EWS/FLI1 Regulates Tumor Angiogenesis in Ewing’s Sarcoma via Suppression of Thrombospondins

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Abstract

Suppression of the expression of antiangiogenic factors has been closely associated with multiple malignancies. Thrombospondins 1 and 2 are members of a family of angiogenic inhibitors that are regulated by several oncogenes. In this study, we investigate the role of thrombospondins in Ewing’s sarcoma and their regulation by EWS/ETS fusion oncoproteins. We show that the EWS/FLI1 fusion suppresses the expression of thrombospondins in both NIH3T3 fibroblasts and Ewing’s sarcoma tumor–derived cell lines. This regulation depends on an intact EWS/FLI1 DNA-binding domain and may involve direct interactions between EWS/FLI1 and thrombospondin promoter regions. Forced expression of thrombospondins in Ewing’s sarcoma cell lines inhibited the rate of tumor formation in vivo and markedly decreased the number of microvessels present in the tumors. These findings suggest that thrombospondins play a biologically significant role in tumor vascularization in Ewing’s sarcoma and suggest potential therapeutic strategies for future therapeutic intervention. [Cancer Res 2007;67(14):6675–84]

Introduction

Human tumorigenesis is a multistep process that involves not only aberrant growth and differentiation of cancer cells but also recruitment of normal cells and establishment of a suitable microenvironment. Interplay between tumor cells, host stroma, blood vessels, inflammatory, and immune systems are necessary for the maintenance and growth of the tumor mass. By modulating the expression and secretion of cytokines and growth factors, tumor cells are able to regulate the structure of the extracellular matrix (ECM) and the composition of neighboring normal cell populations.

Angiogenesis is an essential event during tumorigenesis. It provides tumors with nutrients, delivers oxygen and growth factors, and removes metabolic waste generated by malignant cells. Previous investigations have shown that tumors are unable to grow more than 1 to 2 mm in diameter in the absence of vasculature (1). This is primarily due to hypoxia and can be reversed by promoting neovascularization. Additionally, newly formed blood vessels allow the tumor cells to metastasize to distant sites in the body, underscoring the importance of understanding the regulation of tumor angiogenesis (2, 3).

Thrombospondins are a family of secreted glycoproteins that have been implicated in mediating cell-to-cell and cell-to-matrix signaling pathways. Two members of this family, thrombospondins 1 and 2 (TSP1 and TSP2), negatively regulate angiogenesis by inhibiting endothelial cell migration, promoting apoptosis, and inhibiting accessibility of endothelial cell surface receptors to mobilized growth factors. The biological functions of TSP1 and TSP2 are largely overlapping but their tissue expression patterns differ, particularly during normal development (for review, see ref. 4).

TSP1 and TSP2 have been shown to exert their antiangiogenic effects both by decreasing vascular endothelial growth and by inhibiting activation of growth factors in the ECM. TSP1 and TSP2 bind the CD36 receptor present on the surface of endothelial cells and initiate a proapoptotic signaling cascade. Thrombospondins also interact with as yet unknown receptors to promote antiproliferative effects on vascular endothelial cells that are independent of apoptosis (5–8). Finally, TSP1 and TSP2 directly regulate composition of the ECM by modulating activities of matrix metalloproteinases (MMP). TSP1 antagonizes MMP3 activation of MMP9 and as a result inhibits the release of vascular endothelial growth factor (VEGF) sequestered in the ECM (9, 10).

The inverse relationship between TSP1 and TSP2 expression and the cellular transformation in some model systems suggest that these thrombospondins are important in tumorigenesis. Forced expression of c-MYC, c-MYB, or Id1 can down-regulate TSP1 mRNA expression in murine fibroblast model systems (11–13). In an immortalized human epithelial cell model, activated RAS represses expression of TSP1 through post-translational activation of c-MYC (14, 15). By contrast, forced expression of the PTEN tumor suppressor gene increased TSP1 expression in a human glioma model system (16, 17).

This article describes experiments linking the expression of TSP1 and TSP2 to another human cancer-related oncoprotein, EWS/FLI1. This fusion results from a tumor-specific translocation between chromosomes 11 and 22 that is found in Ewing family tumors (EFT; for review, see ref. 18). Work from several laboratories indicates that EWS/FLI1 acts as a dominant oncogene and its continued expression is necessary to maintain EFT viability. Forced expression of EWS/FLI1 in NIH3T3 cells promotes anchorage-independent growth and accelerates tumorigenesis in immunodeficient mice. Inhibition of EWS/FLI1 through transduction of dominant-negative or antisense constructs or more recently by small interfering RNA (siRNA) knockdown results in growth arrest in most EFT cell lines (19–21).

