Adıyaman University Journal of Science
https://dergipark.org.tr/en/pub/adyujsci
DergiPark AKADEMİK
ISSN 2147-1630
e-ISSN 2146-586X

C/EBPα Mediated Transcriptional Regulation of Human *ADAMTS-3* Gene and Collagen Expression in Osteosarcoma Cells

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Received: 14.06.2024 **Accepted:** 08.07.2024 **Published:** 31.12.2024

Abstract

ADAM Metallopeptidase with Thrombospondin Type 1, Motif 3 (ADAMTS-3) is a procollagen amino proteinase mainly expressed in type II collagen-rich tissues and its primary function is to maturate amino ends of the type II collagen precursors. This maturation process allows correct fibril formation. ADAMTS-3 also has a tumor-suppressive function by regulating the fibronectin expression in the extracellular matrix (ECM.) CCAAT/enhancer-binding protein (C/EBPα) is a transcription factor playing a pivotal role in the cell cycle regulation. Dysregulations in the C/EBPα expression have been reported in solid tumors. C/EBPα expression has been identified to be associated with the metabolism and prognosis of a malignant bone tumor, osteosarcoma (OS). High heterogeneity, metastasis capability, and recurrence lead to poor prognosis and survival rates in OS. Multiple genetic and epigenetic factors affect OS development. Alterations in the ECM elements are closely related to OS development and

* Corresponding Author DOI: 10.37094/adyujsci.1501420

progression. According to the *in-silico* analyses, the ADAMTS-3 promoter includes multiple C/EBPα binding sites suggesting that C/EBPα could have a regulatory effect on ADAMTS-3 transcriptional regulation. In the present study, over-expression of the C/EBPα decreased ADAMTS-3 mRNA and protein levels in Saos-2 and MG-63 osteosarcoma models. Ectopic expression of the C/EBPα also led to alterations in some fibrillar collagen expression levels. Enhanced C/EBPα levels resulted in an increase in the type I and II collagen expression levels but didn't change the type III collagen expression level in Saos-2 cells. On the contrary, increased C/EBPα levels resulted in a decrease in all collagen expression levels in MG-63 cells Co-transfection analysis revealed that C/EBPα negatively regulates ADAMTS-3 promoter activity in both Saos-2 and MG-63 cells. Further EMSA studies indicated that C/EBPα functionally binds to the proximal region of the *ADAMTS-3* gene. Our findings will contribute to understanding C/EBPα mediated regulation of ADAMTS-3 and collagen composition in osteosarcoma cells.

Keywords: *ADAMTS-3*; C/EBPα; Collagen; Transcriptional regulation; Saos-2; MG-63.

Osteosarkoma Hücrelerinde İnsan *ADAMTS-3* Geni ve Kollajen Eksresyonunun C/EBPα Aracılı Transkripsiyonel Regülasyonu

Öz

ADAMTS-3, temelde tip II kollajen bakımından zengin dokularda eksprese edilen ve primer görevi tip II kollajen öncüllerinin amino uçlarını olgunlaştırmak olan bir prokollajen amino proteinazdır. Bu olgunlaşma süreci fibril oluşumunun düzgün olmasını sağlar. ADAMTS-3, hücrelerarası matriksteki (ECM) fibronektin ekspresyonunu düzenleme yoluyla tümör baskılayıcı bir işleve de sahiptir. C/EBPα, hücre döngüsü düzenlemesinde oldukça önemli rol oynayan bir transkripsiyon faktörüdür. Solid tümörlerde, C/EBPα ekspresyonunda düzensizlikler bildirilmiştir. C/EBPα ekspresyonunun, malign bir kemik tümörü olan osteosarkomanın (OS) metabolizması ve prognozu ile ilişkili olduğu tespit edilmiştir. Yüksek heterojenite, metastaz yeteneği ve rekürrens, OS'de kötü prognoz ve düşük sağkalım oranlarına yol açmaktadır. Osteosarkoma oluşumunda birçok genetik ve epigenetik faktör etkilidir. ECM bileşenlerindeki değişiklikler, OS oluşumu ve progresyonuyla yakından ilişkilidir. *In-silico* analizlere göre, ADAMTS-3 promotoru birçok C/EBPα bağlanma bölgesi içermektedir. Bu durum, C/EBPα'nın *ADAMTS-3* geninin transkripsiyonel regülasyonu üzerinde etkili olabileceğini düşündürmektedir Bu çalışmada, C/EBPα'nın over-ekspresyonu, Saos-2 ve MG-63 osteosarkoma modellerinin her ikisinde de ADAMTS-3 mRNA ve protein seviyelerini azaltmıştır. C/EBPα'nın ektopik

