Introduction

Ewing sarcoma is an aggressive and highly metastatic malignancy predominantly afflicting children and young adults. EWS encodes an RNA-binding protein in which function remains largely unknown, whereas the chromosomal translocations, which fuse the N-terminal domain (NTD) of EWS to the DNA-binding domain of the ETS family transcription factors including FLI-1 and other transcription factors such as ATF-1 and WT1, are thought to be responsible for causing Ewing sarcoma family tumors (ESFT). The (11;22)(q24;q12) chromosomal translocation producing the EWS/FLI-1 fusion protein accounts for approximately 85% of ESFT. However, the ectopic expression of EWS/FLI-1 results in growth arrest or cell death rather than the promotion of cellular transformation in cells (1) and in mice (2). Also, EWS/FLI-1 alone was not sufficient to confer sarcomatous change in a transgenic mouse model (3). These results indicated cellular context to be critical for the oncogenic potential of EWS/FLI-1 and additional events are required. Here, we report the identification of the Akt substrate PRAS40 as an EWS target gene. EWS negatively regulates PRAS40 expression by binding the 3' untranslated region in PRAS40 mRNA. ESFT cell proliferation was suppressed by treatment with an Akt inhibitor, and ESFT cell proliferation and metastatic growth were suppressed by siRNA-mediated PRAS40 knockdown. Furthermore, PRAS40 knockdown was sufficient to reverse an increased cell proliferation elicited by EWS knockdown. In support of a pathologic role for PRAS40 elevation in ESFT, we documented inverse protein levels of EWS and PRAS40 in ESFT cells. Together, our findings suggest that PRAS40 promotes the development of ESFT and might therefore represent a novel therapeutic target in this aggressive disease. Cancer Res; 72(5); 1260–9. ©2012 AACR.
targeting PRAS40 might provide an effective novel approach for ESFT treatment.

Materials and Methods

Cell lines and cell culture

The human cervical cancer cell line HeLa S3, tested for 8 short tandem repeat (STR) loci and the amelogenin gene in November 2011, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The HEK293 cell line was purchased from RIKEN Cell Bank (Japan). The ESFT cell lines A673, RD-ES, and SK-N-MC were purchased from American Type Culture Collection, and MHH-ES-1 and TC-71 were purchased from DSMZ (Germany). These cells were tested by the cell banks for 8 STR loci and the amelogenin gene. HEK293 and A673 were grown in DMEM supplemented with 10% FBS, RD-ES was grown in RPMI-1640 supplemented with 15% FBS, SK-N-MC was grown in Minimum Essential Medium Alpha supplemented with 10% FBS, MHH-ES-1 was grown in RPMI-1640 supplemented with 10% FBS, and TC-71 was grown in Iscove's MDM supplemented with 10% FBS. All of the above cell lines were incubated at 37°C with 5% CO₂. The FreeStyle 293-F cell line, which had been screened for viruses, mycoplasma, and sterility, was purchased from Invitrogen and cultured in FreeStyle 293 Expression Medium at 37°C with 8% CO₂. Cell lines purchased were passaged less than 6 months after resuscitation.

MS2 tethering assay

HeLa S3 or HEK293 cells inoculated in 96-well plates were transfected in triplicate with 20 ng of pCIneo-Luc or pCIneo-Luc-12xMS2 and indicated amounts of expression vectors of bacteriophage MS2 coat protein (pMS2-3xFLAG) and MS2–EWS fusion protein (pMS2-EWS-3xFLAG) with Lipofectamine 2000 (Invitrogen). Two nanograms of phRL-SV40 (Promega) was cotransfected to serve as an internal control. The total amounts of plasmids used were kept constant at 62 ng with pMS2-3xFLAG. Luciferase activities were measured 48 hours later.

