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Upregulation of the matrix metalloproteinase-1 gene by the Ewing's sarcoma associated EWS-ER81 and EWS-Fli-1 oncoproteins, c-Jun and p300

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Abstract The mechanisms of action of Ewing's sarcoma (EWS) associated EWS-ETS oncoproteins have largely remained unresolved. Here, we analyzed how two EWS-ETS proteins, EWS-ER81 and EWS-Fli-1, in vitro activate the matrix metalloproteinase (MMP)-1 promoter that is upregulated in a subset of EWSs. EWS-ER81 and EWS-Fli-1 interact with and thereby activate the MMP-1 promoter, which is potentiated by the cofactor p300 and the proto-oncoprotein c-Jun. Further, EWS-ER81 binds to c-Jun in vitro and in vivo. The interaction between c-Jun, p300 and EWS-ER81 or EWS-Fli-1 may also be relevant to the regulation of other yet-to-be-identified genes that are responsible for EWS formation.

Key words: c-Jun; Ewing's sarcoma; Matrix metalloproteinase-1; p300; Transcription

1. Introduction

Ewing's sarcoma (EWS) is a highly aggressive malignancy affecting predominantly children. Several chromosomal translocations specific for EWS have been described and define it as a distinct clinicopathologic entity [1]. As a result of these chromosomal translocations, EWS-ETS fusion proteins are generated in which the N-terminal domain (NTD) is derived from the EWS gene product whereas the C-terminal domain (CTD) consists of a DNA binding domain derived from one out of five ETS transcription factors [1]. EWS-ETS fusion proteins are aberrant transcription factors and oncoproteins that promote anchorage independent cell growth and tumor formation [2,3]. Furthermore, continued expression of EWS-ETS fusion genes is necessary for maintaining EWS histology and growth [4].

Matrix metalloproteinase (MMP)-1, also known as interstitial collagenase, belongs to a class of proteases that regulate the function of many bioactive molecules by proteolytic processing [5]. MMPs also mediate extracellular matrix and basement-membrane degradation during the early stages of tumorigenesis, contributing to the formation of a microenvironment that promotes tumor growth. In later stages of cancer development, MMPs promote metastasis as well as other aspects of tumor growth [6].

Since MMP-1 has been shown to be regulated by many ETS proteins [7] and EWS-ETS fusion proteins are endowed with an ETS DNA binding domain, we hypothesized that MMP-1 is a target gene for EWS-ETS proteins. Indeed, the EWS-ER81 and EWS-Fli-1 fusion proteins can interact with the MMP-1 promoter and collaborate with c-Jun and the cofactor p300 to activate MMP-1 gene transcription in vitro.

2. Materials and methods

2.1. Reporter gene assays

Equal numbers of cells were transiently transfected by the calcium phosphate coprecipitation method [8]. 36 h after transfection, cells were lysed in 25 mM Tris–HCl (pH 7.8), 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM dithiothreitol (DTT) for 5 min. Plates were then scraped and the extract was collected and centrifuged at 20 800×*g* for 1 min. The cleared supernatant was then assayed for luciferase activity [8]. Each of these experiments was repeated at least three times, and the mean values and standard deviations are indicated.

2.2. Glutathione S-transferase (GST) pull-down assays

293T cells were transiently transfected with 6Myc-tagged EWS-ER81, EWS-NTD or ER81-CTD. After 36 h, the cells were harvested in 600 μl of 40 mM HEPES (pH 7.4), 10 mM EDTA, 150 mM NaCl and pelleted for 3 min at 900×*g*. The pellet was resuspended in 80 μl of 10 mM Tris, 30 mM $Na_4P_2O_7$ (pH 7.1), 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM Na_3VO_4, 2 mM DTT, and lysis was allowed to occur for 30 min on ice. Then, debris was removed by centrifugation (10 min, 20 800×*g*, 4°C). Purified GST and GST-c-Jun were tumbled in 20 mM HEPES (pH 7.4), 25 mM NaCl, 0.1% Tween-20, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 mM PMSF, 0.1 mM Na_3VO_4, 2 mM DTT with 25 μl of glutathione agarose beads for 2 h at 4°C, and then again for 2 h after the addition of 10 μl of 293T cell extract. The beads were finally washed four times in binding buffer and bound proteins revealed by anti-Myc Western blotting.

