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ORIGINAL ARTICLE

TNF-α orchestrates ADAM metallopeptidase with thrombospondin type 1 motif 2 upregulation and extracellular matrix remodeling through multimodal signaling in osteosarcoma cells

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Abstract

Background ADAMTS-2 is a key extracellular matrix (ECM) remodeling enzyme increasingly implicated in osteosarcoma biology. Although ADAMTS-2 is known to participate in collagen processing and ECM turnover, the upstream inflammatory cues and transcriptional mechanisms regulating its expression in osteosarcoma remain unclear. Tumor necrosis factor-α (TNF-α), a dominant pro-inflammatory cytokine within the osteosarcoma microenvironment, represents a strong candidate regulator due to its ability to activate several pathways. This study aimed to elucidate the TNF-α–ADAMTS-2 regulatory axis at mechanistic levels.

Methods and results Gene expression profiling revealed that ADAMTS-2 is significantly upregulated in osteosarcoma tissues compared with normal bone and is associated with ECM disassembly and collagen fibril organization pathways. Functional assays in Saos-2 cells demonstrated that TNF-α stimulation markedly increased ADAMTS-2 mRNA (~17-fold) and protein levels (~twofold). Promoter activity assays showed strong TNF-α–mediated activation, with the highest induction in the −180/+112 region. Pharmacological inhibition experiments revealed that MEK, PI3K, JNK, and NF-κB pathways are all required for TNF-α-induced ADAMTS-2 transcription. In silico motif analysis identified STAT3 and NF-κB binding elements within the promoter, and electrophoretic mobility shift assays supported the interaction of these transcription factors with specific promoter regions.

Conclusions This study provides the first mechanistic evidence that TNF-α regulates ADAMTS-2 expression through the coordinated activation of multiple signaling pathways and the engagement of STAT3 and NF-κB transcription factors at the promoter. These findings uncover a previously uncharacterized inflammatory regulatory axis linking TNF-α signaling to ECM remodeling and highlight ADAMTS-2 as a potential mediator of osteosarcoma progression.

Keywords ADAMTS-2 · Osteosarcoma · TNF-α · Inflammation

Abbreviations

ECM	Extracellular matrix
TNF-α	Tumor necrosis factor-α
MMP	Matrix metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
EMSA	Electrophoretic mobility shift assays
DMEM	Dulbecco's modified eagle's medium
FCS	Fatal calf serum

Introduction

Osteosarcoma is a type of bone cancer that primarily affects young individuals and is characterized by the aggressive formation of bone tissue by malignant cells [1]. It is commonly found in the metaphysis of long bones, particularly near the knee joints [2]. Osteosarcoma has a pronounced tendency to spread to distant areas, often resulting in relapses primarily linked to lung metastases, whereas bone metastases or local

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recurrences are comparatively rare. Advancements over the past few decades have led to a five-year disease-free survival rate of 60–70% in patients with osteosarcoma without metastasis. However, in cases of metastasis, prognosis declines sharply, with a five-year survival rate ranging from 10 to 20% [3–5]. Although our understanding of the influence of molecular genetics and the TME on the initiation and progression of osteosarcoma is limited [6], some studies have shown that the tumor microenvironment in osteosarcoma can affect tumor aggressiveness, ability to evade the immune system, and therapeutic response. [7–10].

TNF-α is a multifaceted cytokine that plays crucial roles in both normal physiological processes and pathological conditions of the body. Within the TME, TNF-α plays a significant role in promoting inflammation-associated carcinogenesis [11]. TNF-α plays a dual role in the tumor microenvironment by acting as both a promoter and a suppressor of tumor growth [12, 13]. The response to TNF-α is contingent on the concentration of the cytokines present. At elevated concentrations, TNF-α exerts an antitumor effect by facilitating cellular apoptosis, steering tumor-associated macrophages towards antitumor characteristics, attracting neutrophils and monocytes to the tumor vicinity, spurring macrophage activation, impeding monocyte differentiation into immunosuppressive phenotypes, and triggering the disruption of tumor vasculature. Conversely, low TNF-α expression promotes tumor growth [11, 14]. Additionally, TNF-α triggers the generation of matrix metalloproteinases (MMPs) either within tumor cells or the TME, thus increasing tumor expansion [13, 15]. Therefore, it is possible to manage the metastatic capacity of osteosarcoma by modulating TNF-α activity [16]. Although previous studies have shown that IL-6 can regulate ADAMTS-2 expression in osteoblastic cells, TNF-α is known to be a more dominant proinflammatory regulator in the osteosarcoma microenvironment and to show a high degree of compatibility with the signaling pathways examined in this study (NF-κB, MEK/ERK, JNK, and PI3K/AKT) [17–19]. Moreover, TNF-α has demonstrated clinical relevance in osteosarcoma progression, as shown by Mori et al., who reported that macrophage-derived TNF-α maintains the undifferentiated and tumor-propagating state of osteosarcoma cells. Considering that TNF-α directly affects tumor-specific biological processes such as loss of differentiation, increased metastatic capacity, and remodeling of the bone matrix in osteosarcoma, it is understood that this cytokine occupies a relatively more central position in osteosarcoma biology compared to other cancer types. Together, these features provide a strong rationale for selecting TNF-α as the primary upstream cytokine examined in this study [20].

The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) family, consisting of 19 metalloproteinases with diverse functions in the extracellular environment, has been implicated in numerous conditions, including cancer and osteoarthritis. Evidence indicates that ADAMTSs significantly shape the extracellular microenvironment of tumors [21]. These enzymes can cleave or engage in a range of extracellular matrix elements and signaling molecules, consequently affecting fundamental cellular processes, such as migration, angiogenesis, and proliferation [22, 23]. Within this family, some members exhibit antitumor properties, whereas others expedite disease progression. Additionally, certain members have dual effects [21]. This paradox underscores the need for a comprehensive investigation of this fascinating class of enzymes.

ADAMTS-2 has several important functions in the body. One of its main roles is to cleave specific parts of collagen molecules, particularly types I, II, III, and V collagen [24]. Collagen is a vital protein that provides structure and strength to various tissues in the body, including skin, bones, and cartilage [25]. ADAMTS-2 plays a significant role in the proper assembly and organization of these tissues by cleaving collagen precursors. ADAMTS-2 is known for its role in ECM remodeling [26]. In various cancer types (e.g., osteosarcoma), alterations in the ECM are crucial for tumor growth, invasion, and metastasis [27]. ADAMTS-2's ability to modify ECM components, such as collagens and proteoglycans, could influence the microenvironment that supports osteosarcoma progression [28].

Despite its established role in collagen processing, the regulatory mechanisms controlling ADAMTS-2 expression in osteosarcoma cells remain unclear. Based on this information, the hypothesis of our study is that TNF-α increases ADAMTS-2 expression at the transcriptional level through osteosarcoma-specific proinflammatory signaling networks. Therefore, this study aims to determine the effects of TNF-α on ADAMTS-2 mRNA and protein levels and promoter activity, and to systematically elucidate which intracellular signaling pathways and transcription factors mediate this regulation. We demonstrated that TNF-α stimulation significantly upregulated ADAMTS-2 at both the mRNA and protein levels and activated its promoter. Pathway inhibition assays revealed that this induction was mediated by MEK, PI3K, JNK, and NF-κB signaling cascades. Furthermore, electrophoretic mobility shift assays (EMSA) confirmed the functional interactions of STAT3 and NF-κB transcription factors with multiple ADAMTS-2 promoter regions, supporting the bioinformatic predictions. In silico analysis assessed ADAMTS-2 expression in osteosarcoma versus normal tissues, identified enriched oncogenic pathways, and established its transcriptional association with the NF-κB and STAT networks. Collectively, these findings advance our understanding of TNF-α-driven ECM remodeling in

osteosarcoma and highlight ADAMTS-2 as a potential mediator of tumor progression.