It is currently believed that EWS/FLI1 transforms cells by acting as an aberrant transcription factor to modulate expression of a
cohort of target genes. Our previous microarray studies on gene expression regulation by EWS/FLI1 identified TSP2 as a strongly repressed gene in NIH3T3 cells (22). In the present study, we investigate the role of TSP1 and TSP2 in the oncogenic pathways of EWS/FLI1. We determined that in NIH3T3 cells, EWS/FLI1 directly interacts with the TSP2 promoter region and down-regulates TSP2 expression and that this suppression is DNA binding dependent. Functional studies were also done to determine the biological significance of modulating thrombospondin expression during EFT tumorigenesis. Our results showed that the expression of thrombospondins in EFT cell lines inhibited tumor vascularization and lead to lower rate of tumor formation in immunodeficient mice.

Materials and Methods

Plasmid constructs, retroviruses, and tissue culture. Generation of NH2-terminal FLAG-tagged EWS/FLI1 and EWS/FLI1-KD retroviral constructs has been described previously (23). The full-length murine TSP2 cDNA in the retroviral expression vector pLXSN was a kind gift from Luisa Iruela-Arispe (University of California, Los Angeles, Los Angeles, CA). Murine TSP2 cDNA, a kind gift from Deane F. Mosher (University of Wisconsin, Madison, WI), was cloned into the pflag-Puro retroviral expression vector. EWS/FLI1 short hairpin RNA (shRNA) constructs were cloned into the CSGC lentiviral expression vector, which constitutively expresses green fluorescence protein (GFP) driven by cytomegalovirus promoter. Two EWS/FLI1 RNA interference (RNAi) constructs have been described previously (23). The full-length murine TSP2 cDNA in the retroviral expression vector pLXSN was a kind gift from Luisa Iruela-Arispe (University of California, Los Angeles, Los Angeles, CA). The full-length murine TSP2 cDNA, a kind gift from Deane F. Mosher (University of Wisconsin, Madison, WI), was cloned into the pflag-Puro retroviral expression vector.

Murine TSP2 promoter region was PCR amplified from NIH3T3 genomic DNA and cloned into pGL3-Basic vector, upstream of the firefly luciferase gene. The murine TSP2 promoter region was PCR amplified from NIH3T3 genomic DNA and cloned into pGL3-Basic vector, upstream of the firefly luciferase gene.

Northern blot analysis. Northern blot analysis was done according to Deneen et al. (27). Briefly, nuclear extracts from formaldehyde cross-linked cells were subjected to two rounds of immunoprecipitation using anti-FLAG-M2 agarose beads (Sigma). The cross-linked was reversed and regions of the TSP2 promoter were PCR amplified using P21-dATP nucleotides with the following site-specific primers: −1643–1099, GTACCCCTATGCTCCCTCGTC (forward) and GCCATCTGGTGGGATCTCT (reverse); −1146–684, GCTCCCTGCTCACTTGGTCTC (forward) and ATCTGCGATGGAGGACGAT (reverse); −871–456, GCGTGCAAGCTCTTCGCCTG (forward) and CACACTGCGGGAAGTATT (reverse); and −476–68, AAAATCTCGCCGCGATGTG (forward) and CGGGACCGCATTTAACCATC (reverse). The PCR products were run on a 6% polyacrylamide gel and visualized by autoradiography. A second set of ChIP experiment was done and the enrichment of the TSP2 promoter regions was quantified using RT-PCR with the following: R1 (−1005–915) GCTGGATTTGGACTGGGTAAC (forward) and CGGAGGCACTTGAAGGCAT (reverse); and R2 (−709–628), AGGAGGAGCTCTCCAGC (forward) and TGAAAGGCAGGCGTC (reverse). Standard PCR conditions were 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. All PCR reactions were carried out in Bio-Rad Opticon 2 real-time PCR machine and cycle threshold (CT) values were determined using Option Monitor 2 software. Only PCR amplification with 95% or higher efficiency (determined by the slope of the standard controls) were used to generate the figures. Standard dilutions using each primer set were given an arbitrary copy number, which were then used to determine the copy number of each experimental sample.