Results

Overexpression of EWS represses reporter expression in a tethering assay

We have isolated Xenopus EWS in a yeast 2-hybrid screening with an RNA-binding protein xCIRP2 as a bait (K. Aoki and K. Matsumoto; unpublished data). To investigate which step of
posttranscriptional gene expression is regulated by EWS, we tethered human EWS to firefly luciferase (FLuc) mRNA through specific interaction between the MS2 coat protein and its binding sites (Fig. 1A). Reporter vectors contained an intron in the 5′ UTR with or without 12 copies of MS2-binding sites in the 3′ UTR. Expression of MS2–EWS fusion protein in HeLa S3 cells decreased the FLuc activity of the reporter with MS2-binding sites (FLuc-MS2BS) to 17% compared with expression of MS2 alone, but did not decrease that of the control reporter without MS2-binding sites (FLuc). MS2–EWS expression decreased the luciferase activity of FLuc-MS2BS in a dose-dependent manner (Fig. 1B). In addition, the amounts and sizes of the FLuc-MS2BS and FLuc reporter mRNAs were not affected by the expression of MS2–EWS compared with that of MS2, indicating that MS2–EWS had no effect on the stability or splicing of the reporter mRNAs (Fig. 1C). We repeated a similar set of experiments in HEK293 cells. Expression of MS2–EWS decreased the luciferase activity of FLuc-MS2BS to 32% compared with MS2 expression but did not decrease that of FLuc without affecting either the level or the size of FLuc-MS2BS and FLuc reporter mRNAs (Fig. 1D and E). Taken together, the results shown in Fig. 1 suggest that EWS suppresses a later stage(s) of posttranscriptional gene expression, that is, nuclear export or translation of a reporter mRNA when tethered to it.

PRAS40 mRNA associates with EWS

The results above raise the possibility that EWS suppresses protein production from the mRNA with which it associates. We then searched for mRNAs that bind EWS protein in cells by
RNA immunoprecipitation (RIP)-Chip analysis. We introduced FLAG-EWS expression vector into HeLa S3 cells and subjected the cell lysate to immunoprecipitation with anti-FLAG antibody. RNA extracted from the lysate and the immunoprecipitate of FLAG-EWS–expressing cells was hybridized with DNA microarrays. mRNA which emerged more in the immunoprecipitate than in the lysate (false discovery rate < 0.05) was considered to bind the EWS protein (Supplementary Table S1 and National Center for Biotechnology Information Gene Expression Omnibus Series ID GSE29313). We investigated the nature of the EWS target mRNAs identified by RIP-Chip and selected overrepresented Gene Ontology biologic processes. We found enrichment in the biologic processes of protein folding, spliceosome assembly, and DNA repair (Supplementary Table S2).

