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Advances in Brief

EWS-FLI1, EWS-ERG, and EWS-ETV1 Oncoproteins of Ewing Tumor Family All Suppress Transcription of Transforming Growth Factor β Type II Receptor Gene

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Abstract

Ewing sarcoma-specific chromosomal translocations fuse the *EWS* gene to a subset of *ets* transcription factor family members, most commonly the *FLI1* gene and less frequently *ERG, ETV1, E1A-F, or FEV*. These fusion proteins are thought to act as aberrant transcription factors that bind DNA through their *ets* DNA binding domain. Recently, we have shown (K-B. Hahm *et al.*, Nat. Genet., *23:* 222–227, 1999) that the transforming growth factor β (TGF-β) type II receptor (*TGF-β RII*), a putative tumor suppressor gene, is a target of the EWS-FLI1 fusion protein. Here, we also examined effects of *EWS-ETV1* and *EWS-ERG* on expression of the *TGF-β RII* gene. We show that relative to the control, NIH-3T3 cell lines stably transfected with the *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* gene fusion express reduced levels of TGF-β RII mRNA and protein, and that these cell lines have reduced TGF-β sensitivity. Cotransfection of these fusion genes and the TGF-β RII promoter suppresses TGF-β RII promoter activity and also *FLI1*-, *ERG*-, *or ETV1*-induced promoter activity. These results indicate that transcriptional repression of TGF-β RII is an important target of the *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* oncogene, and that EWS-ets fusion proteins may function as dominant negative forms of *ets* transcription factors.

Introduction

Transcriptional repression of the TGF-β[2] receptors is one of the mechanisms leading to TGF-β resistance. Many human cancer cell lines harbor an apparently normal *TGF-β RII* gene and downstream signaling proteins but express significantly reduced or undetectable levels of TGF-β RII mRNA (1–8). This suggests that altered transcriptional regulation of the *TGF-β RII* gene may be a prevalent mechanism leading to TGF-β insensitivity. Our laboratory has previously cloned and sequenced the 5′ flanking region of TGF-β RII and has characterized two positive regulatory elements. Recently, we have isolated a transcription factor, ERT, that recognizes and binds to the second positive regulatory element of the TGF-β RII promoter (9). The isolation and sequencing of the clone ERT/ESX/ESE-1/ELF3/jen (10–14), which induced greater promoter activity, revealed it to be a member of the *ets* family of transcription factors (15). Our preliminary results demonstrated that most of the *ets* family members that were examined regulate expression of the *TGF-β RII* gene, which suggests that *ets* family members may be important transcriptional factors involved in the regulation of *TGF-β RII* gene expression.

Chromosomal translocations resulting in the expression of chimeric transcription factors are frequently observed in tumor cells (16), and have been suggested to be a common mechanism in human carcinogenesis. Ewing sarcoma and primitive neuroectodermal tumor (PNET) have the t(11;22)(q24;q12) chromosomal translocation that fuses the chromosome 22 *EWSR* gene to the *FLI1* gene on chromosome 11 (17–18). In Ewing sarcoma, EWSR also fused less frequently to the *ERG* gene through a t(21;22) (19), the ETV1 through a t(7;22) (20), the *E1A-F* gene through a t(17;22) (21), or the *FEV* gene through a t(2;22) chromosomal translocation (22). The 11;22 chromosomal translocation specific to Ewing sarcoma generates EWS fusion protein with FLI1, which has been implicated in tumorigenesis. It has been shown that transduction of the *EWS-FLI1* gene can transform NIH-3T3 cells, and that mutants that contain a deletion in either the *EWS* domain or the DNA-binding domain in *FLI1* lose this ability (23, 24). This indicates that the EWS-FLI1 fusion protein may act as an aberrant transcription factor that binds to target genes through the *ets* DNA-binding domain of FLI1 (25). However, the exact mechanism of oncogenesis remains unknown.

