2 cells were plated out of 96-well plates (5 × 10<sup>4</sup> cells/well). 500 U/mL TGF- $\beta$ 1 (Peprotech) was applied to cells after 24 h of serum starvation for 1, 24, 48, and 72 h. Untreated (NT) cells were used as control. The absorbance was determined at 550 nm using a spectrophotometric microplate reader (Thermo Scientific) (Berridge and Tan, 1993; Tokay et al., 2021). The assay was performed at least triplicate. Data was represented as fold value. Fold value was estimated using the following equation OD550 (TGF- $\beta$ 1 treated cells)/OD550 (Not treated (NT) cells).

### 2.2. TGF- $\beta$ 1 Treatment and Pathway Inhibition Studies

In order to define the regulatory effect of the TGF- $\beta$ 1 on ADAMTS-2 expression, Saos-2 cells (2 × 10<sup>6</sup>) were plated 25 cm<sup>2</sup> cell culture flasks and then serum-starved for 24 h with 0.1% BSA. And then cells were stimulated with 500 U/mL TGF- $\beta$ 1 for 72 h. Cells were harvested after 1, 3, 6, 24, 48 and 72 h of incubation times for RNA and protein isolation. In the pathway inhibition studies, Saos-2 cells were treated with specific pathway inhibitors for 30–45 min prior to TGF- $\beta$ 1 stimulation. 1  $\mu$ M of Wortmannin was used to inhibit the PI3K pathway (Cell signaling, CAS 19545-26-7), 10  $\mu$ M of PD98059 was used for MEK

Inhibition (Cell Signaling, CAS 297744-42-4), and 20  $\mu$ M of SP600125 was used to inhibit the JNK pathway (Sigma-Aldrich, CAS 129-56-6). 5  $\mu$ M of SMAD2/3 Inhibitor, SIS3 (CAS no. 1009104-85-1) was used to inhibit Smad2/3. After 6 h of treatment total RNA and protein were isolated for qRT-PCR and western-blot experiments from the harvested cells.

### 2.3. RNA Isolation and Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from both control and TGF- $\beta$ 1 treated Saos-2 cells using GeneJET RNA Isolation kit (Thermo Fisher Sci.) following the instructions. 1  $\mu$ g of total RNA was reverse transcribed into cDNA using Revert Aid Reverse Transcriptase (Thermo Fisher Sci.) and Oligo (dT) as a primer. Real-time PCR was performed using 5  $\mu$ L of RealQ Plus 2× Master Mix Green (Ampliqon) and, 0.5  $\mu$ L of each primer pair (10 ng/ $\mu$ L) in 10  $\mu$ L final volume. Primer sequences for specific genes were as follows: ADAMTS-2 forward: 5'-CTGTGGCGACGAGGT-GCG-3' and reverse 5'-GGTGCACACATAGTC-CCGTCC-3'; Human beta 2 Microglobulin (h $\beta$ 2M) forward 5'-TTTCTGGCCTGGAGGCTATC-3' and reverse 5'-CATGTCTCCATCCCCTACTTA-3'. PCR cycle conditions as follows: A cycle of 95°C for initial denaturation (10 min), 35 cycles of 95°C for denaturation (30 s), and 58°C for annealing (30 s) 72°C for extension (30 s) and a cycle of 72°C for extension (1 min). Each sample was assayed in triplicate, and the Ct value was calculated by the instrument (Light Cycler 485, Roche). The relative change in gene expression between control and TGF- $\beta$ 1 treated cells for each incubation time point was calculated according to 2<sup>- $\Delta\Delta$ Ct</sup> (Livak and Schmittgen, 2001). Human  $\beta$ -2 microglobulin (h $\beta$ -2) is used as an internal control. Fold changes in the ADAMTS-2 mRNA expression were calculated by dividing 2<sup>- $\Delta\Delta$ Ct</sup> value of the TGF- $\beta$ 1 treated cells by control cells.