To verify the results obtained by RIP-Chip, we carried out a real-time PCR analysis with the RNAs from the lysates and the immunoprecipitates of FLAG-EWS–expressing cells. For this analysis, we chose mRNAs that belong to the top 10 in the list (Supplementary Table S1) and, in addition, those implicated in tumorigenesis and/or cell-cycle control. Figure 2A and B show that the mRNAs, which we identified to bind EWS in RIP-Chip, were indeed enriched in the immunoprecipitates. The real-time PCR analysis also confirmed that mRNAs, which were less represented in RIP-Chip (data not shown), were not enriched in the immunoprecipitates (Supplementary Fig. S1). Among the mRNAs bound to the EWS protein, one was AKT1S1 (Akt1 substrate 1), whose protein is named PRAS40 (proline-rich Akt substrate of 40 kDa). For simplicity, we hereafter use PRAS40 for both the mRNA and protein. PRAS40 was originally identified as an Akt substrate which is also a 14-3-3 binding protein (18). PRAS40 associates with the mTORC1 complex. PRAS40 is not only a substrate of Akt1 and mTORC1 but also a functional...
Figure 4. PRAS40 expression is negatively regulated by EWS. A, left, HeLa S3 cells were transfected with an empty vector (-) or the FLAG-EWS (EWS) expression vector. After 48 hours, cell lysates were subjected to Western blotting with antibodies against FLAG, EWS, PRAS40, and β-actin. β-Actin served as a loading control. Right, the quantitative analysis of Western blots (n = 3) is shown. B and C, HEK 293 cells were transfected with an empty vector (-) or the FLAG-EWS (EWS) expression vector. The anchorage-independent growth was analyzed by soft agar assay. The results of 3 independent experiments are shown. Bars, SD. ∗, P < 0.05; Student t test (B). At 48 hours after transfection, cell lysates were analyzed by Western blotting (C). D, HeLa S3 cells were transfected with the FLuc or FLuc-3UTR reporter vector and the indicated amounts of the FLAG-EWS or FLAG-EWS/FLI-1 (E/F) expression vector together with phRL-SV40. FLuc activities were measured and normalized to Renilla luciferase activities. The results of 3 independent experiments are shown. Bars, SD. **, P < 0.1; Student t test (B). E, HeLa S3 cells were transfected with the FLuc or FLuc-3UTR reporter vector and 60 ng of empty vector (-) or the FLAG-EWS (EWS) expression vector. After 48 hours, total RNA was recovered and subjected to Northern blotting with a DIG-labeled FLuc probe. F–H, HeLa S3 cells were transfected with phRL-SV40, and the FLuc or FLuc-3UTR expression vector without (-) or with control (C1 and C2) or EWS (Si1 and Si2) siRNAs. After 48 hours, the expression of EWS, PRAS40, and β-actin was analyzed by Western blotting (F). FLuc activities were measured and normalized to Renilla luciferase activities. Bars, SD (G). Total RNA was recovered and subjected to Northern blotting with a FLuc probe (H). WB, Western blot; NB, Northern blot.

regulator of mTORC1 (19–24). We have chosen PRAS40 mRNA for further study in the subsequent part of this work because ESFT cells treated with an Akt inhibitor showed a significant decline in cell proliferation (see below).

We examined whether the EWS protein bound PRAS40 mRNA directly in vitro. The EWS protein has been shown to bind polyguanylic acids (poly(G)) and polyuridylic acids (poly(U)) (25). Recently, it was shown that G-rich single-stranded DNA and RNA folded in a G-quadruplex specially bind EWS in vitro (26). A region close to the 3′ terminus of PRAS40 3′UTR is G-rich, yet it is not known whether this region can be folded into a G-quadruplex ( Supplementary Fig. S2). Therefore, we tested whether purified EWS binds PRAS40 3′UTR by UV cross-linking analysis (Fig. 2C–H). EWS bound to the 32P-labeled PRAS40 3′UTR RNA probe dependent on the irradiation with UV (Fig. 2C). This shows that the EWS protein associates with PRAS40 3′UTR directly. A 75-kDa band detected by UV cross-linking is likely a degradation product of EWS because the purified EWS contained a similarly migrating protein that was detected by 2 distinct anti-EWS antibodies (Fig. 2E and data not shown). As a control, purified EWS/FLI-1 was also subjected to UV cross-linking, and no binding to the probe was observed, which is probably because EWS/FLI-1 lacks the C-terminal RNA-binding domains of EWS (Fig. 2D). When a 3-fold excess of FLuc-3UTR mRNA, in which PRAS40 3′UTR was cloned downstream of the FLuc coding region (Fig. 2F), was added as a competitor, the amount of EWS bound to the PRAS40 3′UTR probe was decreased to 32%. The amount bound to the probe was decreased only to 67% by the addition of FLuc mRNA, indicating the specificity of the interaction between the EWS protein and the PRAS40 3′UTR (Fig. 2G and H).