2.3. Electrophoretic mobility shift assay

Bacterially expressed, purified GST-EWS-ER81 was incubated on ice for 1 h in 10 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 50 μg/ml bovine serum albumin, 0.1% IGEPAL CA-630, 0.1 μg/μl poly(dI-dC)*poly(dI-dC) with a ^{32}P-labeled MMP-1 probe as described [9]. Where indicated, 0.5 μl of an antibody directed against the C-terminus of ER81 (anti-ETV-1 C20; Santa Cruz) was

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Abbreviations: CTD, C-terminal domain; EWS, Ewing's sarcoma; GST, glutathione *S*-transferase; MMP, matrix metalloproteinase; NTD, N-terminal domain

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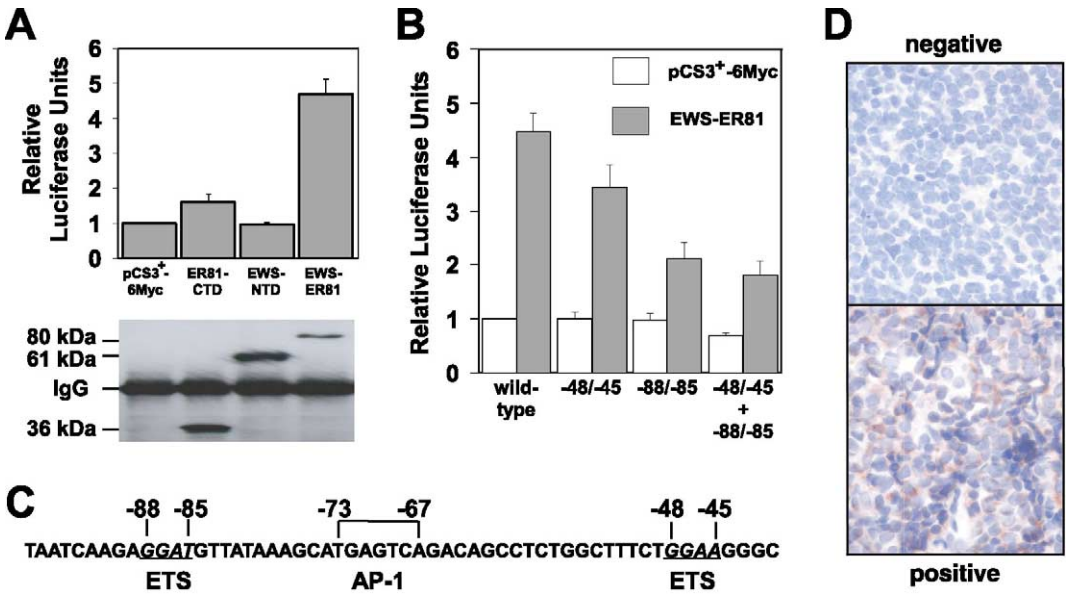

Fig. 1. A: 10 ng of Myc-tagged EWS-ER81, ER81-CTD, EWS-NTD or empty vector pCS3^{+}-6Myc were transfected into RK13 cells. Activation of the cotransfected MMP-1 luciferase reporter plasmid is depicted. The bottom panel shows a respective anti-Myc immunoprecipitation followed by anti-Myc Western blotting. B: MMP-1 luciferase reporter, wild-type or mutated at the ETS sites −88/−85 (GGAT to AAAT) and/or −48/−45 (GGAA to AAAA), was cotransfected with 10 ng of Myc-tagged EWS-ER81 or empty vector pCS3^{+}-6Myc into RK13 cells. C: Partial sequence of the human MMP-1 promoter pointing out the juxtaposed AP-1 and ETS binding sites. D: Immunohistochemical detection of MMP-1 expression (brown color) in two representative EWSs.

added. After incubation, samples were electrophoresed on a 5% polyacrylamide gel in 0.5×TBE at 4°C. The gel was dried and exposed to film.