Recently, we have shown that EWS-FLI1 binds to PRE2 of the TGF-β RII promoter and that EWS-FLI1 suppresses transcription of the *TGF-β RII* gene (26). Introduction of TGF-β RII into Ewing sarcoma cells restores TGF-β sensitivity and blocks tumorigenicity. Overexpression of EWS-FLI1 in non-Ewing sarcoma cells suppresses expression of endogenous TGF-β RII mRNA in these cells, which suggests that transcriptional repression of TGF-β RII is an important target of the *EWS-FLI1* oncogene. In this study, we investigated whether other *EWS-ETS* fusion genes also suppress expression of *TGF-β RII* gene. We demonstrate that overexpression of either EWS-ERG or EWS-ETV1 in non-Ewing sarcoma cells suppresses expression of endogenous TGF-β RII mRNA, and these fusion gene products down-regulate TGF-β RII promoter activity.

Materials and Methods

Cell Culture. Generation of the NIH-3T3 cell lines expressing EWS-FLI1, EWS-ERG, and EWS-ETV1 has been described previously (24). NIH-3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, and HepG2 human hepatoblastoma cells were cultured in MEM supplemented with 10% fetal bovine serum. Expression of EWS-FLI1, EWS-ERG, and EWS-ETV1 proteins was determined by immunoblot analysis using flag-tag antibody as described previously (24).

Plasmids. For the construction of the TGF-β RII promoter-luciferase construct (PRE2-E4Δ38-luc), double-stranded oligonucleotides of PRE2 (+1/+50) of the TGF-β RII promoter were cloned into *Nhe*I and *Xho*I sites of the adenovirus E4 minimal promoter (−38/+38)-luciferase reporter construct. The sequences of the PCR-generated portions of all of the constructs were verified by DNA sequencing. EWS-FLI1, dnFLI1, FLI1, EWS-ERG, dnERG, ERG, EWS-ETV1, and dnETV1 expression constructs were generated by polymerase chain amplification. Human ETV1 cDNA was cloned from human lung total RNA by reverse transcription PCR. Amplified DNA fragments were cloned into pEF-BOS mammalian expression vector (27) using *Bam*HI and

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[2] The abbreviations used are: TGF-β, transforming growth factor β; TGF-β RII, TGF-β type II receptor; PRE2, (the) second positive regulatory element.