### 2.4. Extraction of Proteins and Western Blotting

Cells were washed twice with ice-cold PBS and then lysed in radioimmunoprecipitation (RIPA) assay buffer including protease inhibitors (Tokay and Kockar, 2016). Protein concentration was measured based on the fluorimetric method using Qubit Protein assay kits (Thermo Fisher Sci.) and a Qubit Fluorometer. Equal amounts of proteins (30–50  $\mu$ g) were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After proteins were transferred to a membrane (Immobilon-P PVDF, Millipore), the membrane was incubated with ADAMTS-2 (Abcam, Ab125226, 3  $\mu$ g/mL) or  $\beta$ -Actin (Santa Cruz, sc47778, 1 : 5.000 dilution) specific antibodies. Then, horseradish peroxidase (HRP)-conjugated, anti-rabbit IgG or anti-mouse IgG (1 : 5000 dilution; Sigma) antibodies were applied

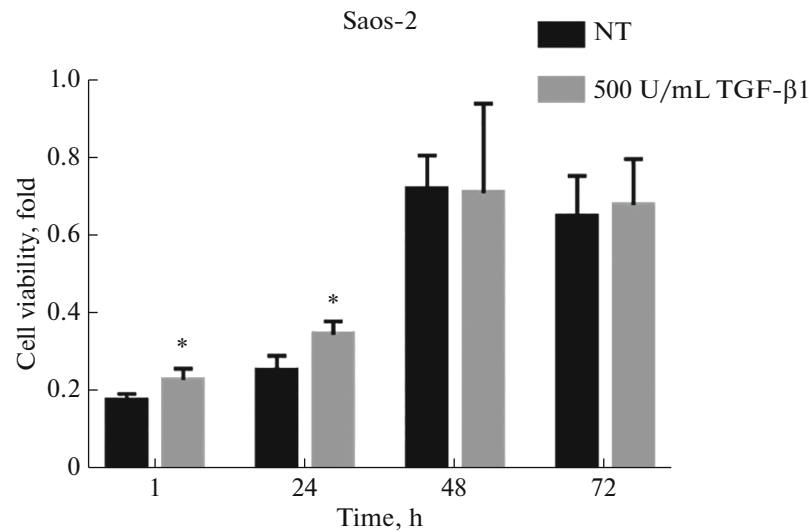


Fig. 1. Cell viability of Saos-2 cells after 500 U/mL TGF-β1 treatment (\* $p \leq 0.05$ ).

to the membrane. Bands were visualized using an enhanced chemiluminescence detection kit (ECL, Pierce) and X-ray-sensitive film (Kodak). Image J software 19 was used for densitometric analysis (Schneider et al., 2021). Fold changes in the ADAMTS-2 protein expression were calculated by dividing densitometry value of the TGF-β1 treated cells by control cells.

### 2.5. Transient Transfection and Dual-Luciferase Reporter Assay

Truncated ADAMTS-2 promoter fragments were cloned into pMetLuc Reporter vector as described previously (Alper and Kockar, 2014). Transient transfection of ADAMTS-2 promoter-reporter plasmids was conducted by the calcium phosphate precipitation method. Briefly, cells were seeded in 48-well plates ( $6 \times 10^4$  cells/well) and 1 μg of ADAMTS-2 promoter constructs and 0.5 μg of a secreted form of human alkaline phosphatase (SEAP) were transiently transfected into Saos-2 cells. SEAP vector was used to determine transfection efficiency. pMetLuc control plasmid was used as positive and empty pMetLuc reporter vector was used as a negative control. To determine the effect of TGF-β1 (Peprotech) on ADAMTS-2 promoter activity, cells were incubated with 500 U/mL of TGF-β1 for 48 h after transfection. Untreated cells were used as control. Ready-To-Glow™ Secreted Luciferase Reporter Systems (TaKara) and Fluoroskan Ascent FL Luminometer (Thermo Electron Co.) were used to measure luciferase and SEAP activities. The relative luciferase activity was calculated by dividing the luciferase value of each well by the SEAP value. Transfection experiments were repeated at least three times (Kockar et al., 2001).