ekspresyonu ayrıca bazı fibriller kollajenlerin ekspresyon seviyelerinde de değişikliklere yol açmıştır. Saos-2 hücrelerinde, artan C/EBPα seviyesi, tip I ve II kollajenlerin ekspresyon seviyelerinde artışla sonuçlanmış ancak tip III kolajen ekspresyon seviyesini değiştirmemiştir.MG-63 hücrelerinde ise tersi şekilde, artan C/EBPα seviyesi tüm kollajen ekspresyon seviyeleri için bir düşüşle sonuçlanmıştır. Ko-transfeksiyon analizi, C/EBPα'nın hem Saos-2 hem de MG-63 hücrelerinde ADAMTS-3 promotör aktivitesini negatif olarak düzenlediğini ortaya koymuştur. EMSA çalışmaları, C/EBPα'nın ADAMTS-3 geninin proksimal bölgesine işlevsel olarak bağlandığını göstermiştir. Bulgularımız, osteosarkoma hücrelerinde ADAMTS-3'ün ve kollajen kompozisyonunun C/EBPα aracılı regülasyonunun anlaşılmasına katkıda bulunacaktır.

Anahtar Kelimeler: ADAMTS-3; C/EBPα; Kollajen; Transkripsiyonel regülasyon; Saos-2; MG-63.

1. Introduction

ADAMTS-3 is a member of ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs type I) protease family and participates in the procollagen amino proteinase (pNP) subgroup. ADAMTS-3 is mainly expressed in type II procollagen-rich tissues such as cartilage and its primary function is to maturate the amino terminus of the procollagen II [1]. This maturation process is very critical for the conformation of the correct fibril structure. In addition, ADAMTS-3 contributes to lymphatic vessel development by activating Vascular Endothelial Growth Factor (VEGF-C) [2]. Recent studies identified that ADAMTS-3 restricts cancer invasion in early breast cancer models by enhancing fibronectin degradation [3].

The C/EBPs (CCAAT/enhancer-binding protein) are modular proteins including six members sharing structural and functional common features. Members of the family modulate a variety of biological processes [4]. C/EBPα belongs to a basic region leucine zipper (bZIP) transcription factor (TF) family. C/EBPα plays a pivotal role in cell cycle regulation, specifically coordinating the proliferation and differentiation of the myeloid progenitors, adipocytes, and hepatocytes as well as lung and placenta cells [5-8]. In recent studies, the tumor suppressor role of the C/EBPα in acute myeloid leukemia (AML) has been identified. Alterations in the C/EBPα expression have been reported in solid tumors such as liver, breast, or lung cancer [9-11]. C/EBPα has been determined as an immune-related antitumor TF in osteosarcoma [12].

Osteosarcoma (OS) is a malignant bone tumor that commonly affects children and adolescents. High heterogeneity, metastasis capability, and recurrence rates lead to poor prognosis and survival rates in OS. Multiple genetic and epigenetic factors affect OS development. Recent

studies revealed that dysregulations in the ECM elements are also closely related to OS progression and migration and chemotherapy response [13-16].

In the present study, we aimed to elucidate transcriptional regulation of ADAMTS-3-a collagen processing matrix proteinase- by C/EBPα and also fibrillar collagens (type I, II, and III) in osteosarcoma cell lines. *In silico* analysis indicated multiple C/EBPα binding sites at the proximal region of the *ADAMTS-3* gene. So, we hypothesized that C/EBPα would have a regulatory effect on ADAMTS-3 promoter activity and ADAMTS-3 expression. In this context, ADAMTS-3 mRNA and protein levels were determined in C/EBPα over-expressed Saos-2 and MG-63 cells. Because fibrillar collagen types I, II, and III are the substrates of the ADAMTS-3, their mRNA levels were also determined to identify the effect of C/EBPα overexpression on collagen composition. Further, the effect of C/EBPα overexpression on the activity of truncated ADAMTS-3 promoter constructs was evaluated. Functional binding of the C/EBPα on the ADAMTS-3 promoter region was identified by Electrophoretic Mobility Shift Assay (EMSA) experiments.

2. Materials and Methods

2.1. Cell Culture and Plasmids

Saos-2 and MG-63 (human osteosarcoma cell line) cells were grown in DMEM (Dulbecco's modified Eagle's medium, Euroclone) supplemented with 10% FCS (fetal calf serum, Sigma) and 2 mM of L-Glutamine (Sigma). All cells were maintained in a humidified incubator with 5%CO_2 at 37˚C. Four different ADAMTS-3 promoter fragments were cloned into the pMeTLuc reporter vector in our previous studies [17]. The human C/EBPα expression plasmid was kindly gifted from Dr. Dipak P. Ramji, Cardiff School of Biosciences.