Material and methods

Bioinformatics and gene expression analyses

The GSE253548 RNA-seq dataset (~50 osteosarcoma and ~40 normal samples) containing osteosarcoma (OS) and normal bone tissues was used. Raw counts were normalized using DESeq2 (v1.xx, R v4.3.1), and low-expression genes were filtered. Differences between the normal and tumor groups were assessed using the Wilcoxon rank-sum test; multiple testing corrections were performed using the Benjamini–Hochberg (BH) method, and an FDR < 0.05 significance threshold was accepted. Differential Gene Expression and Heat Map analyses were performed. The criteria for differential gene expression were set as |log-2FoldChange| > 1 and FDR < 0.05. The expression profiles of the most highly upregulated genes were visualized as heatmaps using heatmap (v1.0.12). Z-score normalization was applied to reduce the effects of outliers. Differentially expressed genes were subjected to Gene Ontology (GO) biological process and KEGG pathway enrichment analyses using clusterProfiler (v4.xx). Significant terms were selected using the p.adjust < 0.05 criterion. Gene–term relationships for GO results were displayed as network diagrams using igraph (v1.5.1), while KEGG results were presented as dot plot graphs. The expression profiles of the ADAMTS/ADAMTSL, NF-κB (NFKB1, NFKB2, RELA, RELB, and REL), and STAT (STAT1–STAT6) gene families were evaluated using the same dataset. Differences between the normal and tumor groups are shown using box plots. Furthermore, the relationship between ADAMTS-2 expression and NFKB1 and STAT3 expression in osteosarcoma samples was determined using Spearman and Pearson correlation analyses.

Cell culture and cytokine treatment

Saos-2 cells, a subtype of human osteosarcoma known for their osteoblastic properties, were cultivated as monolayers in Dulbecco's modified Eagle's medium (DMEM). The culture medium comprised 10% heat-inactivated fetal calf serum (FCS) supplemented with 2 mM L-glutamine. The cells were maintained at 37 °C in a humidified incubator with a CO_2 concentration of 5% (v/v). Before subjecting the cells to TNF-α treatment, they were serum-starved with 0.1% bovine serum albumin (BSA). TNF-α (10 ng/mL) was added to the medium and incubated for 1, 3, 6, 24, or 48 h [29].

RNA extraction and qRT-PCR

RNA was extracted from Saos-2 cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific). RNA concentration was determined using spectrophotometry. Oligo (dT) primers, Reverse Transcriptase (200 U), and ribonuclease inhibitor (40 U) were used to synthesize cDNA from 1 µg of total RNA at 42 °C for 1 h. In a 10 µL final volume, 1 µL cDNA from each group was mixed with 5 µL SYBR® Green PCR Master Mix, along with ADAMTS-2 specific forward and reverse primers (Supp. Table 1). The amplification procedure consisted of an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 1 min. All experiments were conducted with a minimum of three replicates. The human β−2 microglobulin gene was used to normalize the results. The Livak method [30] was used to assess the relative changes in ADAMTS-2 expression [24].

Western blotting

RIPA buffer (10 mM Tris–HCl pH 8, 140 mM NaCl, 1 mM EDTA, 0.1% SDS,1 mM EGTA, 1% Triton-X-100, 0.01% sodium deoxycholate, supplemented with a protease inhibitor cocktail tablet) was used to prepare the protein extracts [31]. A Qubit was used to fluorometrically measure the protein concentration. Protein samples (50 µg) were loaded equally onto the SDS-PAGE. A PVDF membrane (Millipore) was used for protein transfer. Wet transfer was performed at + 4 °C overnight. The membrane was blocked and incubated with ADAMTS-2 antibody (1 µg/mL, ab125226-Abcam) at + 4 °C overnight and β-actin antibody (1:1000, Ab8227-Abcam) for one hour at room temperature, followed by incubation with secondary antibody (1:5000, goat, anti-rabbit) (Ab97069-Abcam) for one hour at room temperature. Fusion FX Vilber Lourmat and ECL substrates (Thermo Fisher Scientific) were used to detect the protein bands. Densitometric analysis was done using Image J.

Immunofluorescence assessment

Saos-2 cells were cultured on coverslips and treated with TNF-α (10 ng/mL) as described previously [32]. ADAMTS-2 antibody (Abcam, Ab125226) was applied to the Saos-2 cells at a concentration of 2 µg/mL and incubated overnight at 4 °C. Subsequently, Alexa Fluor 488 secondary antibody (anti-rabbit; Invitrogen) was applied to the samples for 1 h at room temperature. Fluorescence imaging was performed using an Olympus BX51 microscope equipped with an ED200 fluorescence attachment. An Olympus DP72 microscope was used to capture photographs. The

relative protein expression of ADAMTS-2 was quantitatively assessed using ImageJ software.

Inhibitor treatments

For the inhibition experiments, Saos-2 cells were exposed to specific inhibitors for 30 min. TNF-α (10 ng/mL) was then added to the cells, and the cells were incubated for 6 h. PD98059 (10 µM, Cell Signaling, 9900S) was used to inhibit MEK; Wortmannin (1 µM, Cell Signaling 99,515) was used to inhibit PI3K; SP600125 (20 µM, Santa Cruz, sc200635) was used to inhibit JNK, Bay 11–7082 (5 µM, Santa Cruz, sc-200615) was used to inhibit NF-κB.

Reporter assays

To conduct luciferase reporter assays, we cloned four distinct truncated ADAMTS-2 promoter fragments into the pMetLuc reporter vector: pMET_TS2[−658/+112], pMET_TS2[−530/+112], pMET_TS2[−324/+112], and pMET_TS2[−180/+112], as described in our previous study [33]. For transient transfection assays, 1 µg of ADAMTS-2 promoter constructs and 0.5 µg of pSeap2-Control vector were employed following established protocols [33]. Following a 16-h transfection interval, Saos-2 cells were treated with TNF-α (10 ng/ml). After 48 and 72 h of stimulation, a Fluoroskan Ascent FL Luminometer (Thermo Electron Co.) and Ready-To-Glow™ Secreted Luciferase Reporter Systems (Clontech) were used to quantify SEAP and luciferase activity. The pMetLuc control and pMetLuc reporter vectors served as positive and negative controls, respectively, for assessing transfection efficiency and background activity. SEAP values were used to normalize luciferase values. Transfection experiments were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

MatInspector (Genomatix) software was used to identify potential STAT-3 and NF-κB binding sites within the ADAMTS-2 promoter, and probes covering the ranges probes covering the intervals [−143/−111], [−175/−143], [−190/−184], and [−436/−400] were designed. When Saos-2 cells reached 80% confluence, nuclear extracts were prepared from control and 10 ng/mL TNF-α treated cultures for 24 h, and extracts with determined protein concentration were stored at −80 °C. Double-stranded specific oligonucleotides were biotinylated at the 3' end using terminal deoxynucleotidyl transferase; following incubation at 37 °C, the enzyme was inactivated at 70 °C. DNA–protein binding reactions were set up in a total volume of 20 µL, using 1 × binding buffer, poly(dI·dC), biotinylated probe, and nuclear extract; unlabeled consensus STAT-3 or NF-κB oligonucleotides were added along with the biotin-free oligo of the same region in competitive assays to confirm binding specificity (Supp. Table 2). Reaction mixtures were incubated at room temperature for 2 h, and the resulting complexes were run on a 6% polyacrylamide native gel under 0.5 × TBE conditions at 100 V for approximately 1 h. DNA–protein complexes in the gel were transferred to a nylon membrane soaked in 0.5 × TBE at +4 °C at 100 V for 1 h, and the membrane was cross-linked under UV light for 15 min. EMSA was performed [34] using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) as described in the literature, and biotin-labeled complexes were developed with ECL substrate and detected using a UVP imaging system.

Statistical analysis

Statistical analysis was conducted using Minitab software with a one-way ANOVA. Statistical significance was set at $p \leq 0.05$.