Immunohistochemistry and image analysis. Immunohistochemistry was done using standard methods. Briefly, anti-platelet/endothelial cell adhesion molecule-1 (M20) polyclonal antibody (Santa Cruz Biotechnology) was used at 1:100 dilution to detect CD31 expression in tumor sections. Positive staining was detected using 3,3’-diaminobenzidine (Vector Laboratories) as substrate for peroxidase. Three (3) fields with the strongest staining at ×200 magnification were counted for the number of positive staining events using ImagePro software (Media Cybernetics). The average of the three fields from TSP1-expressing EFT tumors were then compared with those of the control tumors. Statistical analysis was done using two-tailed Student's t test. P < 0.05 was considered as statistically significant.
Results

EWS/ETS fusions down-regulate TSP2 expression in NIH3T3 cells. Previously published microarray data comparing mRNA expression profiles of NIH3T3 cells transduced with EWS/FLI1, EWS/ERG, or EWS/ETV1 were mined to identify candidate genes that were transcriptionally repressed by all three fusions (22). From this listing, TSP2 was found to be strongly down-regulated by all three EWS/ETS fusion proteins (Fig. 1A).

To confirm these initial findings, independent populations of NIH3T3 cells were transduced with replication-deficient retrovirus containing NH2-terminal epitope FLAG EWS/FLI1. After drug selection, stable EWS/FLI1-expressing cell populations were harvested and TSP2 expression was determined by qRT-PCR. This experiment from transduction to TSP2 quantitation was repeated more than 10 times. Although there was some variability among different EWS/FLI1-transduced populations, on average, TSP2 expression was downregulated at least 10-fold compared with empty vector controls (Fig. 1B).

EWS/FLI1 inhibits TSP2 transcription in a DNA binding-dependent manner. The EWS/FLI1 fusion protein is currently thought to promote cellular transformation by acting as an aberrant transcription factor. In this model, EWS/FLI1 modulates the expression of target genes by binding to specific genomic transcriptional regulatory sites. Consistent with this hypothesis, mutation of specific amino acid residues within the ETS domain of EWS/FLI1 abrogates DNA binding and significantly attenuates its ability to transform cells (28).

An EWS/FLI1 mutant was used to test whether site-specific DNA binding was necessary for transcriptional repression of TSP2. As before, retroviral-mediated gene transfer was used to transduce the DNA binding–defective mutant EWS/FLI1aR340N (28). NIH3T3 cells were transduced with retroviruses containing epitope-tagged EWS/FLI1, EWS/FLI1aR340N, or empty vector. Immunoblot analysis of polyclonal cell populations confirmed equivalent protein expression of the transgenes (Fig. 1D). Northern blot analysis revealed that TSP2 levels in cells expressing EWS/FLI1aR340N were comparable with that of the empty vector. In contrast, cells expressing wild-type EWS/FLI1 showed no detectable TSP2 (Fig. 1C). This suggested that EWS/FLI1 represses TSP2 expression in a DNA binding–dependent manner.

Reporter gene assays were done to determine whether the decrease in steady-state TSP2 mRNA expression was at least in part due to transcriptional repression. A region of murine TSP2 genomic locus encompassing the transcription start site to 2,583 bp 5′ that is thought to contain the TSP2 promoter was cloned into an expression vector containing a minimal TATAA box fused with the firefly luciferase gene (29). Equivalent constructs containing one of two 5′-nested deletions of the TSP2 promoter region were also created. These constructs were transiently transfected into NIH3T3 cells that stably express EWS/FLI1, EWS/FLI1aR340N, or empty vector.

Luciferase reporter activities revealed that EWS/FLI1, but not the DNA binding–defective EWS/FLI1aR340N mutant, repressed TSP2 promoter activity by ~50% (Fig. 2). Both 5′-truncated TSP2

![Figure 1.](image-url)
reporter constructs were also repressed by EWS/FLI1, suggesting
that the genomic response sites mediating this effect could reside
within 474 bp 5’ of the TSP2 transcription start site.

**EWS/FLI1 binds to the endogenous TSP2 promoter in
NIH3T3 cells.** EWS/FLI1 has been shown previously to directly
interact with the promoter elements of some of the target genes
that it transcriptionally modulates (27, 30). The need for an intact
DNA-binding domain and reporter gene assays cannot distinguish
whether EWS/FLI1 modulates TSP2 expression by directly
interacting with the TSP2 promoter or indirectly through
intermediate factors.