2.2. Transient transfection and dual reporter assay

Briefly, the cells were plated into 12-well tissue culture plates (25×10^4) 24 h before the transfection. 1μg of ADAMTS-3 promoter-reporter plasmid was transfected into the cells according to the calcium-phosphate precipitation method. SEAP (0.5 μg) (secreted human alkaline phosphatase, Promega) plasmid used for normalization in transfection assays. In addition, 2 μg of C/EBPα expression plasmid was transfected into the cells for co-transfection assays [18]. The luminescence value was measured from the collected medium after 48 h/72 h of transfection using Ready-To-Glow™ Secreted Luciferase Reporter Systems (Takara) and a Fluoroskan Ascent FL Luminometer. pMetLuc control and empty pMetLuc reporter vectors were used as positive and negative controls [19].

2.3. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from control and C/EBPα transfected cell pellets using RNeasy Kit (Thermo Sci.) following the instructions. 1µg of total RNA was reverse-transcribed into cDNA. PCR was performed in 10µl final volume including 1 µL of cDNA template, 5 µL of Light Cycler-FastStart DNA Master SYBR Green I mix (Roche), and 0.5 µL of each pair of primers (10 ng/µL) (Table 1). Cycling conditions were applied as described before. Light Cycler 485 instrument (Roche Diagnostics) was used. Reactions were set up in triplicate for more accurate measurement. hβ-2 (human β-2 microglobulin) was used as an internal control. ΔΔCt method was used to calculate relative changes in *ADAMTS-3* gene expression [20, 21].

2.4. Western blotting

Total protein extraction was performed using RIPA buffer following the previously described instructions. Proteins were loaded on SDS-PAGE with equal concentrations (30-50 µg) for control cells and C/EBPα over-expressed cells. Membranes were treated with polyclonal ADAMTS-3 (3 µg/mL) (Abcam, ab45037), at 4°C for 16 h, or monoclonal β-actin (Santa Cruz Biotech., sc81178) antibody at room temperature for 1 h. Then membranes were washed and treated with HRP-conjugated secondary antibodies at room temperature for 1 h. ECL (Thermo Scientific) was used to visualize the protein bands and the membrane was photographed with Fusion FX Vilber Lourmat. Image J was used for densitometric quantification of protein bands [22, 23].

2.5. Electrophoretic mobility shift assay (EMSA)

EMSA is a core technique used to detect nucleic acid-protein interactions [24]. Here we performed EMSA experiments to identify functional binding of the C/EBPα to the ADAMTS-3 promoter. Nuclear extracts were prepared from control and C/EBPα overexpressing Saos-2 cells as described before [17]. 3' Ends of the Synthetic oligonucleotides (Table 1) were labeled with Biotin-11-UTP and TdT (Terminal Deoxynucleotidyl Transferase) (Thermo Sci.). Nuclear extracts (4 µg) were incubated with biotinylated double-stranded oligonucleotides (20 pmol) in 10% binding buffer and 0.05 µg/mL PolydIdc for 30 min at room temperature. An un-labeled form of the same probe or un-labeled consensus C/EBPα probe (500-fold) was used in competition assays (Table 1). Native polyacrylamide gel (6%) was used to analyze Protein/DNA complexes. [18]. After cross-linking of the complexes to the nylon membrane, biotin signals were detected using a Chemiluminescent Nucleic Acid Detection Module (Thermofisher Sci.) according to the instructions.

2.6. Bioinformatic and statistical analysis

MatInspector (Genomatix Software) was used to determine putative transcription factor-binding sites on the ADAMTS-3 promoter with a threshold of 0.9 [25, 26]. p-values were calculated by using Mini Tab 14 software. $p \leq 0.005$ value accepted as statistically significant. One-way ANOVA analysis was applied between the pairs for statistical significance.

Table 1: Primer sequences used in qRT-PCR and EMSA experiments.