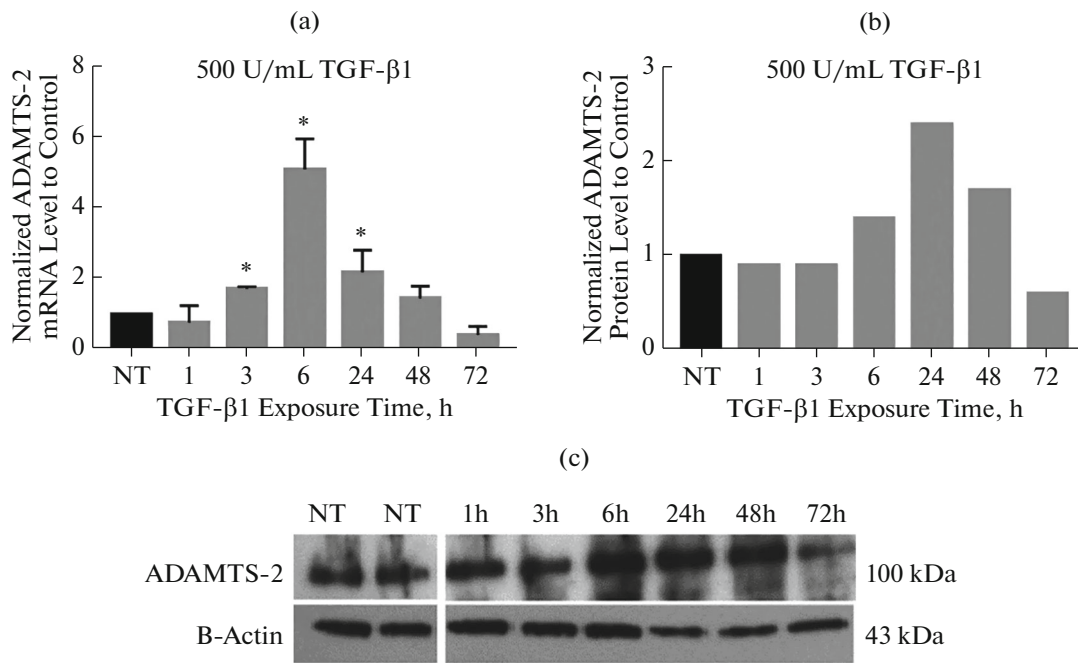
### 2.6. Statistical Analysis

Mini Tab 14 software was used to estimate standard deviations and  $p$  values (Minitab, LLC., 2021). One-way Anova analysis was used to determine statistically significant differences between pairs.  $p$ -value  $< 0.05$  considered statistically significant (Girden, 1992).

## 3. RESULTS

### 3.1. TGF-β1 Induces Saos-2 Cell Proliferation

Saos-2 is a human osteoblastic cell line and it has been extensively used as an in vitro model for osteosarcoma and bone remodeling studies and represents a mature osteoblastic phenotype with a high ALP activity (Czekanska et al., 2012). Because TGF-β1 has a pivotal role in bone remodeling and osteosarcoma progression, various studies have been performed to understand the effect of TGF-β1 on these processes. Dysregulation in synthesis or degradation of the extracellular matrix (ECM) proteins has gained considerable attention for its promise in pathogenic targeting and prognostic value. Therefore determination of the relationship between TGF-β1 and matrix metalloproteinases that are responsible for ECM processing is an important issue (Takeuchi et al., 1995; Corre et al., 2020; Cui et al., 2020). In the present study, at the first step, the effect of TGF-β1 on Saos-2 cell proliferation was investigated. Briefly, 500 U/mL TGF-β1 was applied on serum-starved Saos-2 cells ( $5 \times 10^4$  cells/well) and then an MTT assay was performed. It was found that 500 U/mL TGF-β1 treatment promoted Saos-2 cell proliferation significantly. Maximum increase was observed at 24 h (1.4 fold) incubation time (Fig. 1).



**Fig. 2.** (a) Effect of 500 U/mL TGF- $\beta$ 1 treatment on ADAMTS-2 mRNA and (b) ADAMTS-2 protein expression level. ( $*p \leq 0.05$ ).