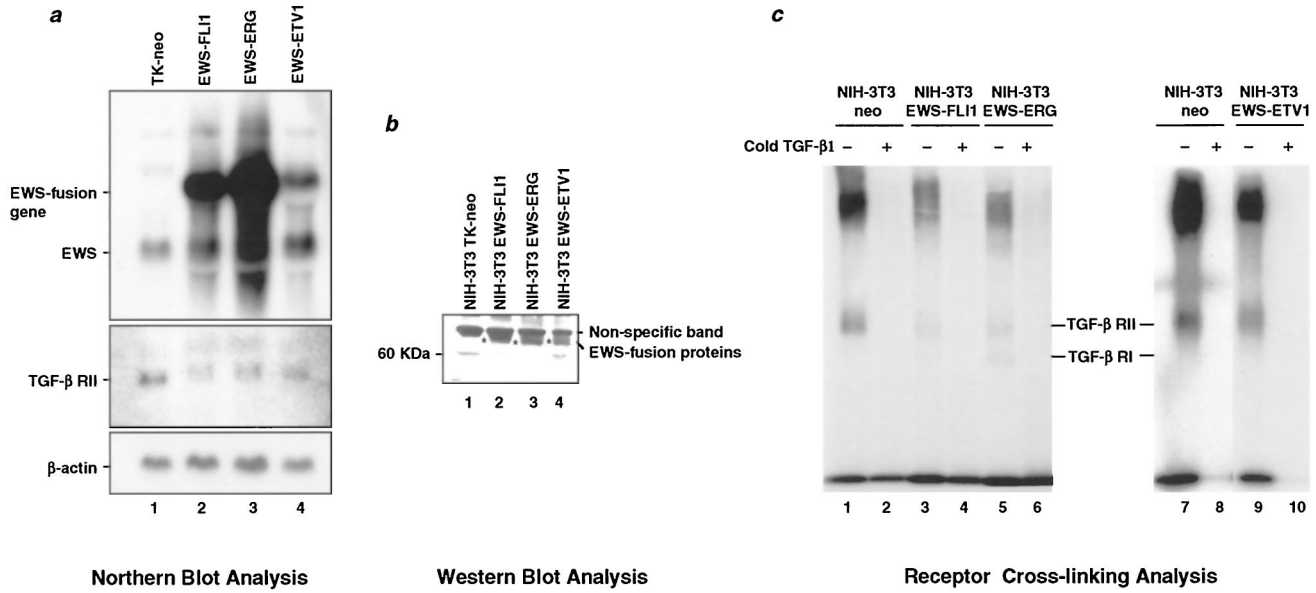

Fig. 1. Expression of TGF-β RII mRNA in NIH-3T3 expressing *EWS-FLI1*, *EWS-ERG*, and *EWS-ETV1*. *a*, Northern blot analysis of TGF-β RII mRNA in the EWS-FLI1-, EWS-ERG-, EWS-ETV1-, and TK-*neo*-NIH-3T3 cell lines. Total RNA was isolated from these cell lines and analyzed by Northern analysis using ^{32}P-labeled TGF-β RII and EWS probes. *b*, the immunoblot analysis of EWS-fusion proteins. Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting with antiflag antibody. $*$, the EWS-fusion protein. *c*, receptor protein cross-linking assay using iodinated TGF-β1. Receptor-ligand binding was performed with 100 pM ^{125}I-labeled TGF-β1 in the presence *(Lanes 2, 4, 6, 8,* and *10*) or absence (*Lanes 1, 3, 5, 7,* and *9*) of 100-fold molar excess of unlabeled TGF-β1. Bound proteins were cross-linked using 300 μM disuccinimidyl suberate, solubilized, and separated with gel electrophoresis.

*Xba*I restriction sites built into the oligonucleotides used for amplification. The sequences of the PCR-generated portions of all of the constructs were verified by DNA sequencing.

Northern Blot Analysis. Total RNA was isolated with guanidinium isothiocyanate/phenol/chloroform. Ten μg of total RNA was electrophoresed on a 1.0% agarose gel containing 0.66 M formaldehyde, transferred to a Duralon-UV membrane, and cross-linked with UV Stratalinker (Stratagene). Blots were hybridized with cDNA probes for EWS, β-actin, and TGF-β RII (28).

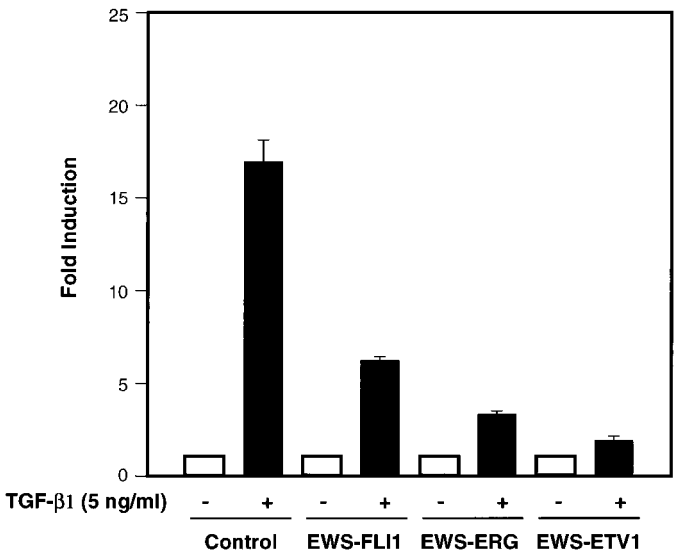

Fig. 2. NIH-3T3 cells expressing *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* gene fusion are less sensitive to TGF-β. NIH-3T3 cells expressing the *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* gene fusion were transfected with a TGF-β responsive element linked to the luciferase gene (*SBE4-lux*), and then cells were incubated in the presence (■) or absence (□) of TGF-β (5 ng/ml). The *neo*-expressing NIH-3T3 cells were used as controls. The data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations with comparable results.