Primer Name	**Sequence**
ADAMTS3 F	5'-TCAGTGGGAGGTCCAAATGCA-3'
ADAMTS3 R	5'-GCAAAGAAGGAAGCAGCAGCC-3'
COL1A1 F	5'-CTAGACATGTTCAGCTTTGTGGACCT-3'
COL1A1 R	5'-GTTGTCGCAGACGCAGATCCG-3'
COL2A1 F	5'-TCGGAGAGTGCTGCCCCATCT-3'
COL2A1 R	5'-GGCAGCAAAGTTTCCACCAAGA-3'
COL3A1 F	5'-AGCTGGCTACTTCTCGCTCTGCT-3'
COL3A1 R	5'-GTTCTGAGGACCAGTAGGGCATGA-3'
β-2Microglobulin F	5'-TTTCTGGCCTGGAGGCTATC-3'
β-2Microglobulin R	5'-CATGTCTCCATCCCACTTAACT-3
C/EBP α consensus F	5'TGCAGATTGCGCAAT3'
C/EBP α consensus R	5'TGCATTGCGCAATCT3'
ADAMTS3[−131/−103] F	5′-GCTCAAATTTCATTTTCATTGAAGCAAAG-3′
ADAMTS3[−131/−103] R	5′-CTTTGCTTCAATGAAAATGAAATTTGAGC-3′
ADAMTS3[−40/12] F	5′-AACTTATTTTGGGCGGGGGGGGTGGGTTTGC-3′
ADAMTS3[−40/12] R	5′-GCAAACCCACCCCCCCCGCCCAAAATAAGTT-3′

3. Results and Discussion

3.1. C/EBPα overexpression leads to decreased ADAMTS-3 mRNA and protein expression in both Saos-2 and MG-63 cells

To evaluate the regulatory effect of C/EBPα on ADAMTS-3 expression in Saos-2 and MG-63 cells, the C/EBPα expression plasmid was transiently transfected into Saos-2 and MG-63 cells. Ectopic C/EBPα expression was confirmed at mRNA and protein levels by qRT-PCR and western-blot assays. C/EBPα mRNA expression level significantly increased in C/EBPα transfected groups up to 2 fold, and protein level up to 2.5 fold after 24 hours of transfection compared to the control groups in Saos-2 cells (Figure 1A and B). Therefore, ADAMTS-3 mRNA and protein levels were analyzed after 24 hours of C/EBPα transfection. C/EBPα reduced

ADAMTS-3 mRNA (5.8 fold) and also protein level (2.6 fold) in a statistically important manner (Figure 1C and 1D). Since the main task of ADAMTS-3 is to process amino ends of the collagens, we also investigated the effect of C/EBPα on the expression of type I, II, and III collagens. C/EBPα overexpression induced mainly collagen types I (2 fold) and II (1.3 fold) and didn't lead to any changes in collagen III expression level (Figure 1E). Next, we assessed alterations in ADAMTS-3 expression in a different osteosarcoma model, MG-63. Although both Saos-2 and MG-63 cells are osteosarcoma models, they differ in origin and gene expression profiles. For instance, while Saos-2 cells have epithelial characteristics, MG-63 cells are fibroblastic. These cells also differ in their collagen compositions ([27, 28]. As can be seen in Figure 2A, C/EBPα expression was significantly increased up to 6.5-fold after 6 h of transfection relative to non-transfected groups. C/EBPα overexpression significantly decreased ADAMTS-3 mRNA (0.024-fold at 6 h and 0.26-fold at 24 h) and protein expression (0.6-fold) levels similar within Saos-2 cells (Figure 2B and 2C) Secondly, the effect of the C/EBPα protein on collagen expression patterns were investigated. Quite different from Saos-2 cells, it was determined that C/EBPα significantly suppressed collagen I, II, and III expressions in MG-63 cells (Figures 2D and 2E). As a main substrate of the ADAMTS-3, the collagen II protein expression level was also confirmed in C/EBPα overexpressing MG-63 cells and a slight decrease was observed (Figure 2F).

Regulation of the ADAMTS family members with CEBP transcription factors is mainly studied in vascular diseases because of the ECM processing activity in the vessel wall. For example, C/EBPβ has been identified as the chief mediator in IL-1β and TNF-α mediated regulation of ADAMTS-1 in vascular cells. Therefore ADAMTS-1 has been suggested as a promising candidate for the development of novel therapeutics for vascular disorders [29]. Abnormalities in the signaling and structural components of the ECM have been known as the leading causes of osteosarcoma formation and progression [14]. Wei and colleagues identified that type I collagen could facilitate malignant OS development and induce the capability of cell proliferation and tumorigenesis [13]. Because of the collagen processing activity of the ADAMTS-3, which are the basic components of the ECM, determining the factors affecting its expression level could be important in terms of OS progression. In our previous studies, we determined that ADAMTS-3 is abundantly found in osteosarcoma cell lines Saos-2 and MG-63 [30]. Previous studies identified that type I collagen levels were higher in Saos-2 cells when compared to type II and III collagen levels. Here we determined that C/EBPα expression resulted in a significant decrease in ADAMTS-3 and an increase in both type I and II collagen levels in Saos-2 cells. Therefore, it can be suggested that the increase in type I collagen level observed as a result of C\EBPα-mediated downregulation of ADAMTS-3 would be critical for OS. Previous

studies identified that MG-63 cells have higher type I and III collagen levels than type II. C\EBPα overexpression in MG-63 cells resulted in a decrease in the expression of all collagens types (type I-II and III). This result may be due to the heterogeneity of the gene expression profiles due to the maturation status of the osteoblastic cell lines.