3. Results

3.1. EWS-ER81 and EWS-Fli-1 oncoproteins activate the MMP-1 promoter

Five different ETS transcription factors can be fused to EWS to generate oncogenic EWS-ETS fusion proteins [1]. One of these is EWS-ER81 (also called EWS-ETV-1) in which the last 164 amino acids of ER81, including its ETS DNA binding domain, are fused to the N-terminal 264 amino acids of EWS [10]. When cotransfected with a luciferase reporter gene driven by the human MMP-1 promoter containing sequences from −525 to +15 [9] EWS-ER81 raised luciferase activity by 4.7-fold over the vector control (Fig. 1A, top panel), whereas neither ER81-CTD nor EWS-NTD alone had any dramatic effect on MMP-1 promoter activity. This was not due to the fact that ER81-CTD and EWS-NTD were less expressed than EWS-ER81 (Fig. 1A, bottom panel). To a comparable level as observed with EWS-ER81 and similar to recently reported transfection experiments with a different MMP-1 (−517 to +63) reporter plasmid [11], another EWS-ETS fusion protein, EWS-Fli-1, in which the first 264 EWS amino acids are fused to the last 212 amino acids of Fli-1, enhanced MMP-1 promoter activity (see Fig. 3A).

DNA binding sites for ETS proteins are characterized by a 5′-GGAA/T-3′ core sequence [12], and two of these ETS sites have been identified in the MMP-1 promoter at positions −88 to −85 and −48 to −45 (see Fig. 1C). Indeed, mutation of the −88/−85 ETS site decreased MMP-1 promoter activity by 54% in the presence of EWS-ER81, whereas mutation of the −48/−45 ETS site barely reduced the ability of EWS-ER81 to activate the MMP-1 promoter (Fig. 1B). Similar results were obtained with EWS-Fli-1 (data not shown). Altogether, EWS-ETS fusion proteins activate the MMP-1 promoter in vitro primarily through the −88/−85 ETS site.

Furthermore, we obtained 30 EWS specimens with permission of the Mayo Institutional Review Board and stained them with an anti-MMP-1 antibody as previously described [13]. MMP-1 staining was observed in 3 out of 30 tumors

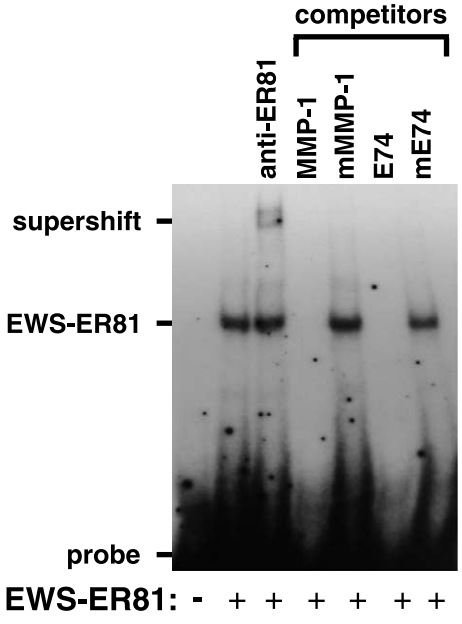

Fig. 2. Binding of EWS-ER81 to a ^{32}P-labelled MMP-1 promoter fragment (−94 to −62) was determined by electrophoretic mobility shift assays. Where indicated, these assays were performed in the presence of an anti-ER81 antibody or a 100-fold excess of non-labeled competitor oligonucleotides (MMP-1 or E74), wild-type or mutated (prefix 'm') at the ETS core sequence (GGAT to AAAT).