Receptor Cross-linking. Cells were plated at a density of 1×10^6 cells/well in 6-well dishes. Receptor-ligand binding was performed with 100 pM ^{125}I-labeled TGF-β1 in the presence or absence of 100-fold molar excess of unlabeled TGF-β1 (29). Bound proteins were cross-linked using 300 μM disuccinimidyl suberate, solubilized, and separated with gel electrophoresis.

Transient Transfection and Luciferase Assay. NIH-3T3 cells expressing *neo*, *EWS-FLI1*, *EWS-ERG*, or *EWS-ETV1* were seeded in 6-well plates at 1.2×10^6 cells/well and transiently transfected with *SBE4-Lux* (30) using lipofectin (Life Technologies, Inc.). After 12 h, complete media were added, and the cells were incubated for an additional 24 h. The cells were treated with 5 ng/ml TGF-β1 for an additional 24 h. To examine *TGF-β RII* gene regulation, the TGF-β RII promoter was cotransfected into either NIH-3T3 or HepG2 cells with the vectors described in Fig. 4. Luciferase activity was determined in the cell lysate using an assay kit (Analytic Luminescence Lab) and a Dynatech Laboratories ML3000 luminometer. Activities were normalized on the basis pf β-galactosidase expression from pSVβ-galactosidase in all of the luciferase reporter experiments. All of the experiments were repeated at least three times, and similar results were obtained each time.

Results and Discussion

In this study, we used NIH-3T3 cell lines overexpressing *EWS-FLI1*, *EWS-ERG*, and *EWS-ETV1* to examine the effects of these *EWS* fusion genes on expression of *TGF-β RII* gene. These cell lines express varying levels of EWS-FLI1, EWS-ERG, and EWS-ETV1. Because the COOH-terminus of these EWS fusion proteins contains flag tag, we examined the level of these fusion gene products. EWS-fusion proteins are detected by Western blot analysis using flag-tag antibodies (Fig. 1*b*). Northern blot analysis showed that mRNA levels of TGF-β RII were decreased in these cell lines compared with the *neo*-expressing NIH-3T3 cells (Fig. 1*a*). However, TGF-β RII expression was not completely suppressed in these cells. Because we did not select an individual clone but rather a pool of G418-resistant clones, we cannot exclude the possibility that, although some of the clones may have become resistant to neomycin, they did not express the EWS-ETS fusion proteins. This may have resulted in the relatively low degree of suppression of endogenous TGF-β RII mRNA. The EWS-ERG mRNA level in the EWS-ERG-expressing cells was much