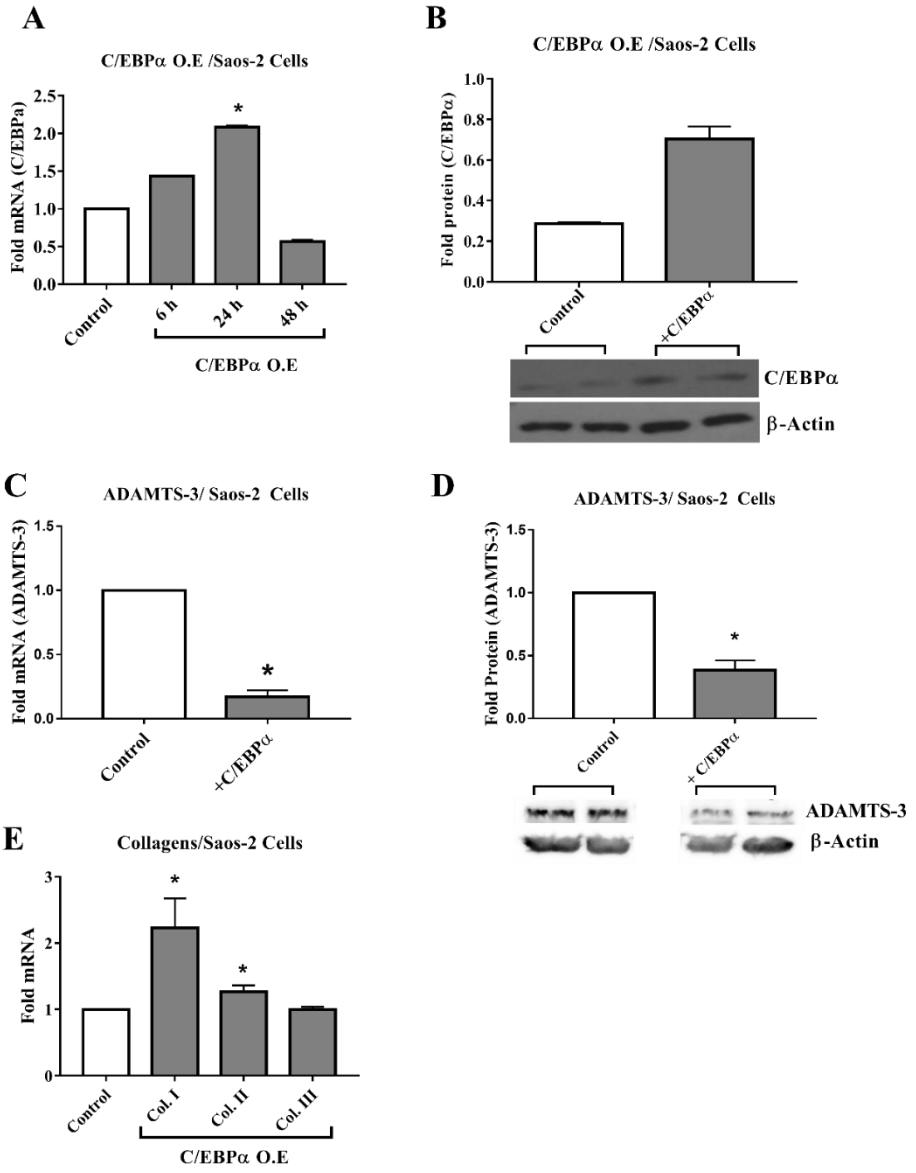

Figure 1: A-B C/EBPα mRNA and protein levels after transfection of C/EBPα expression plasmid. **C-D** ADAMTS-3 mRNA and protein levels in C/EBPα over expressed Saos-2 cells after 24 h of transfection. **E** Fibrillar collagen (type I, II, and III) levels in C/EBPα over expressed Saos-2 cells. Asterisks indicate $p \leq 0.05$.