ChIP assays were done to determine whether EWS/FLI1 binds to
the TSP2 promoter in vivo. As before, polyclonal populations of
NIH3T3 cells that were stably transduced with either FLAG-tagged
EWS/FLI1, EWS/FLI1R340N, or empty vector were prepared. Cells
were then formaldehyde cross-linked *in situ*, and chromatin was
harvested and subjected to two sequential immunoprecipitation
with monoclonal anti-FLAG–bound beads. 32P-doped PCRs with
site-specific primers were used to identify genomic regions of the
TSP2 promoter that were enriched in the EWS/FLI1 immunopre-
cipitated chromatin relative to the R340N mutant or empty vector
preparation. Three primer pairs spanning over 1,100 bps upstream
of the TSP2 transcription start site were used to amplify by PCR.
One region covering promoter location −817 to −456 bp showed
significant enrichment with EWS/FLI1 but not with the R340N
mutant or empty vector (Fig. 3A). This suggests that EWS/FLI1 is
able to directly interact with the TSP2 promoter within this broad
region and that this interaction is dependent on an intact DNA-
binding domain.

To confirm these results, an additional ChIP assay was done
using chromatin harvested from independently NIH3T3 popula-
tions transduced with either EWS/FLI1 or empty vector. Chromatin
preparation and immunoprecipitation conditions were kept the
same as the previous assay. To more precisely measure differences
between EWS/FLI1 and empty vector immunopurified chromatin,
qRT-PCR was done using four different primer pair sets from the
region 1,000 bp upstream of the TSP2 transcription start. Two
primer pairs that amplify the TSP2 promoter within 720 bps
upstream of the transcription start site showed at least a 2-fold
enrichment in the EWS/FLI1 preparation compared with empty
vector (Fig. 3B). Primer pairs between promoter locations −1050 to
−915 and −206 to −118 showed no enrichment. Together, these
ChIP data suggest that EWS/FLI1 interacts with region within the
TSP2 promoter that is centered around −700 to −400 of the
transcriptional start site.

**EWS/FLI1 down-regulates TSP1 in human EFT cell lines.** The
cell of origin for EFTs has yet to be clearly defined. Although EWS/
FLI1-transduced NIH3T3 cells do display some of the cellular
characteristics of EFT-derived lines, they are still an approximation.
Lacking a biologically validated human cell line into which EWS/
FLI1 could be transduced, an alternative strategy was used to
determine whether EWS/FLI1 could transcriptionally modulate
thrombospondins in a native EFT context. Endogenous EWS/FLI1
expression was knocked down by transduction of shRNA
constructs into EFT cell lines. Genes whose expression increased
in these transduced cells would by inference be candidate EWS/
FLI1 down-regulated target genes.

Expression of endogenous thrombospondins in EFT cell lines
transduced with shRNA or empty vector constructs was assayed by
qRT-PCR. To avoid misinterpretation of potential idiosyncratic
effects, these experiments were done in redundant fashion. Two
different EFT cell lines (A4573 and TC32) were chosen that could
be efficiently transduced with recombinant lentiviruses. Two
different shRNA constructs were created [EF4 (24) and 818] that
were under the transcriptional control of two different polymerase
III promoters. Normal EWS protein is expressed in EFT cells but
normal FLI1 is not (18). Therefore, shRNA constructs were targeted
to FLI1 coding (818) or 3’-untranslated (EF4) sequences. These
shRNAs were placed 3’ to either U6 (818) or H1 (EF4) promoters
and then cloned into the lentiviral vector CSGC (31).

Transduction of either shRNA generated a consistent and
reproducible biological response in both EFT cell lines. After 7 to
9 days, cells transduced with either shRNA but not empty vector
underwent cell growth arrest and displayed a large flat morphology
consistent with cellular senescence (data not shown). To assess
changes in thrombospondin expression before these global effects,
EFT cells were harvested 48 h after high efficiency (>85%,
determined by GFP expression from the lentiviral vector)
transduction by shRNA or empty vector lentiviruses. Immunoblot
analysis revealed decreased protein expression of endogenous
EWS/FLI1 by either 818 or EF4 shRNAs (Fig. 4A).