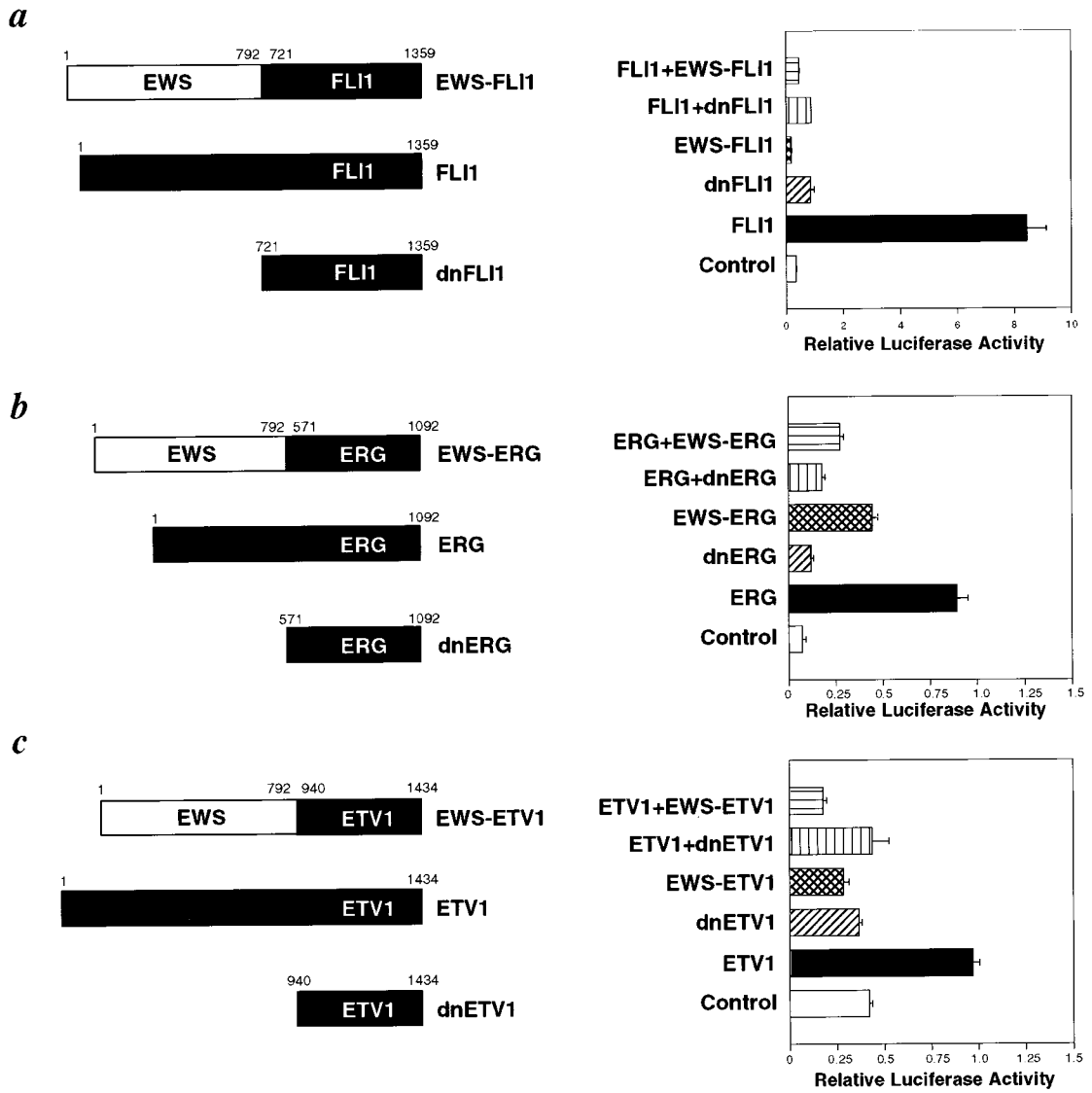

Fig. 3. Regulation of the TGF-β RII promoter by EWS-FLI1, FLI1, dnFLI1, EWS-ERG, ERG, dnERG, EWS-ETV1, ETV1, and dnETV1. *Left*, a schematic representation of the constructs used. Regulation of activity of the PRE2 of TGF-β RII promoter by EWS-FLI1 (*a*), EWS-ERG (*b*), or EWS-ETV1 (*c*). The TGF-β RII promoter construct was cotransfected with either a control vector or vectors described *in the left panel* into either NIH-3T3 or HepG2 cells, which were then harvested after 36 h and assayed for luciferase activity. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations, with comparable results.

greater than that of EWS-FLI1 in EWS-FLI1-expressing cells, yet the TGF-β RII mRNA level was lower in the EWS-FLI1 expressing cells than in those expressing EWS-ERG. Also, EWS-ERG mRNA levels were much higher than the EWS-ETV1 mRNA level in the EWS-ETV1-expressing cells, whereas TGF-β RII mRNA levels were slightly lower in the EWS-ETV1-expressing cells than in the EWS-ERG-expressing cells. This suggests that EWS-FLI1 and EWS-ETV1 have a greater effect on transcriptional repression of the *TGF-β RII* gene than EWS-ERG. On the other hand, immunohistochemical analysis of TGF-β RII protein expression in *EWS-ERG* gene fusion-positive primary Ewing sarcoma samples demonstrated virtually no protein expression (data not shown) as previously found for EWS-FLI1 (26), which indicated that EWS-ERG is able to have a similar biological effect on TGF-β RII protein expression as EWS-FLI1. Moreover, receptor cross-linking assays further demonstrated that cell lines expressing the various fusion genes express significantly lower TGF-β RII protein levels compared with those of the control (Fig. 1*c*).