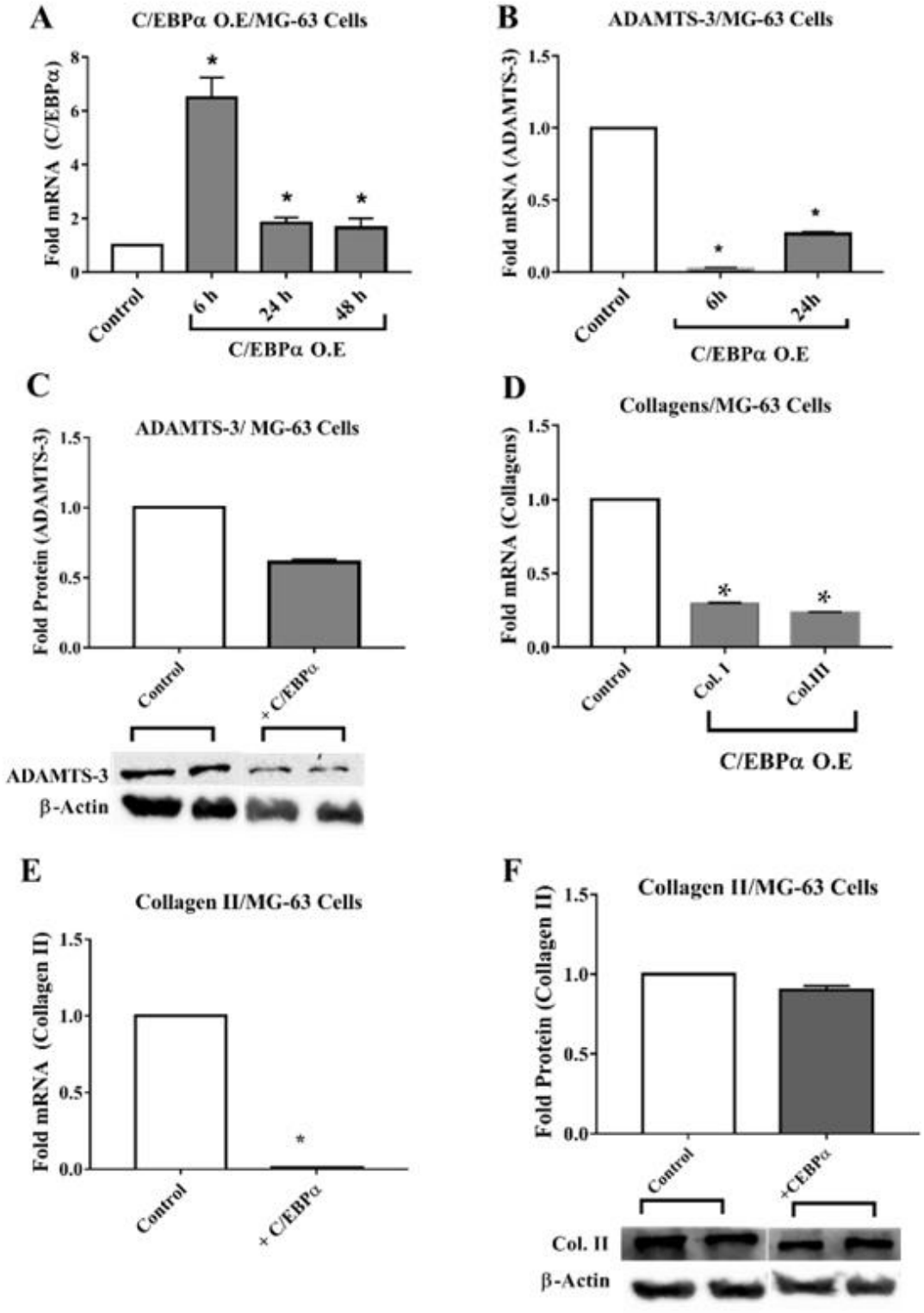

Figure 2: A C/EBPα mRNA levels after transfection of C/EBPα expression plasmid. **B-C** ADAMTS-3 mRNA and protein levels in C/EBPα over expressed MG-63 cells after 6 h of transfection. **D-E** Fibrillar collagen (type I, II, and III) mRNA levels in C/EBPα over expressed MG-63 cells. F. Type II collagen Protein expression level in C/EBPα over expressed MG-63 cells. Asterisks indicate $p \leq 0.05$.