Results

Upregulation of ADAMTS-2 in osteosarcoma patients and its association with extracellular matrix remodeling

To understand the role of ADAMTS-2 in osteosarcoma, we first compared the expression levels of the gene in tumor and normal tissues. The analysis revealed that ADAMTS-2 was expressed at significantly higher levels in osteosarcoma tissues than in normal bone tissues (log2(TPM + 1) mean: 3.68 ± 0.52 vs. 2.40 ± 0.38; Wilcoxon rank-sum, W = 714.5, p = 0.021) (Fig. 1A). This finding suggests that ADAMTS-2 could be a potential candidate gene for osteosarcoma progression. To elucidate the molecular context of the increase in ADAMTS-2 expression in tumor biology, differential gene expression analysis was performed. The analysis revealed significant transcriptomic differences between normal bone tissue and osteosarcoma, with the majority of the 12 most highly upregulated genes meeting the FDR < 0.05, |log2FC| > 1 threshold being associated with ECM regulation and cellular adhesion processes. The heatmap demonstrated that these genes exhibited significantly higher expression in tumor samples than in normal tissues, identifying new candidates that may contribute to matrix remodeling in osteosarcoma (Fig. 1B). Following these results, GO enrichment analysis was performed to understand the biological processes associated with the differentially increased genes. Genes such as MMP13, COL11A1, and NDNF were found to be prominent in the processes of 'extracellular

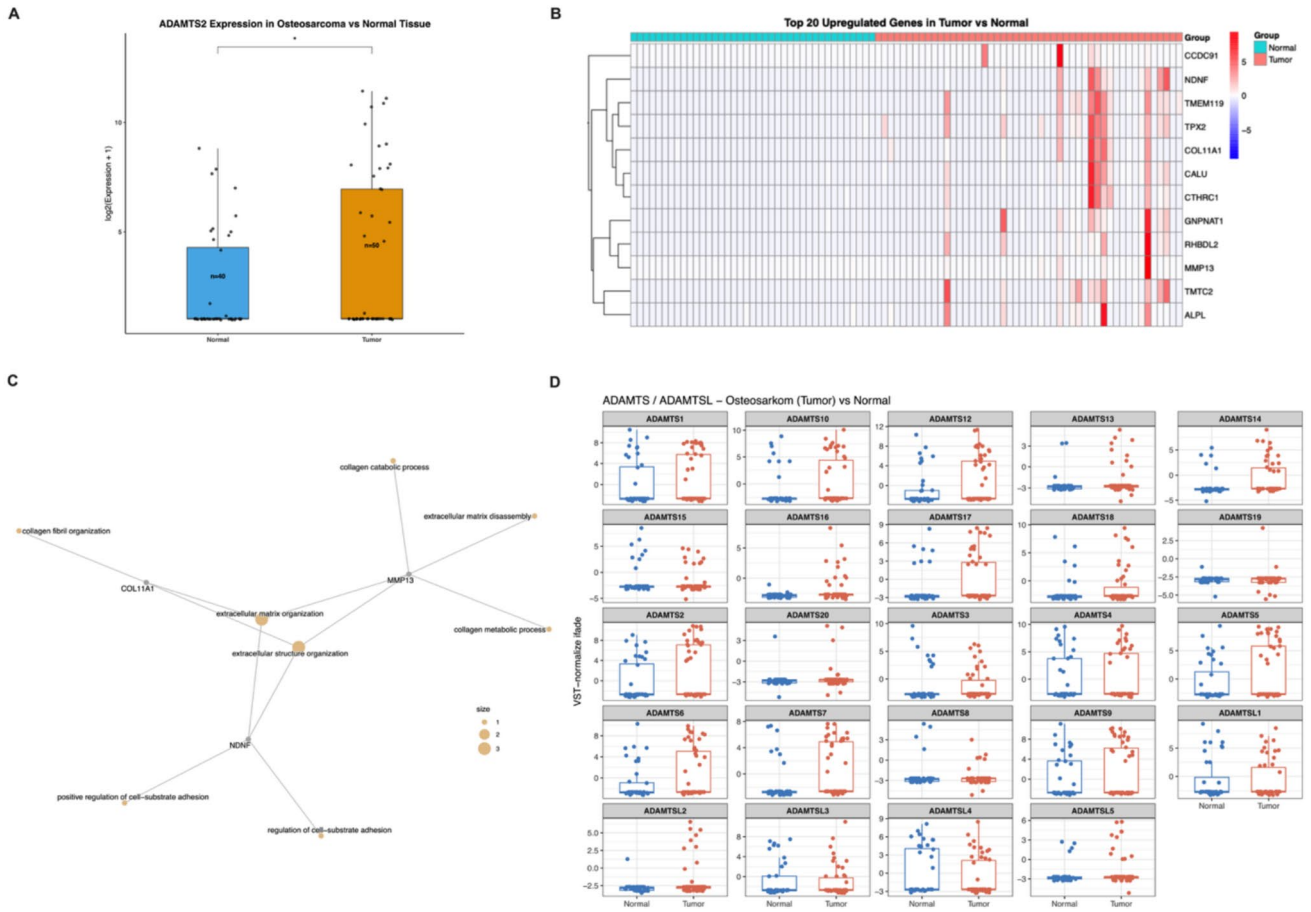

Fig. 1 Increased ADAMTS-2 gene expression in osteosarcoma and its relationship with the extracellular matrix. **A**. When comparing normal bone tissue with osteosarcoma samples, ADAMTS-2 gene expression was significantly higher in osteosarcoma tissues (Wilcoxon test, $p < 0.021$). **B.** A heatmap showing the 12 genes with the greatest increase in differential gene expression analysis. Most of these genes are associated with cell adhesion and ECM organization. **C.** GO analysis revealed that the upregulated genes were particularly enriched in biological processes such as 'extracellular matrix organization', 'collagen fibril organization, and 'collagen catabolic process.' The relevant gene–term interaction network indicated that genes such as MMP13 and COL11A1 play critical roles in ECM remodeling. **D.** Expression profiles of the ADAMTS/ADAMTSL gene family in normal and osteosarcoma tissues. Specifically, ADAMTS-2, ADAMTS-6, ADAMTS-7, ADAMTS-12, ADAMTS-14, ADAMTS-18, and ADAMTS-20 showed a marked increase in tumor tissues, while some members (e.g., ADAMTSL4) exhibited lower expression (Wilcoxon test; FDR < 0.05)

matrix organization', 'collagen fibril organization', 'collagen catabolic process', and 'extracellular matrix disassembly.' The gene–term interaction network suggested that these genes may contribute to tumor progression by disrupting the structural integrity of the extracellular matrix in the tumor microenvironment (Fig. 1C). Finally, to determine whether the increase in ADAMTS-2 expression was part of a general reorganization at the ADAMTS family level, all family members were examined. Analysis revealed that many ADAMTS members were significantly upregulated in osteosarcoma compared to normal tissues. Specifically, ADAMTS-2, ADAMTS-6, ADAMTS-7, ADAMTS-12, ADAMTS-14, ADAMTS-18, and ADAMTS-20 were markedly upregulated in tumor tissues, whereas some members such as ADAMTS-L4 were downregulated, indicating that the ADAMTS family undergoes heterogeneous but robust reorganization in osteosarcoma (FDR < 0.05) (Fig. 1D). Overall, these findings suggest that ADAMTS-2 is not merely an individual difference in osteosarcoma but a prominent component of a broader molecular network contributing to matrix remodeling and ECM degradation processes.

Effect of TNF-α on ADAMTS-2 gene expression

ADAMTS-2 may play different roles in cancer progression depending on the type and stage of cancer. ADAMTS-2 suppresses tumor growth and invasion in colorectal cancer but promotes tumor invasion and metastasis in breast cancer [35]. In this study, we examined the effect of TNF-α on the transcriptional regulation of ADAMTS-2 in Saos-2 cells. Within this scope, we performed immunofluorescence analysis of the protein levels of ADAMTS-2 in Saos-2 cells. To

achieve this, Saos-2 cells were treated with 10 ng/mL TNF-α for 24 h, and the untreated cells were used as controls. Our immunofluorescence assay observations consistently mirrored those of western blot analysis, demonstrating that TNF-α stimulation contributed to a noticeable enhancement in ADAMTS-2 protein expression (Fig. 2A). Furthermore, to expand our exploration, we performed western blotting to measure the effect of TNF-α on ADAMTS-2 expression. Treatment with 10 ng/mL TNF-α induced a 2.1-fold increase in the protein expression level of ADAMTS-2 at 3 h and a 2.2-fold increase at 6 h (Fig. 2B). We used qRT-PCR to measure the effect of TNF-α on ADAMTS-2 expression. Our research revealed a substantial correlation between the mRNA and protein expression of ADAMTS-2 in Saos-2 cells. Notably, the most striking 17-fold increase was observed at the 3rd hour (Fig. 2C). The pronounced increase observed during the early phase (1–3 h) likely reflects the immediate transcriptional response to TNF-α, driven by rapid activation of promoter-proximal transcription factors such as NF-κB and AP-1. Although the magnitude of induction decreases at later time points, the sustained elevation observed between 24–48 h may reflect the contribution of additional regulatory processes. These may include TNF-α–associated autocrine or paracrine signaling effects, delayed activation of downstream response pathways, or time-dependent changes in mRNA stability, which together could