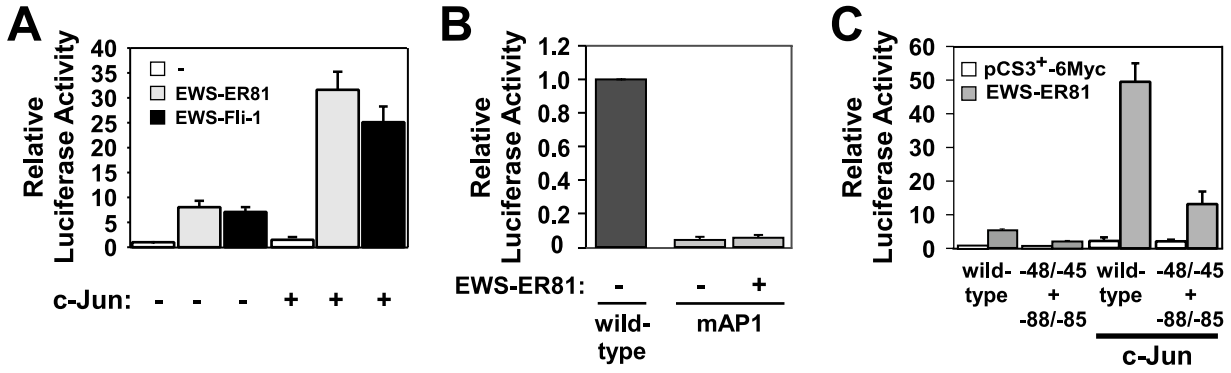

Fig. 3. A: The MMP-1 luciferase reporter was cotransfected with 10 ng EWS-ER81 or 250 ng EWS-Fli-1 in the absence and presence of c-Jun (600 ng). Due to different vector backbones, different amounts of EWS-ER81 and EWS-Fli-1 expression vector were utilized to obtain optimal MMP-1 activation. B: Effects of mutating residues within the AP-1 binding site (−70 to −68 from GTC to TGG; mAP1) in the MMP-1 promoter on EWS-ER81-mediated transcription in RK13 cells. C: MMP-1 luciferase reporter, wild-type or mutated at the ETS sites −88/−85 and −48/−45, was cotransfected with 10 ng of Myc-tagged EWS-ER81 or empty vector pCS3^{+}-6Myc and 600 ng of c-Jun expression vector into RK13 cells.

(Fig. 1D), indicating that MMP-1 is expressed in a subset of EWSs in vivo.

3.2. EWS-ER81 binds to the MMP-1 promoter

Next, we tested DNA binding of EWS-ER81 to the MMP-1 promoter employing electrophoretic mobility shift assays with a ^{32}P-labeled oligonucleotide spanning MMP-1 promoter nucleotides −97 to −62. EWS-ER81 was indeed capable of binding this MMP-1 promoter fragment, and inclusion of an anti-ER81 antibody resulted in supershift formation (Fig. 2). Further, this DNA binding was specific since the non-radiolabeled MMP-1 oligonucleotide suppressed the formation of the EWS-ER81/^{32}P-DNA complex, whereas a mutated MMP-1 oligonucleotide did not. Similarly, an established ER81 binding site, E74, but not a mutated E74 oligonucleotide that is unable to associate with ER81 [14], competed with the radiolabeled MMP-1 oligonucleotide for EWS-ER81 binding (Fig. 2). Together with our reporter gene studies, these data show that EWS-ER81 can bind to the ETS site at −88 to −85 in order to activate the MMP-1 promoter in vitro. However, and consistent with our transfection analysis, no binding of EWS-ER81 to the ETS site at −48 to −45 of the MMP-1 promoter was detectable (data not shown).

3.3. c-Jun collaborates with EWS-ER81 and EWS-Fli-1

The MMP-1 promoter contains an AP-1 binding site between nucleotides −73 to −67 which is in close proximity to the ETS site at −88/−85 (see Fig. 1C). Furthermore, AP-1 has been shown to activate the MMP-1 promoter and play crucial roles in tumorigenesis [15]. Thus, we analyzed whether EWS-ER81 and EWS-Fli-1 may collaborate with AP-1 to stimulate the MMP-1 promoter. Indeed, whereas either c-Jun, a major component of AP-1, or EWS-ER81 alone activated MMP-1 transcription less than eight-fold, together c-Jun and EWS-ER81 dramatically activated MMP-1 promoter activity by 32-fold; similar results were obtained for EWS-Fli-1 (Fig. 3A).

We wondered whether endogenous AP-1 is necessary for EWS-ER81 to mediate MMP-1 promoter activation. Thus, we mutated the AP-1 site within the MMP-1 promoter and observed a reduction of promoter activity by more than 10-fold (Fig. 3B), with EWS-ER81 being unable to stimulate this mutated MMP-1 promoter. These data implicate that endogenous AP-1 critically contributes to the stimulation of MMP-1 transcription by EWS-ER81.