3.2 C/EBPα negatively regulates ADAMTS-3 promoter activity in both Saos-2 and MG-63 cells

C/EBPα has a basic leucine zipper (bZIP) domain and binds the CCAAT sequence in the promoter regions of the target genes [31]. Scanning of 5' upstream of the *ADAMTS-3* gene revealed putative binding sites for the C/EBPα transcription factor and these regions were schematically indicated in Figure 3A. Truncated ADAMTS-3 promoter-reporter constructs were prepared in our previous studies and named pMET_TS-3[–1340/+40], pMET_TS-3[–879/+40], pMET_TS-3 [–576/+40] and pMET_TS-3 [–131/+40] [17]. To determine the regulatory effect of the C/EBPα on the *ADAMTS-3* promoter, luciferase-based *ADAMTS-3* promoter constructs were transiently transfected together with the C/EBPα expression plasmid in Saos-2 and MG-63 cells. Ectopic increase of the C/EBPα, decreased ADAMTS-3 promoter activity up to 0.2-fold for pMET_TS-3[–1340/+40], and approximately 0.4-fold for pMET_TS-3[–899/+40], pMET_TS-3 [–576/+40] and pMET_TS-3 [–131/+40] in Saos-2 cells. The decrease in the activities of the promoter constructs was statistically significant except for the pMET_TS-3[–879/+40]. In MG-63 cells, C/EBPα also downregulated ADAMTS-3 promoter activity with all constructs. Maximum decrease was observed with pMET_TS-3 [–576/+40] (up to 0.2-fold) in a statistically important manner.

When we compared C/EBP binding regions of the ADAMTS-3 promoter constructs, additional binding regions at the -269, -266, and -159 bp positions were determined on the pMET_TS-3 [–576/+40]. The binding of the C/EBPα to these regions was found to make an additional contribution to the reduction of ADAMTS-3 promoter activity in MG-63 cells. Similarly, the C/EBPα binding region at the -1142 bp position on the pMET_TS-3[–1340/+40] promoter fragment was found to make an additional contribution to the reduction of ADAMTS-3 promoter activity in Saos-2 cells.

In the second step, EMSA assays were performed to determine whether C/EBPα functionally binds to the ADAMTS-3 promoter. Probes [-131/-103] and [-40/-12] were designed that specific to ADAMTS-3 promoter. Three DNA-protein complex formations were observed when the biotinylated probes [-131/-103] and [-40/-12] were included in the binding reactions with Saos-2 nuclear extracts (Figure 3D and 3E; lanes 2). Competition assays were performed to determine the specificity of the complexes using the unlabelled (cold) forms of the same oligonucleotides. It was determined that while Complex1 (C1) indicates non-sequence-specific DNA-protein interaction, Complex 2 (C2) and C3 (Complex 3) were sequence-specific since they disappeared in competition assay (Figure 3D and 3E; lanes 3). When the C/EBPα unlabelled

consensus probe was included in the reactions as a competitor, C2 and C3 disappeared indicating functional binding of the C/EBPα to specific regions (Figure 3D and 3E; lanes 4).