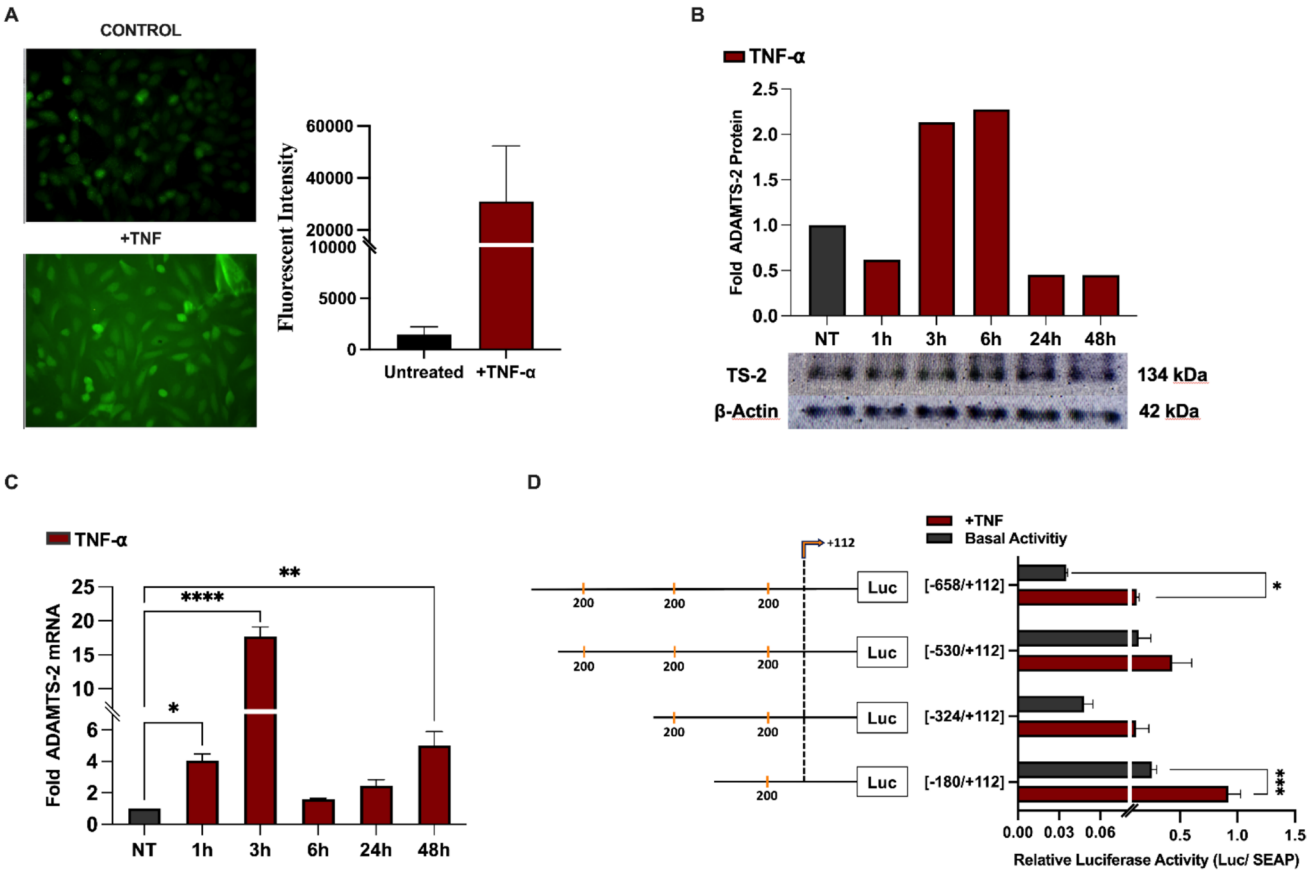

Fig. 2 Effect of TNF-α on ADAMTS-2expression and promoter activity in Saos-2 cells. **A**. Immunofluorescence analysis was conducted to evaluate ADAMTS-2 protein levels in Saos-2 cells. The cells were cultivated on coverslips and treated with TNF-α (10 ng/mL). The corrected total cell fluorescence (CTCF) values of ADAMTS-2 were calculated using ImageJ software for the quantitative evaluation of ADAMTS-2 relative protein expression. Untransfected cells were used as the control group for the analysis. **B.** The effect of TNF-α stimulation on ADAMTS-2 protein levels was examined. To ensure accurate comparison, normalization was performed using β-actin. The resulting graph provides a visual representation of the fold increase in ADAMTS-2 protein levels in cells subjected to TNF-α treatment compared to their untreated counterparts. Quantitative analysis was performed using ImageJ software. **C.** To examine their effects, Saos-2 cells were treated with TNF-α. To assess the impact on ADAMTS-2 mRNA levels, we performed qRT-PCR. For proper comparative analysis, the measured mRNA levels were normalized using the reference gene hβ2, which ensured reliable and standardized measurements across different samples. Untreated cells were used as the control group to establish a baseline, allowing us to discern the specific effect of TNF-α treatment on ADAMTS-2 mRNA levels. **D.** To understand the effects of TNF-α on ADAMTS-2 promoter activity, Saos-2 cells were transiently transfected with ADAMTS-2 promoter constructs and subsequently treated with 10 ng/mL TNF-α for 48 h. The untreated groups served as controls, reflecting the basal promoter activity. In this analysis, luciferase activity was normalized to SEAP activity to ensure accurate comparisons. The reported data represent the mean of three individual measurements and are presented as mean ± standard deviation (SD). Asterisks (*) represent statistical significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$

support the maintenance of ADAMTS-2 expression during prolonged exposure. Subsequently, the effect of TNF-α on ADAMTS-2 promoter activity was investigated because the ADAMTS-2 promoter region contains potential transcription factor-binding sites involved in TNF-α signaling. Transient transfection was employed to introduce ADAMTS-2 promoter constructs, namely, pMET_TS2[−658/+112], pMET_TS2[−530/+112], pMET_TS2[−324/+112], and pMET_TS2[−180/+112], into Saos-2 cells. Following a 16-h transfection period, TNF-α was administered. Luciferase and SEAP activities were assessed after 48 h in both cytokine-treated and untreated cells. Remarkably, TNF-α induced a significant upregulation across all constructs, with the pMET_TS2[−180/+112] promoter activity displaying a substantial up to 4.4-fold increase, which was statistically significant. Additionally, a moderate but statistically significant enhancing effect of TNF-α was observed on pMET_TS2[−658/+112] promoter activity (Fig. 2D).

Pathway analysis of TNF-α mediated ADAMTS-2upregulation

Following the determination that TNF-α plays an important role in regulating ADAMTS-2 expression and promoter activity, signal pathway inhibitors contributing to TNF-α-mediated ADAMTS-2 upregulation were investigated in Saos-2 cells. For this purpose, MEK (PD98059), JNK (SP600125), PI3K (Wortmannin), and NF-κB (BAY11-7082) inhibitors were tested in transient transfection experiments using the [−656/−112] ADAMTS-2 promoter reporter construct. We observed that TNF-α increased ADAMTS-2 [−656/−112] promoter activity approximately 2.5-fold. Following the application of pathway inhibitors, we observed that the inducer effect of TNF-α on ADAMTS-2 promoter activity was reversed by all inhibitors used (Fig. 3A). By extending these promoter-level findings, the effects of the same inhibitors on ADAMTS-2 protein expression were evaluated by Western blot and normalized to β-actin levels. According to the data obtained, ADAMTS-2 protein expression, which was consistently suppressed by TNF-α, was consistently suppressed by all pathway inhibitors, with the most pronounced decrease observed with the NF-κB inhibitor BAY11-7082 (Fig. 3B). Additionally, ADAMTS-2 mRNA levels were examined by qRT-PCR in the same experimental setup and normalized to those of hβ−2. The data showed that TNF-α increased ADAMTS-2 mRNA levels approximately 20-fold, and this induction was largely abolished by inhibitors of MEK, JNK, PI3K, and NF-κB (Fig. 3C). These findings indicate that ADAMTS-2 protein levels are regulated by TNF-α through the participation of multiple signalling pathways.