### 3.2. TGF- $\beta$ 1 Significantly Up-Regulates Endogenous ADAMTS-2 mRNA and Protein Levels in a Time-Dependent Manner in Saos-2 Cells

To investigate the regulatory effect of the TGF- $\beta$ 1 on endogenous ADAMTS-2 mRNA and protein expressions, Saos-2 cells were stimulated with 500 U/mL of TGF- $\beta$ 1 for 72 h. The regulatory effect of the TGF- $\beta$ 1 on ADAMTS-2 mRNA level was determined by qRT-PCR strategy using specific primer pairs for ADAMTS-2. TGF- $\beta$ 1 increased ADAMTS-2 mRNA level approximately 1.7 fold after 3 h of stimulation and the maximum increase was observed (up to 5.1 fold) after 6 h. The inducing effect of the TGF- $\beta$ 1 on ADAMTS-2 mRNA level was gradually decreased after then (Fig. 2a). In addition, western blot studies were conducted to determine the effect of TGF- $\beta$ 1 on the ADAMTS-2 protein level. Consistent with the mRNA data, TGF- $\beta$ 1 increased ADAMTS-2 protein level up to 1.4 fold after 6 h of stimulation. The maximum increase was observed after 24 h (up to 2.4 fold). The up-regulatory effect of the TGF- $\beta$ 1 on ADAMTS-2 protein level was started to decrease after 48 h and diminished after 72 h of incubation (Fig. 2b).

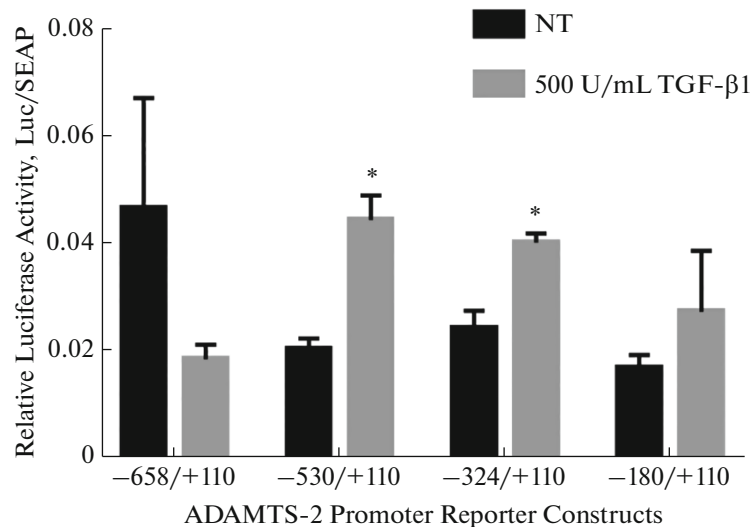
### 3.3. TGF- $\beta$ 1 Induces ADAMTS-2 Promoter Activity in Saos-2 Cells

Next, transient transfection studies were conducted using four DNA constructs, containing 5' truncations of the ADAMTS-2 gene promoter in the pMetLuc vector, pMET\_TS2\_180 (-180/+112),

pMET\_TS2\_324 (-324/+112), pMET\_TS2\_530 (-530/+112), pMET\_TS2\_760 (-658/+112). Here it was aimed to determine the responsible promoter region for the TGF- $\beta$ 1 mediated induction of ADAMTS-2 expression. Therefore, ADAMTS-2 promoter fragments were transiently transfected into the Saos-2 cells and then the reporter gene activity in cells that were either left untreated or exposed to TGF- $\beta$ 1 was determined. A TGF- $\beta$ 1 dependent induction in promoter activity was obtained with pMET\_TS2\_530 (-530/+112), pMET\_TS2\_324 (-324/+110) and, pMET\_TS2\_180 (-180/+110) promoter constructs (Fig. 3).