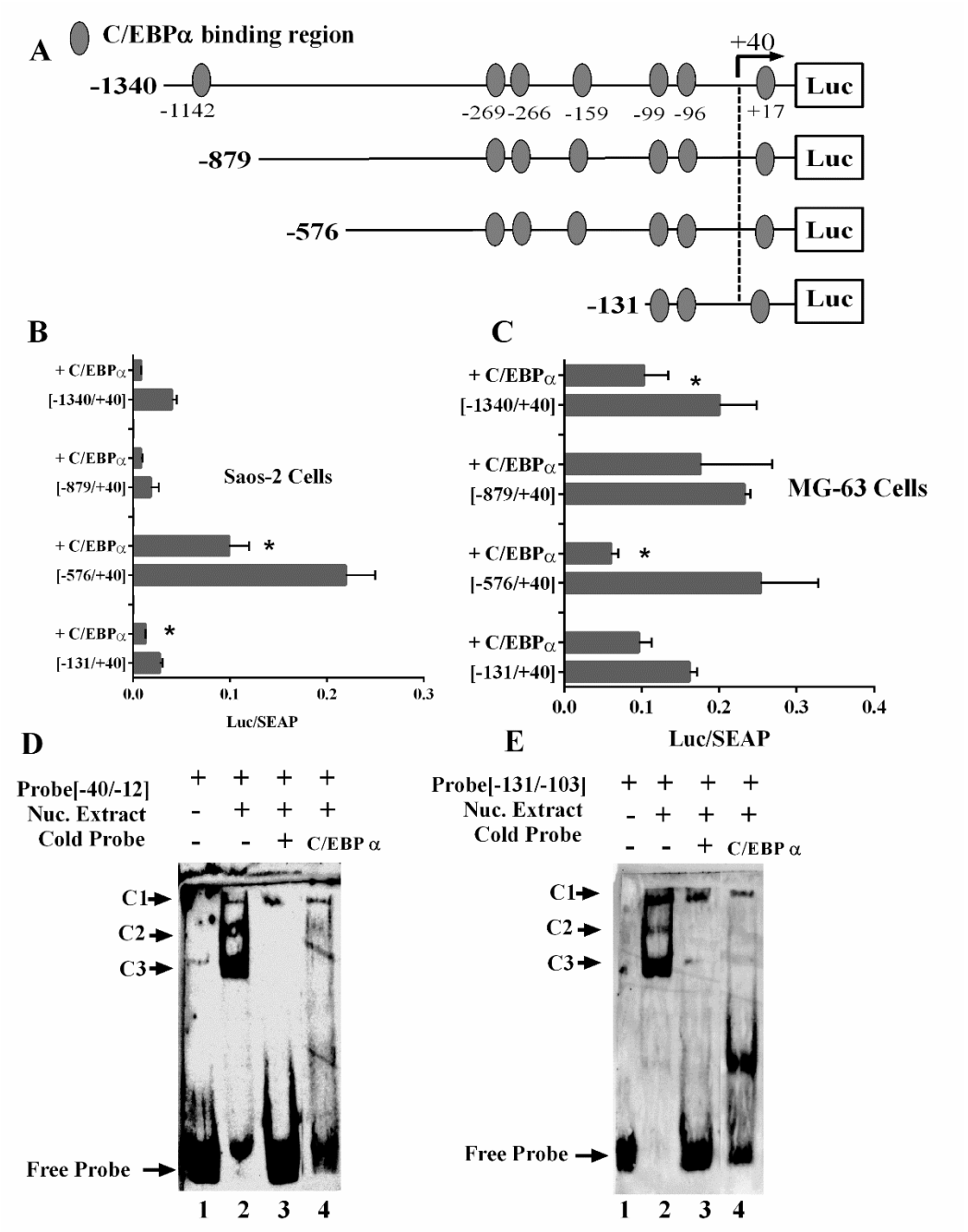

Figure 3: A. Scheme for ADAMTS-3 promoter-reporter constructs used in transfection assays. C\EBPα binding regions were indicated with a circle. **B-C.** Relative Luciferase activities of ADAMTS-3 Promoter reporter constructs in C\EBPα over-expressed Saos-2 and MG-63 cells **D-E.** *In vitro* binding analysis of C\EBPα to ADAMTS-3 promoter region by EMSA. Asterisks indicate p≤0.05.

4. Conclusion

The extracellular matrix (ECM) is an important constituent of the tumor microenvironment. In addition to the structural and biochemical supportive function of its cellular components, the ECM coordinates cellular behaviors. Aberrant changes in the ECM composition enable cells to gain malignant and metastatic characteristics. The contribution of the ECM to osteosarcoma progression and metastasis has been reported. Among the ECM components, collagen I level has been found to increase in OS patients and elevated collagen levels promoted OS progression, invasion, and migration through MMP-2 activation.

Our findings indicated that ADAMTS-3 could be transcriptionally regulated by C/EBPα transcription factor. C/EBPα significantly decreased ADAMTS-3 expression level in osteosarcoma cells. Because ADAMTS-3 can process ECM components, we suggest that changes in the ADAMTS-3 expression levels could be critical for the ECM remodeling in osteosarcoma progression. Further, we determined that decreased ADAMTS-3 levels increased collagen I and II expression levels in Saos-2 cells. So it can be suggested that C/EBPα-mediated down-regulation of ADAMTS-3 could be responsible for decreased collagen I and II expression levels in Saos-2 cells. Saos-2 and MG-63 cells have differently composed ECM [27]. While collagen I and III expression levels are high in Saos-2 cells, collagen II expression levels are quite low. In MG-63 cells, similar to Saos-2 cells, collagen I and III levels were higher than collagen II but generally collagen II and III levels were higher than those in Saos-2 cells. On the contrary, downregulation of the ADAMTS-3 in MG-63 cells resulted in a decrease in both collagen I and III levels. So, it can be concluded that maturation level, heterogeneity, and differences in the ECM composition could be responsible for the different responses of collagen expression levels to ADAMTS-3 decrease in Saos-2 and MG-63 cell models.

Acknowledgments

Saos-2 cells were kindly provided by Dr. Kenneth Wann and human C\EBPα expression plasmid was kindly provided by Dr. Dipak P. RAMJI, (Cardiff, School of Biosciences, Cardiff UK).MG-63 cells were kindly provided by Dr. Berivan ÇEÇEN, (Dokuz Eylül University, İzmir, TÜRKİYE). This work was supported by the Scientific and Technological Research Council of Turkey (TUBITAK), Project number; 114Z025.

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