In contrast to our NIH3T3 model, TSP2 was not detected by qRT-
PCR in either parental or shRNA-transduced EFT cells (data not
shown). However, FLI1 shRNA transduction resulted in strong
up-regulation of TSP1, a close structural and functional homologue of TSP2 (Fig. 4B). TSP1 expression in control vector-transduced cells was undetectable by qRT-PCR in both EFT cell lines, suggesting that TSP1 expression is completely repressed. This indicates that in a native EFT context, TSP1 is a target gene for down-regulation by EWS/FLI1. To show that the observed effects on TSP1 are not due to off target effects of the shRNAs, we transduced our shRNA constructs into a rhabdomyosarcoma cell line (SJCRH30) that lacks an EWS/FLI1 fusion. TSP1 expression was undetectable in the control or FLI1 shRNA-transduced cells, suggesting that the up-regulation of TSP1 in EFT cells with FLI1 shRNA was specific to EWS/FLI1 knockdown. Furthermore, EWS/FLI1 protein expression in TC32 cells with EF4 was only 20% to 30% decrease compared with empty vector control (Fig. 4A) and exhibited only a moderate up-regulation of TSP1 compared with the other FLI1 shRNA-treated cells (Fig. 4B), suggesting a dose-dependent down-regulation of TSP1 by EWS/FLI1.

**Forced expression of thrombospondins inhibits EFT xenograft growth.** TSP1 and TSP2 are highly conserved structurally and functionally. The primary function of these secreted proteins is to modify ECM. In some cancer model systems, expression of these proteins has been reported to inhibit malignant growth by decreasing the rate of neovascularization (10, 32–34). The strong suppressive effect of EWS/FLI1 on TSP1 or TSP2 suggests that decreased expression of these target genes may be promoting tumorigenesis in our EFT and NIH3T3 models. To test this explicitly, EFT cells were transduced with TSP1 or TSP2 and the effects on tumorigenic growth in immunodeficient mice was determined.

Full-length TSP1 and TSP2 cDNAs were cloned into retroviral expression vectors. Given that thrombospondins are secreted proteins, the murine counterparts of these genes were used because the intent was to assay their *in vivo* effects in a mouse host. TSP1 or TSP2 were then transduced into the TC71 EFT cell line by retroviral-mediated gene transfer. This particular cell line was chosen because it had been previously validated for use as a viable xenograft model system (35). Cells transduced with either thrombospondin showed a transient decrease in growth rate. However, within 1 week after antibiotic selection, there was no apparent difference in growth rate in tissue culture or cell cycle profiles, between thrombospondin-transduced cells versus empty vector controls in spite of high levels of thrombospondin expression (Supplementary Figs. S1 and S2).

**Figure 3.** ChIP assay showing *in vivo* interaction of EWS/FLI1 and regions of the murine TSP2 promoter. A, 32P-labeled PCR analysis of the murine TSP2 promoter regions following a two-step anti-FLAG immunoprecipitation of nuclear lysates from NIH3T3 cells expressing epitope-tagged EWS/FLI1, EWS/FLI1R340N, or empty vector. Chromosome 6 (chr6) ChIP is included for loading control. upp1, positive control, showing enrichment with EWS/FLI1 immunoprecipitation, but not with R340N mutant or empty vector control. Filled circles above the horizontal line, putative FLI1 binding sites on the plus strand of the TSP2 promoter; filled circles below the horizontal line, binding sites on the minus strand of the TSP2 promoter. R1, R2, R3, and R4, the products of the qRT-PCR amplification reaction. B, qRT-PCR analysis of an independent ChIP experiment showing enrichment of the same TSP2 promoter region as shown in (A). These experiments were done in triplicates and are statistically significant ($P < 0.05$).
Polyclonal transduced TC71 populations were injected s.c. into SCID mice and rates of tumor formation were assessed. TSP1-expressing TC71 cells formed tumors at a significantly slower rate than empty vector controls (Fig. 5A). To show that growth inhibition was not specific to a particular cell line, a second EFT line (TTC6647) was transduced with TSP1 and implanted into SCID mice in similar fashion. Once again, forced expression of TSP1 inhibited tumor growth of TTC6647 cells (Fig. 5B). Finally, forced expression of TSP2 also inhibited growth of TC71 xenografts (Supplementary Fig. S3).

In many physiologic contexts, thrombospondins are thought to inhibit angiogenesis. To determine whether this was a possible underlying cause of EFT in vivo growth inhibition, capillary density analysis was done on TC71 tumor explants with and without TSP1 expression. Tumor slices were immunohistochemically stained with anti-CD31, examined under light microscopy, and median capillary density was determined using image analysis software. These studies revealed a 2.5-fold decrease in vascular density in tumors from TSP1-transduced TC71 cells compared with empty vector controls (Fig. 6).