In silico analysis on the association of ADAMTS-2 expression with NF-κB and STAT signalingpathways in osteosarcoma

When examining the pathway-level distributions of upregulated genes in osteosarcoma, the ECM–receptor interaction,

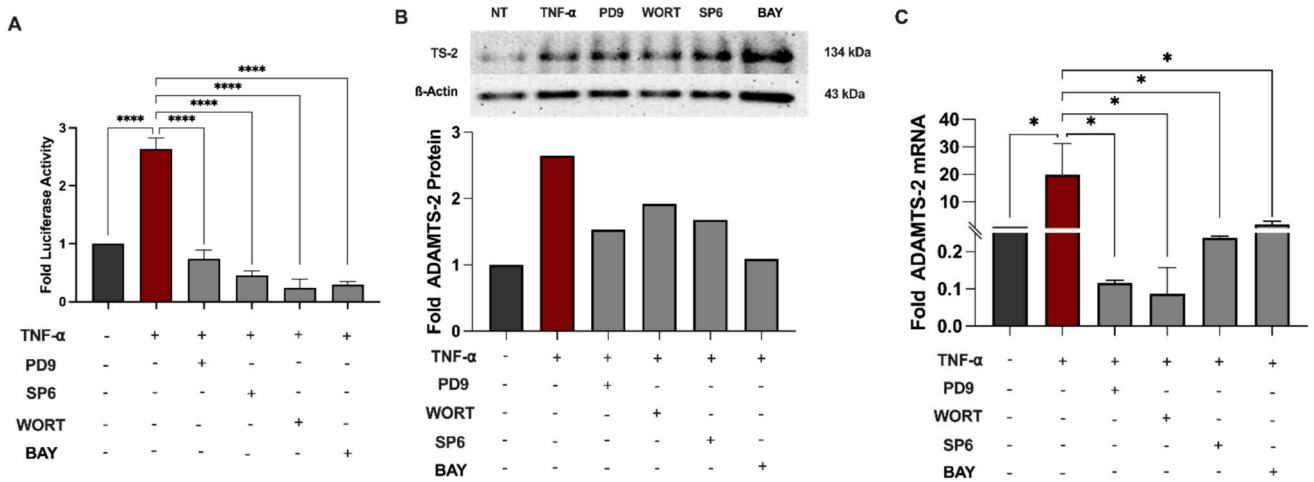

Fig. 3 Influence of MEK, PI3K, JNK, and NF-κB pathways on TNF-α-induced modulation of ADAMTS-2 mRNA and protein levels and promoter activity. **A**. Activation of the human ADAMTS-2 promoter by TNF-α involves the MEK, PI3K, JNK, and NFKB pathways. Saos-2 cells were treated with specific inhibitors before stimulation with TNF-α. Comparative fold changes were calculated. The values are reported as mean ± SE from three independent experiments, each conducted in triplicate. **B.** The MEK, PI3K, JNK, and NF-κB pathways were involved in the induction of ADAMTS-2 protein expression by TNF-α. Protein levels were assessed by western blotting and normalized to β-actin. Quantification of the bands was performed using ImageJ software **C.** The induction of ADAMTS-2 mRNA expression by TNF-α is mediated through the MEK, PI3K, JNK, and NFKB pathways. Saos-2 cells were exposed to the targeted inhibitors before stimulation with TNF-α. mRNA levels were quantified using qRT-PCR after a 6-h stimulation period, with normalization to hβ−2 levels. The presented results represent the mean ± SE of three experiments conducted in triplicate. Asterisks (*) represent statistical significance. *$p<0.05$ and ****$p<0.0001$

focal adhesion, and cytokine–receptor interaction pathways were identified as the most enriched processes. In addition, the TNF, NF-κB, JAK-STAT, and IL-17 signaling pathways were found to be significantly enriched. Furthermore, alterations in apoptosis-related genes revealed that transcriptional networks in tumor tissue extend not only to the remodeling of the extracellular matrix but also to the cell death mechanisms (Fig. 4A). The NF-κB family consists of five members that form different heterodimers in the canonical and non-canonical pathways. In the canonical response, the RELA (p65) and NFKB1 (p50/p105) heterodimer plays a dominant role, whereas in the non-canonical pathway, RELB and NFKB2 (p100/p52) are prominent. The literature indicates that Inhibition of RELA or RELB reduces osteosarcoma cell proliferation, and NF-κB members play a functional role in tumor progression [1]. Therefore, it is conceivable that ADAMTS-2 may be associated with both NF-κB and STAT transcription factors. In family level analyses, NF-κB genes exhibited higher expression in osteosarcoma than in normal tissues. This increase was particularly pronounced for RELA (p65) and NFKB2 (p100/p52), whereas only an upward trend was observed for NFKB1 (p50/p105) (Fig. 4B). Subsequently, when the STAT family was examined, the STAT genes were found to be significantly upregulated in osteosarcoma. STAT1, STAT2, and STAT3 levels were markedly increased in tumor tissues (FDR < 0.05) (Fig. 4C). Considering these findings, the relationship between ADAMTS-2 expression and the signaling pathways was evaluated. Correlation analyses revealed a significant positive correlation between ADAMTS-2 and NFKB1, and a weaker but still positive correlation with STAT3 (Fig. 4D). Overall, these data indicate that ADAMTS-2 is particularly associated with the NF-κB and STAT signaling networks in osteosarcoma, and that this relationship may contribute to the remodeling of the tumor microenvironment in the context of TNF-mediated inflammatory response and ECM remodeling.

Unveiling transcription factor binding dynamics in ADAMTS-2 promoter under TNF-α stimulation

Bioinformatic analyses were employed to identify potential transcription factors that might play a role in the upregulation of ADAMTS-2 in response to TNF-α in the ADAMTS-2 promoter region. The MatInspector tool indicated multiple binding sites that are potentially targeted by STATs and NF-κB within the promoter region of ADAMTS-2, indicating their potential involvement in inflammation-related pathways. To investigate the functional binding of these transcription factors in the presence of TNF-α, EMSA probes were designed for four different regions: [−143/−111], [−175/−143], [−190/−184], and [−436/−400] within the ADAMTS-2 promoter sequence. These probes were then analyzed to assess their interactions with unlabeled STAT-3 and NF-κB consensus binding sites. When the biotinylated probes [−143/−111] and [−436/−400] were incubated with Saos-2 nuclear extracts, the distinctive formation of a single complex was observed (Fig. 5A, lane 2,6; Fig. 5D, lane 2,6). To confirm the specificity of these complexes, unlabeled versions of the [−143/−111] and [−436/−400] probes were introduced. Subsequent competition assays demonstrated the complete abolishment of these complexes. This occurred when unlabeled STAT-3 or NF-κB consensus oligonucleotides were introduced, regardless of whether the nuclear extracts were from the control (Fig. 5A and 5D, lanes 3, 4, and 5) or TNF-α-stimulated group (Fig. 5A and 5D, lanes 7, 8, and 9). This outcome suggests that STAT and NF-κB may bind to the ADAMTS-2 promoter regions.

Furthermore, nuclear extracts from both TNF-α-treated and untreated Saos-2 cells were used to compete with the biotinylated ADAMTS-2 [−175/−143] probe, employing non-biotinylated [−175/−143], STAT-3, and NF-κB probes. The density of the formed complexes decreased, although the complexes did not completely disappear. Finally, focusing on the [−190/−184] probe, EMSA analysis revealed the emergence of three different complex formations when nuclear extracts were employed, regardless of the presence of TNF-α (Fig. 5C, lanes 2 and 6). In competitive assays, the strength of these complexes diminished upon the introduction of an excessive quantity of unlabelled [−190/−184] probes and STAT-3 and NF-κB consensus oligonucleotides. This reduction in intensity was observed in both control and TNF-α-stimulated nuclear extracts. Notably, complex 2 exhibited a remarkable outcome: complete abolition when treated with either unlabelled STAT-3 or NF-κB consensus oligonucleotides. These findings support the possibility that STAT-3 and NF-κB with the promoter of ADAMTS-2 when TNF-α-treated Saos-2 nuclear extracts were employed in the binding reactions, and a more robust complex formation was observed (Fig. 5C, lane 6), thereby indicating the stimulating impact of TNF-α on the binding of STAT-3 and NF-κB proteins within the [−190/−184] region.