### 3.4. TGF- $\beta$ 1 Up-Regulates ADAMTS-2 Expression via Both Smad-Dependent/Independent Pathways

To further clarify signaling molecules that are responsible for the TGF- $\beta$ 1 mediated activation of the ADAMTS-2 expression we conducted pathway inhibition studies. Saos-2 cells were pretreated for 30–45 min with specific pathway inhibitors as described above and then TGF- $\beta$ 1 (500 U/mL) was added and cells were incubated for an additional 6 h. Smad3 Inhibitor (SIS3), MEK Inhibitor (PD98059), JNK inhibitor (SP600125), and PI3K Inhibitor (Wortmannin) diminished the activating effect of TGF- $\beta$ 1 on ADAMTS-2 mRNA expression (Fig. 4a). We also performed pathway inhibition studies at the protein level to confirm mRNA data. Interestingly, while Smad3 inhibitor (SIS3) and MEK inhibitor (PD98059) couldn't block the activating effect of the TGF- $\beta$ 1 on



**Fig. 3.** Effect of 500 U/mL TGF- $\beta$ 1 on ADAMTS-2 promoter activity. The cells that were not administered TGF- $\beta$ 1 cytokine were used as the control. The data are the mean of three independent values  $\pm$ SD (\* $p \leq 0.05$ ).

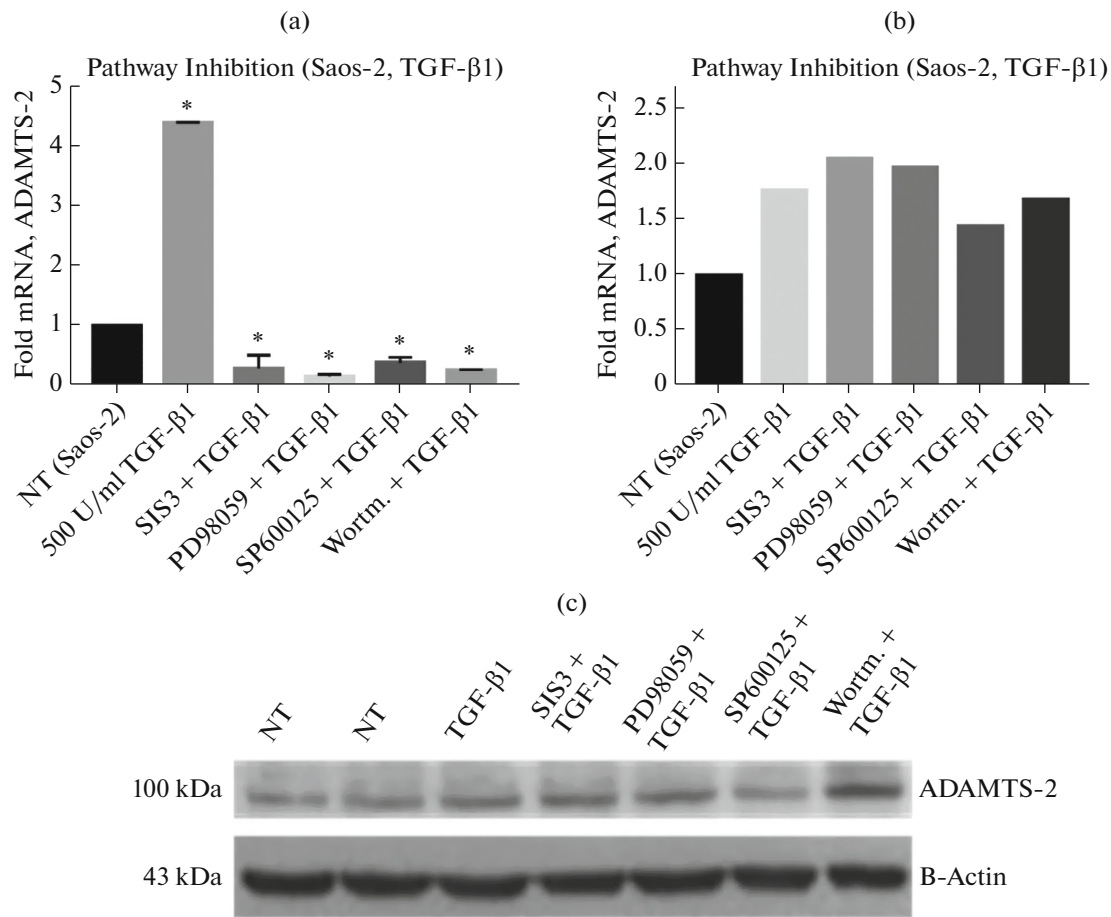
ADAMTS-2 expression, JNK and PI3K inhibitor reduced activating effect of the TGF- $\beta$ 1 at protein level (Figs. 4b and 4c). Fold changes were represented as the ratio of ADAMTS-2 transcript levels in the TGF- $\beta$ 1 treated SW480 cells relative to the control (NT) cells. Fold changes of the ADAMTS-2 levels in the inhibitor treated SW480 cells were estimated by dividing TGF- $\beta$ 1 treated SW480 cells to observe blocking effect of the inhibitor on ADAMTS-2 expression.