PI3K/Akt inhibitors suppress the growth of ESFT cells

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is constitutively activated in Ewing sarcoma cells (27, 28), and PI3K inhibitors suppress their proliferation (28, 29). PI3K inhibitors augment apoptosis in ESFT cells induced by drugs now clinically used to treat Ewing sarcoma: actinomycin D (30) and doxorubicin (31). To clarify whether the PI3K/Akt inhibitors influence the proliferation of ESFT cells, we treated A673 cells with a PI3K inhibitor, LY294002 or Wortmannin, or an Akt inhibitor, API-2 (triciribine). Cells treated with API-2 showed a
Expression of PRAS40 Is Regulated by EWS

To investigate whether EWS affects PRAS40 protein expression, we overexpressed FLAG-EWS in HeLa S3 cells. The level of the PRAS40 protein upon transfection of the FLAG-EWS expression vector was reduced compared with that on transfection of the empty vector (Fig. 4A). To verify the effect of EWS on the protein expression of PRAS40 and to investigate the biologic consequences, we overexpressed FLAG-EWS in HEK293 cells and carried out an anchorage-independent proliferation assay (Fig. 4B). The HEK293 cells transfected with the FLAG-EWS expression vector showed an obvious decline in cell proliferation to 70% (P < 0.05). Consistent with the results in HeLa S3 cells (Fig. 4A), the level of the PRAS40 protein was markedly decreased upon the overexpression of FLAG-EWS (Fig. 4C).

We next examined whether the downregulation of PRAS40 protein expression caused by the overexpression of EWS is attributable to the binding and repression of PRAS40 3’UTR. Transfection with an increasing amount of the FLAG-EWS expression vector suppressed the luciferase activity of the FLuc-3UTR reporter compared with transfection with an empty vector, but did not suppress that of the FLuc reporter (Fig. 4D). In contrast, overexpression of FLAG-EWS/FLI-1 did not show any difference between the luciferase activities of the FLuc-3UTR and FLuc reporters. Furthermore, overexpression of EWS did not show any effect on the amount and size of either FLuc-3UTR or FLuc reporter mRNA (Fig. 4E).

To confirm the repression of PRAS40 expression by EWS further, we next knocked down the expression of EWS with siRNAs. The levels of EWS were significantly reduced in HeLa S3 cells transfected with EWS siRNA (S1 and S2), but not in those transfected with control RNA (C1 and C2). Upon the knockdown, the levels of PRAS40 were increased (Fig. 4F). The luciferase activity of the FLuc-3UTR reporter was augmented about 2- to 2.7-fold by the knockdown of EWS compared with that in the absence of siRNA transfection (Fig. 4G) without any effect on the level of either FLuc-3UTR or FLuc reporter mRNA (Fig. 4H). The results of both the overexpression and knockdown experiments indicate that EWS suppresses the production of PRAS40 protein.

EWS protein expression is downregulated in some ESFT cells

In ESFT cells, as at least 1 EWS allele is fused to transcription factor genes by chromosome translocation, the native EWS protein is produced from the remaining allele. We examined the protein levels of EWS, EWS/FLI-1, and PRAS40 in ESFT cell lines. Human umbilical vein endothelial cells (HUVEC) were used as a control because there is a high degree of similarity in gene expression between ESFT cells and HUVECs (33). The results suggest that EWS is expressed at lower levels in 4 out of 5 ESFT cell lines that express EWS/FLI-1 than in HUVECs. Significantly, in ESFT cell lines (A673, RD-ES, MHH-ES-1, TC-71, and SK-N-MC), the protein level is higher than that in HUVECs (Fig. 5). These results suggest the pathologic consequences of the abnormal expression of EWS and PRAS40 in ESFT cells.

PRAS40 knockdown suppresses the proliferation and metastatic potential of ESFT cells

API-2 was shown to reduce the phosphorylation of PRAS40, and PRAS40 promotes the tumorigenesis and chemoresistance of melanoma cells (32). We also found that API-2 inhibited the proliferation of ESFT cells (Fig. 3). Therefore, we next examined the role of PRAS40 in the proliferation of ESFT cells. To do this, we knocked down the expression of PRAS40 with siRNAs in A673 cells, in which a lower level of EWS and a higher level of PRAS40 were expressed than in HUVEC (Fig. 5). The protein levels of PRAS40 were significantly reduced in A673 cells transfected with 2 PRAS40 siRNAs (#1 and #2), but not in those transfected with scrambled siRNA (Fig. 6A). Simultaneously, we found that the activated form of caspase 3, cleaved caspase 3, was markedly increased in the PRAS40-knockdown cells. These results provide an interpretation for the previous finding that PI3K inhibitors augment the apoptosis in ESFT cells induced by actinomycin D (30) and doxorubicin (31). A673 cells transfected

significant decline in proliferation compared with the control (DMSO), as did those treated with LY294002 or Wortmannin. All of the inhibitory effects were dose dependent (Fig. 3A).