Discussion

Osteosarcoma is an aggressive primary bone tumor that primarily affects adolescents and young adults and exhibits rapid cellular proliferation and high metastatic potential. The progression of the disease is closely related to the uncontrolled proliferation of osteoblasts, which regulate bone formation and ECM dynamics [36–39]. Therefore, proteases that remodel the ECM, particularly members of the ADAMTS family, are emerging as critical regulators in

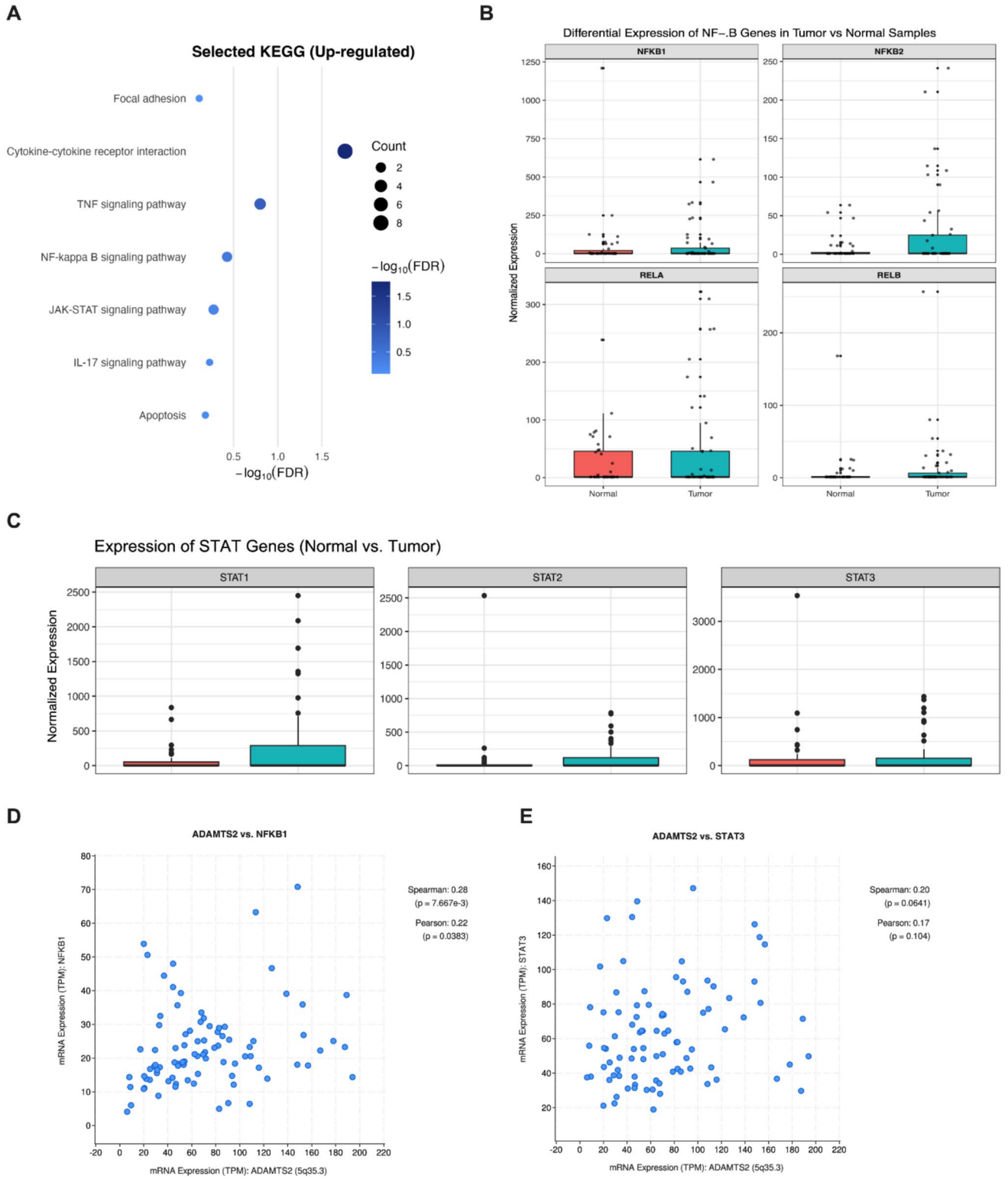

Fig. 4 Relationship between ADAMTS-2 and NF-κB and STAT pathways in osteosarcoma. **A**. KEGG pathway analysis of differentially upregulated genes revealed significant enrichment in ECM–receptor interaction, focal adhesion, cytokine–receptor interaction, TNF, NF-κB, JAK-STAT, IL-17, and apoptosis signaling pathways. **B.** Expression analysis of the NF-κB gene family revealed a marked increase in osteosarcoma tissues compared to normal tissues, particularly for RELA (p65) and NFKB2 (p100/p52), with an upward trend in NFKB1 and RELA. **C.** Expression analysis of the STAT family showed that STAT1, STAT2, STAT3, and STAT6 were significantly upregulated in osteosarcoma, whereas STAT5A and STAT5B showed a more limited tendency to increase. **D-E.** Correlation analyses revealed that ADAMTS-2 gene expression was positively correlated with NFKB1 and STAT3 expression