## DISCUSSIONS

ADAMTSs are extracellular metalloproteinases having a broad substrate spectrum. Substrate cleavage mechanisms that were catalyzed by ADAMTSs are essential for ECM formation and remodeling in physiological cases. However, ADAMTS-mediated processing of ECM components can also lead to some pathological situations such as arthritis.

These enzymes can also contribute to the modification of the tumor microenvironment by cleaving or interacting with ECM components or regulatory factors. Hence, they affect cell adhesion, migration, proliferation, and angiogenesis. In addition, it has been reported that different *ADAMTS* genes were overexpressed, mutated, or silenced with epigenetic mechanisms in different tumor types, suggesting the direct relevance of these metalloproteases in cancer development (Theret et al., 2021; Rose et al., 2021). Despite the importance of ADAMTS proteases in normal and pathological conditions, little is known about their transcriptional activators or repressors. Some studies have been conducted at transcriptional/translational levels about transcription factor, hormone, and cytokine-mediated regulation of ADAMTS members to

some extent. For example, because of the critical roles in osteoarthritis progression, the effects of the pro-inflammatory cytokines on ADAMTS-4 and ADAMTS-5 expressions were investigated. IL-1 $\beta$  increased ADAMTS-4 expression in human chondrocytes. IL-6 and TNF- $\alpha$  also increased ADAMTS-4 and ADAMTS-5 expressions in synovial cells (El Mabrouk et al., 2007; Mimata et al., 2012; Uchida et al., 2017). It was determined that, ADAMTS-16, which plays a role in kidney morphogenesis, is positively regulated by TGF- $\alpha$  in chondrocyte cell lines (SurrIDGE et al., 2009; Schnellmann et al., 2018). In the ADAMTS family, ADAMTS-2 was originally known for its ability to cleave the amino-terminal of the fibrillar collagen precursors which is the fundamental step for the correct fibril accumulation (Jiang et al., 2009; Rose et al., 2021). In addition, mutations and deficiencies in the ADAMTS-2 gene correlated with Ehlers-Danlos syndrome and male infertility, respectively (Takeda 2009; Satz-Jacobowitz et al., 2021). Increased ADAMTS-2 expression has been demonstrated in acute myocardial infarction and fibroblast cells of gastric cancer tissue (Hubmacher et al., 2015; Jiang et al., 2019). In our previous studies, transcriptional regulation of ADAMTS-2 by inflammatory cytokines was studied in detail. We elucidated that IL-6 and IL-1 $\beta$  could induce ADAMTS-2 expression in osteosarcoma models, Saos-2 and MG-63 (Alper and Kockar, 2014; Alper et al., 2015). Wang and colleagues determined that TGF- $\beta$ 1 is able to promote the secretion of ADAMTS-2 in MG-63 cells (Wang et al., 2003). In the present study, the regulatory effect of TGF- $\beta$ 1 on ADAMTS-2 transcriptional regulation was elucidated. Determining the effect of TGF- $\beta$ 1 on ADAMTS-2 promoter activity and the signaling pathways that were effective in TGF-



**Fig. 4.** Pathway Inhibition Studies. (a) Effect of pathway inhibitors at ADAMTS-2 mRNA level ( $*p \leq 0.05$ ). (b, c) Effect of pathway inhibitors at ADAMTS-2 protein level.