Next, we assessed the ability of c-Jun to activate MMP-1 gene transcription upon mutating both ETS sites at −88 to −85 and −48 to −45. As shown in Fig. 3C, c-Jun was able to enhance EWS-ER81 dependent transcription of the mutated

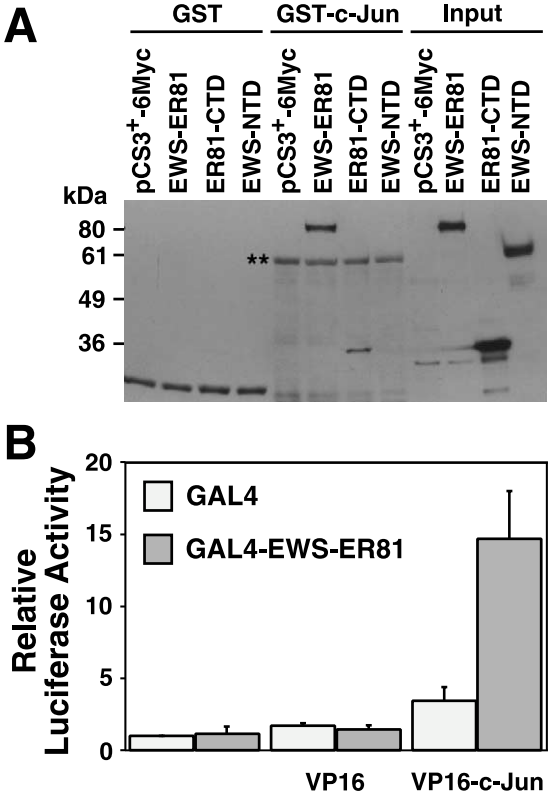

Fig. 4. A: Extracts from 293T cells transfected with Myc-tagged EWS-ER81, ER81-CTD, EWS-NTD or empty vector pCS3^{+}-6Myc were incubated with either GST or GST-c-Jun bound to glutathione beads. Interacting proteins were detected by anti-Myc immunoblotting. Asterisks indicate GST or GST-c-Jun that cross-reacted with the anti-Myc antibody. B: A GAL4 DNA binding site-containing luciferase reporter construct was cotransfected with 50 ng of either the GAL4 DNA binding domain or GAL4-EWS-ER81 and VP16 or VP16-c-Jun into 293T cells. Resulting luciferase activities were determined.