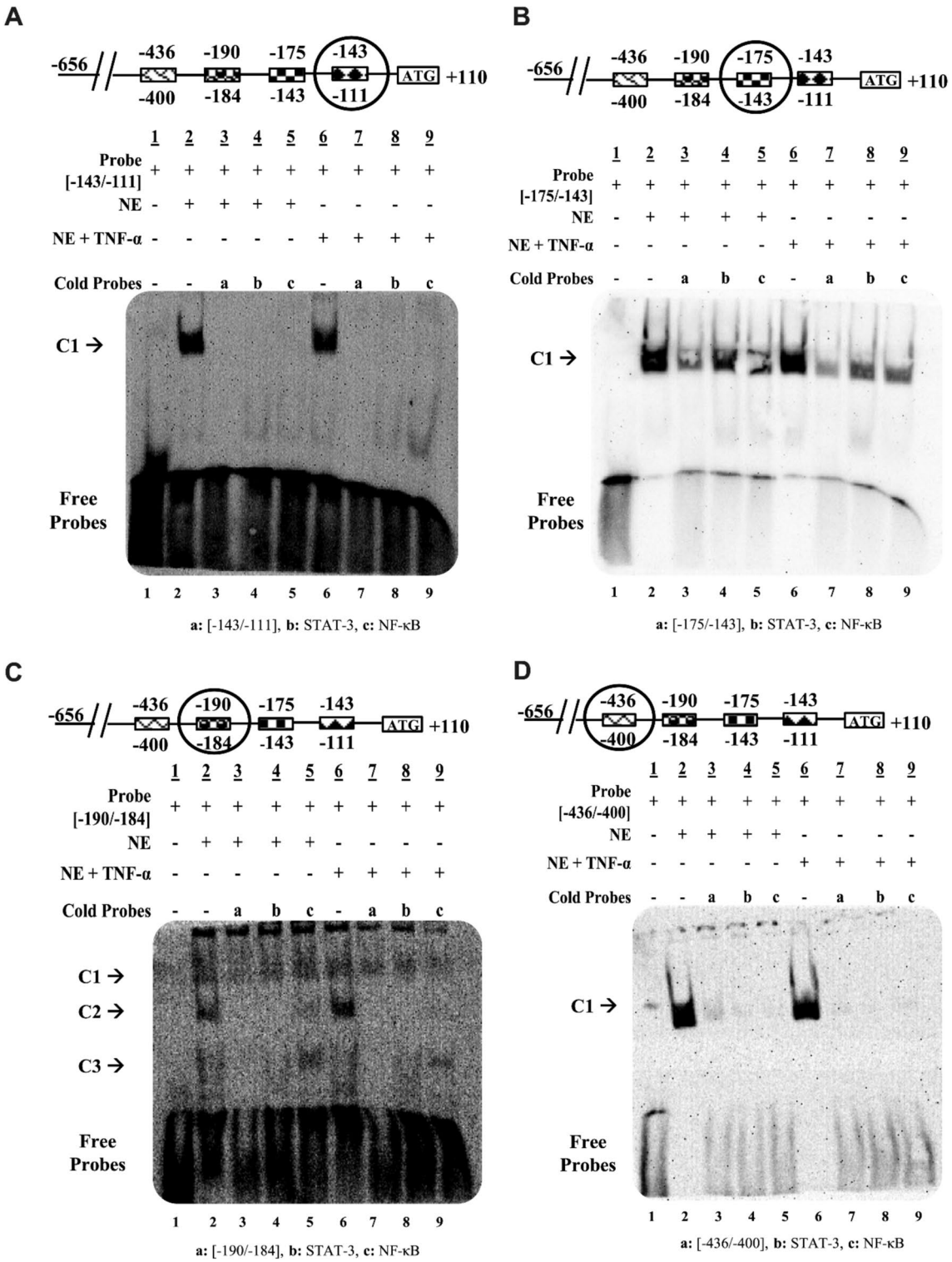

Fig. 5 EMSA was conducted to explore the binding of STAT-3 and NF-κB transcription factors to the ADAMTS-2 promoter region in the presence and absence of TNF-α. **A**. The binding competition between the biotin-labeled probe [−143/−111] and unlabeled STAT-3 and NF-κB transcription factors was assessed using Saos-2 nuclear extracts with or without TNF-α. **B.** A similar approach was used with nuclear extracts from TNF-α-treated and untreated Saos-2 cells, and non-biotinylated STAT-3 and NFκB probes competed against the biotinylated ADAMTS-2 [−175/−143] probe. **C.** We analyzed transcription factor binding using the biotin-labeled ADAMTS-2 [−190/−184] probe and unlabeled STAT-3 and NFκB transcription factors within Saos-2 nuclear extracts, accounting for the presence or absence of TNF-α in the medium. **D.** Binding analysis of transcription factors was carried out using the biotin-labeled ADAMTS-2 [−436/−400] probe, and the competition with unlabeled STAT-3 and NFκB transcription factors was examined using Saos-2 nuclear extract under both TNF-α-present and TNF-α-absent conditions

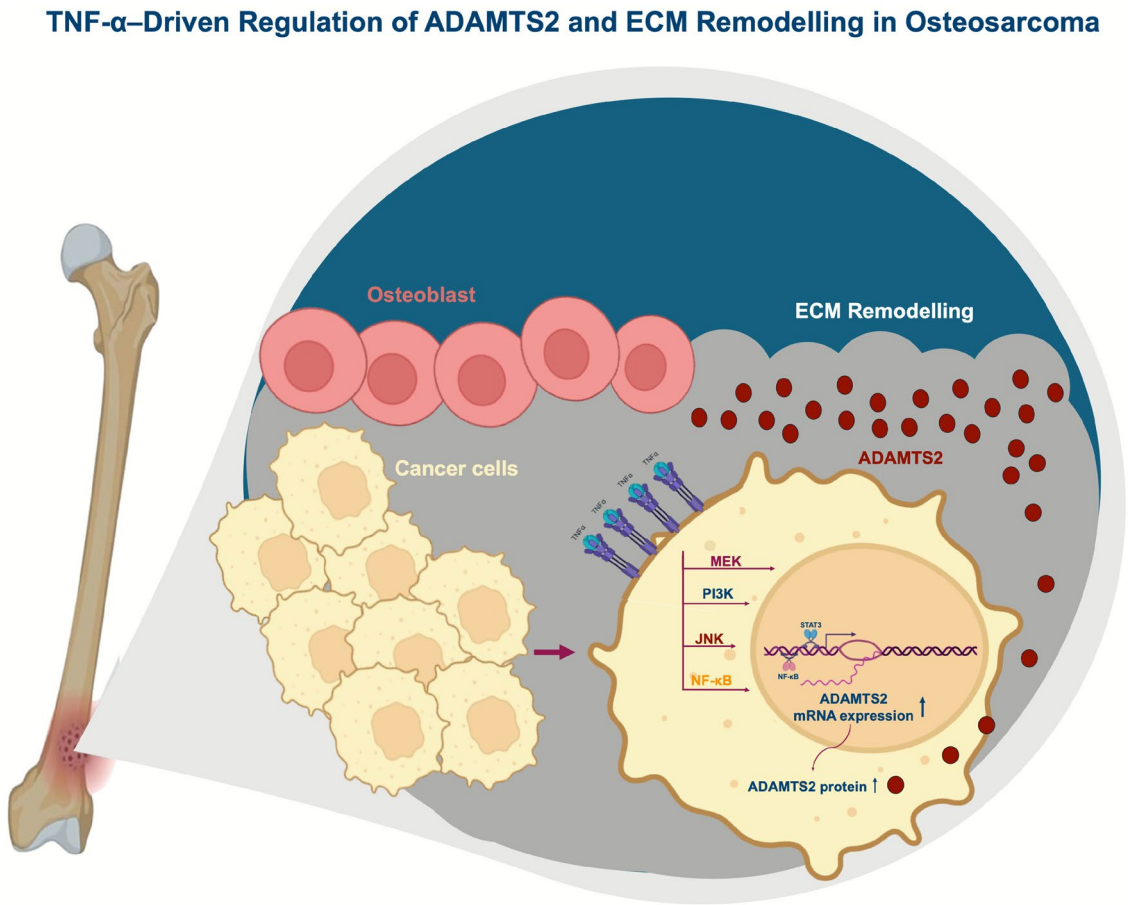

Fig. 6 Schematic representation of the proposed TNF-α–driven regulatory mechanism controlling. ADAMTS-2 expression and extracellular matrix (ECM) remodelling in osteosarcoma. TNF-α stimulation activates multiple intracellular signaling pathways, including MEK/ERK, PI3K/AKT, JNK, and NF-κB, leading to the activation of transcription factors such as NF-κB and STAT3. These factors are suggested to regulate ADAMTS-2 transcription, resulting in increased ADAMTS-2 mRNA and protein expression. Elevated ADAMTS-2 levels contribute to ECM remodelling within the osteosarcoma microenvironment, potentially facilitating tumor progression

osteosarcoma biology. Different members of the ADAMTS family perform distinct yet interconnected functions in osteosarcoma. For example, low expression of ADAMTS-7 has been associated with poor prognosis and has been shown to suppress tumor progression through COMP-mediated mechanisms [40]. ADAMTS-1 has been reported to increase three-dimensional tumor growth by regulating type I collagen processing, while decreasing cell migration [41]. Furthermore, it is known that ADAMTS-2 and ADAMTS-3 affect collagen metabolism by being regulated by cytokines such as IL-6 and transcription factors such as SP1 and USF1 [33, 34, 42]. These collective findings demonstrate that the ADAMTS family has gene-specific but functionally interconnected roles in osteosarcoma Fig. 6.

ADAMTS-2, one of the key components of ECM remodeling, holds particular importance in osteosarcoma. In addition to being an essential procollagen N-proteinase for collagen biogenesis, ADAMTS-2 is an active regulator of the tumour microenvironment, as demonstrated by recent studies [24, 43]. Increased ADAMTS-2 expression in colorectal cancer has been associated with invasion and metastatic spread and has been proposed as a biomarker for distinguishing high-risk patient subgroups [44]. Similarly, a marked increase in ADAMTS-2 in HGSOC models supports proliferation, migration, and invasion, suggesting that the gene functions through common mechanisms across tumor types [45]. Furthermore, its increased expression in both tumor and stromal cells in gastric cancer indicates that ADAMTS-2 may have a dual role in regulating the microenvironment [46]. Consistent with these literature findings, bioinformatic analyses performed in our study demonstrated that ADAMTS-2 is significantly upregulated in osteosarcoma tissues. Furthermore, it was determined that the increase in ADAMTS-2 shows strong parallels with gene sets associated with collagen degradation, ECM remodeling, and cellular adhesion. These results support the notion that ADAMTS-2 may be a central regulator of matrix remodeling in osteosarcoma. The fact that ADAMTS-2 has such a closely related expression

pattern to the ECM suggests that the gene may be involved in upstream inflammatory signaling pathways. These results support the notion that ADAMTS-2 may be a central regulator of matrix remodeling in osteosarcoma. The fact that ADAMTS-2 exhibits such a closely related expression pattern to the ECM raises the critical question of which upstream inflammatory signals trigger this gene. Although some members of the ADAMTS family have been reported in the literature to respond to pro-inflammatory cytokines, it has not been clearly defined which specific signaling pathways ADAMTS-2 responds to.