We also treated 3 other ESFT cell lines, SK-N-MC, MHH-ES-1, and TC-71 (Fig. 3B-D), with LY294002, Wortmannin, or API-2. Similar to A673 cells, these cell lines exhibited a significant reduction in cell proliferation on API-2 treatment compared with DMSO treatment, as with LY294002 or Wortmannin (Fig. 3E). API-2 inhibits the phosphorylation of Akt (32), thus downstream of Akt would be important to the proliferation of ESFT cells. This prompted us to elucidate the role of PRAS40 in ESFT cells.

Expression of PRAS40 is repressed by EWS

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with PRAS40 siRNA exhibited a remarkable decline to 26% to 44% in anchorage-dependent cell proliferation compared with no siRNA transfection, whereas those transfected with scrambled siRNA did not show any significant change (Fig. 6B). The anchorage-independent cell proliferation was as low as about 10% of no siRNA control, whereas the proliferation of cells transfected with scrambled siRNA was about 80% of that without siRNA transfection (Fig. 6C). Similar results were obtained in the 2 other ESFT cells (MHH-ES-1 and SK-N-MC cells; Supplementary Fig. S3A–S3C and data not shown).

Ewing sarcoma is highly metastatic, and about 25% of patients exhibit metastatic disease at diagnosis (34). In addition, a PI3K inhibitor suppressed the migration of Ewing sarcoma cells (28). To examine whether PRAS40 plays a role in the metastasis of ESFT cells, we subjected the A673 and MHH-ES-1 cells transfected with siRNAs to a cell migration assay (Fig. 6D and Supplementary Fig. S3D) and a cell invasion assay (Fig. 6E and Supplementary Fig. S3E). The migrating or invading A673 cells transfected with PRAS40 siRNA significantly decreased (20%–25% or 23%, respectively), compared with no siRNA transfection, whereas those transfected with scrambled siRNA did not show any remarkable change. Thus, the data showed that knockdown of PRAS40 repressed the proliferation and metastatic potential of ESFT cells.
Expression of PRAS40 Is Regulated by EWS

We then examined the effect of EWS knockdown on the cell proliferation. A673 cells depleted of EWS exhibited a significant increase in anchorage-independent cell proliferation compared with control siRNA-transfected cells (Fig. 7A and B). As was seen in HeLa S3 cells, PRAS40 expression was increased upon EWS knockdown (Fig. 7A). The EWS siRNAs used here (S1 and S2) target the middle region of EWS so that only native EWS but not EWS/FLI1 was depleted (Fig. 7A and C and data not shown). Interestingly, the increased cell proliferation was abrogated by the simultaneous knockdown of PRAS40 (Fig. 7D). Therefore, the stimulated cell proliferation in the EWS-depleted cells may be partially mediated by the increase in PRAS40 expression.

Discussion

Ewing sarcoma is characterized as a highly malignant tumor. With conventional treatment, the 5-year disease-free survival rate for patients with localized Ewing sarcoma is only 60% to 70% and that for individuals with metastases drops to less than 20% (35). Therefore, novel treatments are needed urgently. Insulin-like growth factor receptor inhibitors exhibit remarkable activity toward Ewing sarcoma (36, 37), but many patients develop resistance to the therapy and disease recurrence within several months (38). A small molecule, YK-4-279, blocking the interaction of EWS/FLI-1 with RNA helicase A inhibits the growth of Ewing sarcoma cells and orthotopic xenografts (39). Targeting of EWS/FLI-1 by RNA interference results in growth inhibition in Ewing sarcoma cells (40, 41). However, the specificity, toxicity, and clinical utility of YK-4-279 and the siRNAs remain to be clarified.