To investigate whether reduced expression of TGF-β RII receptor results in reduced TGF-β responsiveness, a reporter construct, SBE4-luciferase, which contains multiple TGF-β responsive elements was transiently transfected into *EWS-FLI1*-, *EWS-ERG*-, and *EWS-ETV1*-expressing cells and control cells. Exogenous TGF-β induced luciferase activity greater than 12-fold in *neo*-expressing NIH-3T3 cells, whereas the induction levels in fusion gene-expressing cells were much lower than those of the control cells (Fig. 2). Although the mRNA level of TGF-β RII was much lower in the EWS-FLI1-expressing cells than in the EWS-ERG or EWS-ETV1-expressing cells, the fold induction of the EWS-FLI1-expressing cells was much greater than that of the EWS-ERG- and EWS-ETV1-expressing cells, which suggested that EWS-ERG and EWS-ETV1 might also regulate other components of the TGF-β signaling pathway in addition to TGF-β RII transcription.

When ERG, ETV1, or FLI1 are fused to EWS, their NH_2-terminal portions (which each contain a transactivational domain) are lost and their COOH-terminal portions (which contain the *ets* DNA binding domain) become fused to NH_2-terminal EWS. We, therefore, generated expression constructs containing the portions of ERG, ETV1, and FLI1 present in EWS-ERG, EWS-ETV1, or EWS-FLI1, respectively.

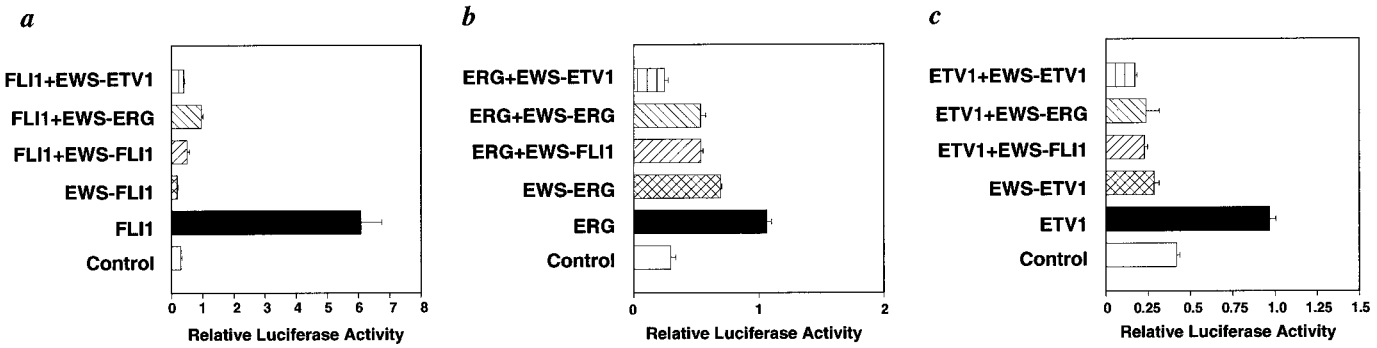

Fig. 4. Suppression of the FLI1-, ERG-, or ETV1- induced TGF-β RII promoter activity by EWS-FLI1, EWS-ERG, or EWS-ETV1. Regulation of activity of the PRE2 of TGF-β RII promoter by EWS-FLI1 (*a*), EWS-ERG (*b*), or EWS-ETV1 (*c*). The TGF-β RII promoter construct was cotransfected with either a control vector or vectors (*described on the left side of* a) into HepG2 cells, which were then harvested after 36 h and assayed for luciferase activity. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations, with comparable results.