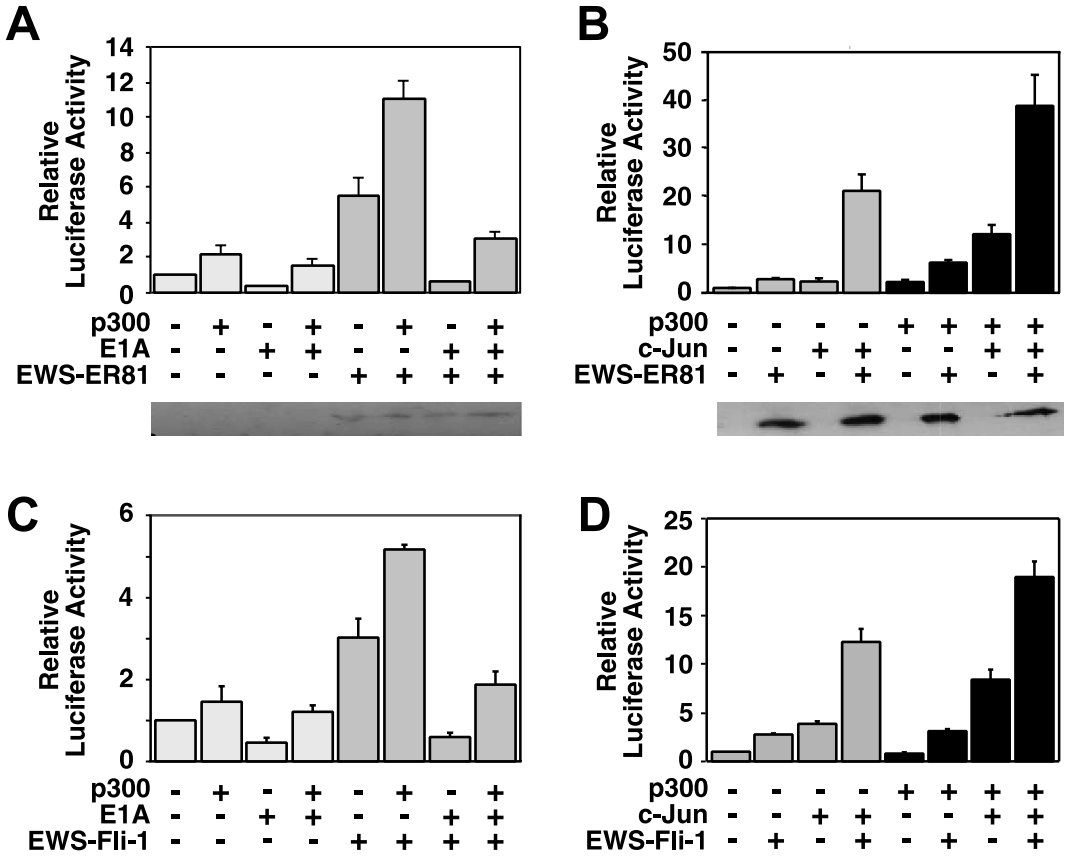

Fig. 5. A: 10 ng of 6Myc-EWS-ER81 were expressed together with p300 (2.5 μg) and E1A (20 ng) as indicated in RK13 cells. Activation of the cotransfected MMP-1 luciferase reporter is presented. The bottom panel shows comparable expression of EWS-ER81 by anti-Myc Western blotting. B: Similar, coexpression of 6Myc-EWS-ER81 (150 ng), p300 (250 ng) and c-Jun (250 ng) and their effects on MMP-1 promoter activity. In order to detect maximal cooperation, different amounts of expression plasmids were utilized than before. C,D: Analogous to A and B with EWS-Fli-1 instead of EWS-ER81.

MMP-1 promoter, albeit to a lesser degree relative to the wild-type promoter. These data imply that EWS-ER81, even when not bound to the above mentioned ETS sites of the MMP-1 promoter, is able to collaborate with c-Jun.

One way of doing so would be through a physical interaction between EWS-ER81 and c-Jun. To test for this, we employed a GST pull-down assay. Whereas EWS-ER81 was not bound by the GST moiety itself, GST-c-Jun interacted with EWS-ER81 (Fig. 4A). No interaction was detected with EWS-NTD, but weak binding of ER81-CTD to GST-c-Jun was noticeable. Thus, the EWS-ER81 fusion protein interacts more strongly with c-Jun than its C-terminal portion.

To validate that EWS-ER81 and c-Jun also interact in vivo, we performed a mammalian two-hybrid analysis. Here, EWS-ER81 was fused to the DNA binding domain of the yeast protein GAL4 and its transcriptional activity measured with a GAL4 binding site-containing luciferase reporter (Fig. 4B). The GAL4-EWS-ER81 fusion protein itself was transcriptionally inactive in this assay. We then coexpressed c-Jun that was N-terminally tagged with the very potent transactivation domain of the herpes simplex virus VP16 protein. VP16 and VP16-c-Jun alone were incapable to strongly activate GAL4 binding site-driven transcription. However, when coexpressed with GAL4-EWS-ER81, a robust transcriptional activation was observed with VP16-c-Jun but not VP16 (Fig. 4B). These results indicate that EWS-ER81 can interact with c-Jun in vivo.

3.4. EWS-ER81, EWS-Fli-1 and p300

A variety of transcription factors were shown to interact with the coactivator p300 to mediate RNA polymerase II-dependent gene transcription [16]. Because both EWS and ER81 bind to p300 [17,18], we wished to address the question whether p300 is also able to interact with the EWS oncoprotein EWS-ER81 and thereby facilitate EWS-ER81-mediated transcription. Indeed, p300 was able to potentiate MMP-1 activation mediated by EWS-ER81 by two-fold (Fig. 5A). Next, we assessed whether EWS-ER81-mediated transcription was dependent on endogenous p300. To this end, we employed the adenoviral protein E1A that sequesters and thereby functionally inactivates p300 [16]. E1A dramatically reduced MMP-1 promoter activity in the presence of EWS-ER81, suggesting that endogenous p300 is required for EWS-ER81 to efficiently activate MMP-1 transcription. Consistently, the repressive effect of E1A could be alleviated by overexpression of p300 (Fig. 5A), indicating that E1A indeed acts to a great extent by sequestering p300. However, we were unable to demonstrate any direct binding of p300 to EWS-ER81 (data not shown).