Recently, increasing attention has been given to the role of RNA-binding proteins in tumorigenesis. To find a novel approach to the treatment of Ewing sarcoma, here we explored the function of native EWS. EWS showed a significant repression of protein production in a tethering assay (Fig. 1), which suggests that MS2–EWS suppressed either mRNA nuclear export or translation, or both. Our preliminary data from in situ hybridization combined with immunofluorescence for MS2–EWS revealed that FLuc-MS2BS mRNA localized to the nucleus of cells expressing MS2–EWS. This finding needs to be studied further. In any case, we hypothesized that EWS binds to a subset of mRNAs and suppresses protein production from them. A number of mRNAs that bound to EWS participate in DNA repair and mRNA splicing (Supplementary Tables S1 and S2). A study with EWS knockout mice showed impaired meiosis and premature senescence of embryonic fibroblasts, suggesting a role for EWS in DNA recombination repair (42). Therefore, further study of the role of the mRNA targets related to DNA repair would help verify the mechanism by which EWS contributes to DNA repair. EWS has already been implicated in mRNA splicing as it interacts with several splicing factors and regulates alternative splicing through these protein–protein interactions (10–14). If we assume that EWS regulates the protein production from mRNAs encoding splicing factors, our data, together with these previous reports, suggest multiple layers of control of mRNA splicing by EWS.

We showed the pharmacologic inhibition of ESFT cell proliferation by an Akt inhibitor (API-2) as well as by PI3K inhibitors (LY294002 and Wortmannin; Fig. 3) consistent with previous reports (28, 29), which indicate the PI3K/Akt pathway to be critical to the proliferation or survival of ESFT cells. The inhibitory action of API-2 toward all the Akts results in growth inhibition of ESFT cells. The previous reports (28, 29), which indicate the PI3K/Akt pathway to be critical to the proliferation or survival of ESFT cells. The inhibitory action of API-2 toward all the Akts results in growth inhibition of ESFT cells. The previous reports (28, 29), which indicate the PI3K/Akt pathway to be critical to the proliferation or survival of ESFT cells. The inhibitory action of API-2 toward all the Akts results in growth inhibition of ESFT cells.
binding to the 3′UTR. In good agreement with this, whereas EWS was expressed at a lower level, PRAS40 was expressed at a higher level in ESFT cell lines (Fig. 5), which implied a pathologic role for EWS and PRAS40 in ESFT. We showed that silencing PRAS40 reduced greatly the oncogenic potential including proliferation, migration, and invasion of ESFT cells (Figs. 6 and 7 and Supplementary Fig. S3), which indicates that PRAS40 acts as an oncogene in ESFT in which the level of EWS is decreased possibly by haploinsufficiency (Fig. 7E). Silencing of PRAS40 has been shown to result in impaired TORC1 signaling and cell death (19, 45). PRAS40 also plays an important role in cell survival among different species (20). PRAS40 promotes the tumorigenesis of melanoma by deregulating apoptosis (30). Our results are consistent with these observations. Based on this, we consider that PRAS40 is a candidate target for ESFT treatment. The phosphorylation of PRAS40 results in its release from mTORC1 (22). The release of PRAS40 seems to lead to mTORC1 kinase activity toward 4EBP1 and S6K1 (22, 23). Therefore, functions of PRAS40 in cell proliferation and survival in mTOR-dependent and/or mTOR-independent pathways should be clarified. The present results warrant further study on the molecular mechanisms by which PRAS40 is involved in the proliferation and metastatic potential of ESFT cells. We will investigate the role of other mRNAs bound to EWS, the protein expression of which is regulated by EWS. Elucidation of the dysregulation of the entire mRNA target network by poorly expressed native EWS in ESFT may provide new insights into the molecular mechanisms and the treatment of ESFT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

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PRAS40 Is a Functionally Critical Target for EWS Repression in Ewing Sarcoma

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