Osteosarcoma is a tumor type that is highly sensitive to cytokine imbalances due to its distinct inflammatory microenvironment. The decisive effects of IL-1β and IL-6 on proliferation, migration, and invasion have been demonstrated in previous studies [47]. TNF-α is a key regulator of the osteosarcoma microenvironment, and its activation triggers signaling networks that shape the aggressive behavior of tumor cells [48–54]. Although it is known that TNF-α modulates some members of the ADAMTS family, the specific signaling axes it activates in ADAMTS-2 are not yet understood. To address this knowledge gap, our study provides a comprehensive assessment of how TNF-α regulates ADAMTS-2 expression in osteoblastic Saos-2 cells in a time-dependent and mechanistic manner. Our expression study clearly demonstrates that the marked increase observed in both ADAMTS-2 protein levels and ADAMTS-2 mRNA expression following TNF-α application indicates that this gene elicits a potent and time-dependent inflammatory response in osteosarcoma cells. qRT-PCR analyses revealed that mRNA levels began to rise at 1 h and reached approximately 18–20-fold at 3 h. This early peak suggests that TNF-α triggers rapid transcriptional activation and that ADAMTS-2 may be among the early inflammatory response genes. However, the biphasic kinetic profile observed in ADAMTS-2 mRNA expression is noteworthy; the sharp increase in the early phase is followed by a more moderate second rise in subsequent hours. This dynamic response is thought to be related to the activation of TNF-α's multistage signaling networks.

To determine which upstream signaling mechanisms drive the time-dependent increase observed in ADAMTS-2 in response to TNF-α, luciferase reporter assays were performed using the promoter region pMET_TS2[−658/+112]. These analyses clearly demonstrate that ADAMTS-2 promoter activation is not governed by a single signaling pathway, but rather results from the coordinated interplay of multiple signaling mechanisms. The MEK/ERK pathway is the primary regulator of transcriptional activation in TNF-α signaling. This pathway triggers the expression of ECM-related genes by activating transcription factors such as AP-1, SP1, and Elk-1 through ERK-dependent phosphorylation events [35, 55]. This finding is consistent with studies reporting that IL-1α regulates ECM-associated metalloproteinase genes via the MEK/ERK-mediated signaling network. Indeed, Alper et al. demonstrated that IL-1α increases ADAMTS-2 expression by activating the MEK/ERK and PI3K pathways in osteoblast-like cells [56]. The PI3K/AKT pathway is an important signaling branch in the TNF-α response. Previous studies have reported that primary osteoblasts increase the expression of metalloproteinase genes through the interaction between the TNF-α-mediated PI3K/AKT axis and NF-κB activation [57]. These results enabled us to investigate potential transcription factors involved in the upregulation of ADAMTS-2 by TNF-α in osteosarcoma, further deepening our understanding of inflammation-related pathways. The JNK pathway is generally a key component of TNF-α-mediated stress and inflammation signaling. JNK activation increases the transcriptional activity of the AP-1 complex via c-Jun phosphorylation [18]. Similarly, the literature reports that JNK activation induces the expression of ADAMTS-4 and MMP-9 [58]. The NF-κB pathway is the best-defined signaling axis for TNF-α. TNF-α induces the transcription of NF-κB-dependent genes by causing the degradation of IκB and translocation of the p65/p50 dimer to the nucleus via the TNFR1-TRAF2-IKK complex [17]. Similarly, Tian et al. reported that TNF-α activates ADAMTS-4 and ADAMTS-5 via the NF-κB and MAPK pathways [59]. Notably, our findings provide the first comprehensive evidence that TNF-α regulates ADAMTS-2 transcription through the integrated activation of the MEK/PI3K/JNK/NF-κB signaling network, rather than via an isolated pathway, thereby constituting the fundamental mechanistic novelty of this study.

Bioinformatics analyses revealed high-probability binding sites for STAT3 and NF-κB in the ADAMTS-2 promoter region, suggesting that these transcription factors may play a role in regulating gene expression in response to TNF-α. KEGG analyses revealed that genes co-expressed with ADAMTS-2 were significantly enriched in the ECM–receptor interaction, focal adhesion, TNF, NF-κB, JAK–STAT, and IL-17 signaling pathways. Similarly, it has been reported that ADAMTS-8, another member of the ADAMTS family, is upregulated in colorectal cancer through the activation of NF-κB and STAT3 signalling pathways via TNF-α [60], which induces ADAMTS-7 expression via NF-κB signalling in osteoarthritis models [61]. However, specifically regarding ADAMTS-2, although data indicate that IL-6 increases its expression in osteosarcoma via JNK signalling [62], the relationship between TNF-α and ADAMTS-2, as well as the molecular pathways through which this interaction occurs, has not been sufficiently clarified in the literature. In silico analyses conducted to address this gap revealed that ADAMTS-2 was positively correlated with both NF-κB and

STAT3. Furthermore, the observed increase in RELA (p65) and NFKB2 (p100/p52) in osteosarcoma tissues supports both canonical and non-canonical NF-κB activation, while the upregulation of STAT1, STAT2, and STAT3 suggests that JAK/STAT and NF-κB signaling may intersect ADAMTS-2 regulation. These findings suggest that TNF-α–mediated inflammatory signalling may be associated with ECM remodeling and cell adhesion through ADAMTS-2. Finally, EMSA analyses performed to experimentally validate our in silico data suggested that NF-κB and STAT3 may potentially interact with specific regions of the ADAMTS-2 promoter in osteosarcoma cells, and that the intensity of these interactions increases following TNF-α stimulation. The specificity and efficiency of the DNA–protein interaction were validated through multiple EMSA-based approaches. Competition assays using an unlabelled (cold) version of the probe effectively abolished complex formation, confirming the sequence-specific nature of the binding. Furthermore, competition with NF-κB and STAT3 consensus oligonucleotides markedly reduced or eliminated the shifted bands, indicating that the observed complex is predominantly formed by NF-κB or STAT3 transcription factors. These EMSA findings collectively support the involvement of these factors in mediating TNF-α–responsive promoter binding. When these results are considered together, it is suggested that ADAMTS-2 may function as both a target and a potential effector of inflammatory transcriptional networks in osteosarcoma. Importantly, this study provides the first systematic evidence suggesting that TNF-α regulates ADAMTS-2 expression through the simultaneous activation of multiple signaling pathways, possibly involving STAT3 and NF-κB at promoter-proximal regions. This mechanistic insight constitutes the central innovation of our work. Our research not only fills existing gaps in knowledge regarding the molecular basis of the TNF-α–ADAMTS-2 interaction but also provides a new perspective on understanding inflammation-mediated tumor progression. These findings shed light on a potential role for inflammatory signalling in osteosarcoma pathogenesis and provide a valuable biological basis for the development of targeted therapeutic strategies in the future.

Conclusions

This study demonstrates that multiple signaling pathways may play a role in regulating ADAMTS-2 expression in osteosarcoma cells in response to TNF-α. Our findings reveal that TNF-α treatment is associated with a time- and pathway-dependent increase in ADAMTS-2 mRNA, protein, and promoter activity levels. The partial or significant reduction of this increase by MEK/ERK, PI3K/AKT, JNK, and NF-κB inhibitors suggests that the effect of TNF-α on ADAMTS-2 may not be reducible to a single mechanism and that multiple signalling components may contribute together. Bioinformatics analyses pointed to a possible relationship between ADAMTS-2 and the NF-κB and STAT3 signalling axes; EMSA studies provided results suggesting that these transcription factors may interact with specific promoter regions. However, advanced functional experiments are needed to confirm these interactions.

Overall, this study demonstrates that multiple signaling pathways and transcription factors may be involved in the transcriptional regulation of ADAMTS-2 by TNF-α, shedding light on the role of ADAMTS-2 in osteosarcoma biology within the context of inflammation-driven ECM remodeling. This study provides a foundation for future mechanistic investigations aimed at elucidating the molecular basis of this axis.

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Data availability The data supporting the findings of this study are available from the corresponding author upon reasonable request. request.

Declarations

Competing interest The authors declare no competing interests.

Ethics approval This article does not contain research in which animals or humans were used, so ethical approval was not required for this study.

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