To analyze the ability of EWS-ERG, ERG, dnERG, EWS-FLI1, FLI1, dnFLI1, EWS-ETV1, ETV1, and dnETV1 to regulate the TGF-β RII promoter, NIH-3T3 cells or HepG2 human hepatoblastoma cells were cotransfected with a TGF-β RII promoter-luciferase construct and an expression vector for these genes. We have previously shown (26) that FLI1 induced TGF-β RII promoter activity; however, EWS-FLI1 reduced not only TGF-β RII basal promoter activity but also FLI1-induced promoter activity. In Fig. 3, we confirmed these previous findings. The dnFLI1, which contains only the FLI1 portion of EWS-FLI1, slightly induced basal promoter activity but suppressed FLI1-induced promoter activity (Fig. 3*a*). Interestingly, contrary to EWS-FLI1, EWS-ERG induced TGF-β RII promoter activity but to a much lesser extent than ERG alone did (Fig. 3*b*). The dnERG, which only contains the ERG portion of EWS-ERG, only slightly induced TGF-β RII promoter activity. EWS-ERG or dnERG suppressed ERG-induced TGF-β RII promoter activity. Comparison of the amino acid sequences of the ERG domain found in EWS-ERG with the FLI1 domain of EWS-FLI1 showed an 85% sequence homology. However, the FLI1 domain contained an extra 36 amino acids at the NH_2 terminus. It would be of interest to evaluate whether these extra amino acids contributed to the suppressive activity of TGF-β RII transcription. We also examined the effect of EWS-ETV1 on TGF-β RII promoter activity. ETV1 induced TGF-β RII promoter activity 2.7-fold, whereas EWS-ETV1 reduced TGF-β RII basal promoter activity and ETV1-induced promoter activity (Fig. 3*c*). The dnETV1 also suppressed ETV1-induced promoter activity. The different results obtained from ERG, ETV1, and FLI1 suggest different effects on regulation of TGF-β RII gene expression.

In Ewing sarcoma, gene fusions with *EWS* involve only one allele of each *ets* partner gene. Therefore, Ewing sarcoma cells would theoretically still have the capacity to express the other allele of the wild-type *ets* gene (as well as other *ets* transcription factors). With this in mind, we examined whether the suppression by EWS-Ets fusion proteins is specific to the ETS component or whether there is broad cross-reactivity for other Ets binding sites. Our results show that each *EWS* fusion gene can suppress FLI1-, ERG-, and ETV1-induced TGF-β RII promoter activity (Fig. 4). These findings suggest a more general ability of EWS-Ets fusion proteins to suppress wild-type Ets protein activity.

In summary, EWS-ETV1 and EWS-ERG, like EWS-FLI1, suppress TGF-β RII transcription. Because FLI1, ETV1, and ERG each induce TGF-β RII promoter activity, it is likely that these fusion gene products may act in a dominant negative form. The FLI1 domain (dnFLI1) in EWS-FLI1 induces TGF-β RII basal promoter activity 2.5-fold, whereas EWS-FLI1 suppresses basal promoter activity. This result suggests that the EWS domain in EWS-FLI1 may confer suppressive activity. However, in the case of EWS-ERG, dnERG slightly induced TGF-β RII basal promoter activity, whereas EWS-ERG induced TGF-β RII basal promoter activity to a much greater extent. This result suggests that the three-dimensional structures of these fusion products may also contribute to the regulation of gene expression.

Despite the recognition that virtually all of the Ewing family tumors express *EWS-ETS* gene fusions encoding chimeric oncoproteins, few physiological target genes of these aberrant transcription factors have been identified (31, 32). Recently, we have demonstrated that transcriptional repression of TGF-β RII is a major target of the *EWS-FLI1* oncogene. The fact that other EWS fusion genes, such as *EWS-ERG* and *EWS-ETV1*, also repress TGF-β RII expression suggests that inactivation of the TGF-β RII may be an important step in Ewing sarcoma tumorigenesis.

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EWS-FLI1, EWS-ERG, and EWS-ETV1 Oncoproteins of Ewing Tumor Family All Suppress Transcription of Transforming Growth Factor β Type II Receptor Gene

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