$\beta$ 1-mediated regulation of ADAMTS-2 are the most remarkable aspects of this study. Saos-2 osteosarcoma model was used as the model which has the most mature osteoblastic phenotype among the osteosarcoma cell models. Osteosarcoma is one of the cancer types having the highest heterogeneity level in humans. This heterogeneity can be observed not only in micro-environmental components but also at the genomic, transcriptomic, and epigenetic levels. Also, osteosarcoma cell models have different characteristics in heterogeneity, surface receptor, maturity, and gene expression profiles. Since the regulation of ADAMTS genes is cell and tissue-specific, the cytokine response is likely to vary depending on the cell type (Pautke et al., 2004; Poos et al., 2015). While TGF- $\beta$ s function as a tumor suppressors and as a tumor promoters, they act only as tumor promoter in sarcomas through their pro-metastatic effects. An increase in TGF- $\beta$  expression is correlated with high-grade osteosarcoma and lung metastasis. TGF- $\beta$ s exert their function through Smad and non-Smad pathways. In Smad dependent pathway Smad2/3 forms a heterodimeric complex with Smad4 and this

complex translocate into the nucleus to regulate target gene expression. In the non-Smad pathways other kinases such as ERK1/2, p38, and AKT are activated (Verrecchia and R dini, 2018; Liu et al., 2018; Clayton et al., 2020). Dong and colleagues reported that TGF- $\beta$ 1-induced EMT (epithelial-mesenchymal transition) and cell migration in an osteosarcoma model, U2OS through the activation of the PI3K/Akt, ERK, and p38 MAPK signaling pathways (Dong et al., 2018). Here, TGF- $\beta$ 1 treatment increased ADAMTS-2 mRNA level in Saos-2 cells significantly starting early stages of stimulation and continuing up to 48 h of incubation. Compared with ADAMTS-2 mRNA expression, the increasing effect of the TGF- $\beta$ 1 on ADAMTS-2 protein level was observed in later incubation times (6 h) and maintained up to 48 h. Transient transfection studies that were performed under TGF- $\beta$ 1 stimulation revealed that TGF- $\beta$ 1 could activate ADAMTS-2 expression through (-530/+112), (-324/+110), and (-180/+110) promoter regions. These results provide strong evidence that TGF- $\beta$ 1 regulates ADAMTS-2 expression at the transcriptional level.



TGF- $\beta$ 1 Pathway inhibition studies revealed that both canonical (Smad-dependent) and non-canonical (non-Smad) pathways are responsible for TGF- $\beta$ 1 mediated activation of ADAMTS-2 expression at the mRNA level. Pathway inhibition studies that were performed at the protein level indicated that TGF- $\beta$ 1 uses non-canonical JNK and PI3K signaling pathways to increase ADAMTS-2 expression in Saos-2 cells. This result indicated that in addition to transcriptional mechanisms, posttranslational regulations also play a role in TGF- $\beta$ 1 mediated up-regulation of ADAMTS-2.

In conclusion, TGF- $\beta$ 1 is the strong positive regulator of ADAMTS-2 expression both at mRNA and protein levels in Saos-2 cells. TGF- $\beta$ 1 exerts its inducing effect through specific regions in the ADAMTS-2 promoter. Similar with proteolytic enzyme MMPs, an increase in the ADAMTS-2 could be critical for the invasiveness of osteosarcoma.

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#### COMPLIANCE WITH ETHICAL STANDARDS

The author declares no conflicts of interest. The article contains no research in which animals or human participants were used.

#### AUTHOR CONTRIBUTIONS

In the approved manuscript, the design of the study, the acquisition of data, or the analysis and interpretation, the preparation of the draft, and editing were performed by Meltem Alper.

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