How could then p300 be recruited to the MMP-1 promoter? One way would be through AP-1 whose components, c-Fos and c-Jun, have been shown to interact with p300 [16]. Consistent with such a view, E1A was even able to repress the MMP-1 promoter in the absence of EWS-ER81 (Fig. 5A), probably by preventing the collaboration between endogenous

AP-1 and p300. To further explore this conjecture, we coexpressed c-Jun and p300 and observed that they indeed collaborated to induce MMP-1 transcription (Fig. 5B). Additionally, p300 was able to enhance MMP-1 promoter stimulation jointly mediated by c-Jun and EWS-ER81. Thus, EWS-ER81 collaborates with c-Jun and the c-Jun-associated cofactor, p300, in order to upregulate the MMP-1 gene in vitro. As shown in Fig. 5C,D, an equivalent conclusion can be drawn for EWS-Fli-1.

4. Discussion

EWSs harbor tumor-specific EWS-ETS proteins. These fusion proteins are essential for initiating and maintaining the tumor phenotype, yet only little is known about the mechanisms of EWS-ETS action in the process of cell transformation [1]. In this report, we demonstrated that two EWS-ETS oncoproteins, EWS-ER81 and EWS-Fli-1, collaborate with the proto-oncoprotein c-Jun and the cofactor p300 in regulating gene transcription.

c-Jun is a critical regulator of cell proliferation and a proto-oncoprotein [15]. Our data show that c-Jun functionally cooperates with EWS-ER81 and EWS-Fli-1, and even physically interacts with EWS-ER81. Interestingly, c-Jun may also be upregulated in EWS [19]. As such, EWS-ER81, or EWS-Fli-1, and c-Jun might be destined to collaborate in EWS. Even if gene promoters have only a binding site for either EWS-ER81 or c-Jun, these two proteins may co-regulate transcription as long as the protein binding to DNA is capable of recruiting the other through protein–protein interactions. This might be the reason why the MMP-1 promoter is still somewhat inducible by EWS-ER81 even when the ETS sites at both −88/−85 and −48/−45 are mutated.

Immunohistochemical analysis revealed that MMP-1 is expressed in 10% of EWSs, indicating that MMP-1 might indeed be upregulated by EWS-ETS oncoproteins in tumors. The reason why 90% of EWSs display no MMP-1 upregulation could be due to the fact that AP-1 components such as c-Jun are not always upregulated or that the protein levels of EWS-ETS oncoproteins differ, thereby not allowing an efficient activation of MMP-1 transcription in all EWSs.

In addition to MMP-1, several other MMPs are overexpressed in EWS, namely MMP-2, -3 and -9 [11,19,20] that can be regulated by c-Jun [21,22]. Interestingly, the binding site for c-Jun is also very close to an ETS site in the MMP-9 promoter, suggesting that closely spaced binding sites for c-Jun and EWS-ETS oncoproteins may often be responsible for the upregulation of an MMP gene in EWS. In conclusion, collaboration between EWS-ETS oncoproteins and c-Jun may be widespread and identification of further common target genes might reveal important regulatory mechanisms associated with EWS.

The coactivator p300 promotes gene transcription by bridging between DNA binding transcription factors and the basal transcription machinery, by providing a scaffold for integrating transcription factors, and by modifying transcription factors and chromatin through acetylation [16]. Previous reports have shown that both EWS and ER81 interact with p300 [17,18], but we have been unable to detect a physical interaction between EWS-ER81 and p300. This could be due to the fact that the EWS-ER81 fusion protein contains only the NTD of EWS, which does not interact with p300 [17], and the CTD of ER81, which lacks ER81 amino acids 249–313 that may be required for the interaction with p300 [18]. Nevertheless, our analysis shows that EWS-ER81 (as well as EWS-Fli-1) and p300 functionally collaborate on the MMP-1 promoter in vitro. One potential mechanism for this collaboration is that c-Jun co-recruits p300 and EWS-ER81 (or EWS-Fli-1), thereby allowing for a functional collaboration between EWS-ER81 (or EWS-Fli-1) and p300. The formation of such a ternary complex consisting of EWS-ER81 (or EWS-Fli-1), c-Jun and p300 may be instrumental for the dysregulation of many genes and thereby provide one mechanism leading to cell transformation and ultimately to the EWS phenotype.

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