Discussion

We have shown that EWS/FLI1 strongly represses the expression of thrombospondins in two different model systems: TSP2 in murine NIH3T3 and TSP1 in human EFT cell lines. The fact that both endogenous TSP2 and a TSP2 model reporter construct are repressed by EWS/FLI1, but not by a DNA binding–defective point mutant, suggests that EWS/FLI1 is acting by actively inhibiting the transcription of this target gene. This hypothesis is bolstered by our ChIP data, which suggest that EWS/FLI1 physically interacts with the TSP2 promoter in cells. Taken as a whole, these results support the current notion that EWS/FLI1 exerts its biological effects by binding to specific genomic response sites and modulating the transcription of adjacent target genes.

At the same time, these data need to be interpreted with certain caveats in mind. First, EWS/FLI1 ChIP assays are idiosyncratic and technically demanding. For this reason, we have adopted a stringent sequential ChIP-ChIP procedure using epitope-tagged constructs and very avid serologic reagents. Although it does seem that EWS/FLI1 binds to the TSP2 promoter in cells, our qRT-PCR data indicate low-level enrichment, suggesting that these interactions may be weak and/or transient. In addition, although mapping precise binding site locations by ChIP is very difficult, it seems that EWS/FLI1 binds to a region that is slightly 5′ to that predicted by our reporter gene assays. Therefore, it is difficult to gauge the physiologic significance of these interactions at this point. Second, although EWS/FLI1 down-regulates expression of endogenous TSP2 over 30-fold, it only inhibits the corresponding reporter construct by about half. This quantitative disparity between these two responses has been seen with other EWS/FLI1 direct target genes and may simply reflect the inability for small episomal reporter constructs to faithfully mimic the effects of EWS/FLI1 at endogenous gene loci. Model reporter constructs with better dynamic range will need to be developed to map EWS/FLI1 response sites with greater precision.

Although the TSP1 and TSP2 genes encode proteins that are structurally and functionally highly similar, their respective
Putative promoter regions are very dissimilar. Considering this plus the low specificity of the FLI1 core binding site (GGAA), it is very difficult to predict potential functional EWS/FLI1 response sites in the TSP1 gene in EFT cells from our TSP2 studies in NIH3T3 cells.

It may be that EWS/FLI1 down-regulates TSP2 levels by modulating mRNA processing or metabolism. MYB down-regulates TSP2 in NIH3T3 cells by decreasing its transcript half-life and increasing TSP2 degradation (36). In similar fashion, MYC increases TSP1 mRNA turnover and resulting in a decrease in steady-state expression in human endothelial cell models (11). It is notable that the 3′-untranslated regions of both mouse TSP1 and TSP2 transcripts contain multiple AU-rich motifs that have been associated with regulation of mRNA stability (37). However, the functional significance of these sequences has not yet been determined. It has also been shown previously that MYC activation by RAS leads to TSP1 suppression (15). Finally, EWS/FLI1 has been shown to alter splicing of model mini-gene constructs (38). To what extent modulation of these alternative physiologic processes is responsible for EWS/FLI1 down-regulation of TSP1 and TSP2 deserves further investigation.

siRNA-mediated knockdown of endogenous EWS/FLI1 has been used by several investigators to identify candidate target genes (24, 30). Interrogating the one microarray data set that is publicly available, we found that one of the two shRNA constructs used in this study resulted in a 50-fold up-regulation of TSP1 (24). A caveat to this study is that it exclusively used the A673 EFT cell line that shows an unusual response to EWS/FLI1 knockdown. In contrast to the vast majority of EFT cell lines, down-regulation of EWS/FLI1 in A673 does not always result in growth arrest on plastic but primarily a loss of anchorage-independent growth in soft agar.

In addition to variations in cell context, there are three potential concerns when implementing any RNAi strategy. First,

![Figure 5](image_url). Kaplan-Meier survival curve representing the proportion of mice without a 1.5 cm (in diameter) tumor at a given time when injected with TC71 or TC71-TSP1 EFT cells (A) and TTC6647 or TTC6647-TSP1 EFT cells (B). The data in (A) are representative of two independent experiments, where the requisite cell lines were independently produced. n = 10 (A) and n = 5 (B). P for these experiments indicates that the observed differences are statistically significant (P < 0.01).
it is likely that most if not all shRNA constructs reduce the expression of genes other than the one that is primarily being targeted. However, the fact that TSP1 was consistently up-regulated in two different EFT cell lines by two different shRNA constructs that each targeted a distinct location within the EWS/FLI1 transcript makes it very unlikely that this is simply an off-target effect. Second, siRNA knockdown EWS/FLI1 is toxic to EFT cells and induces apoptosis or senescence depending on experimental conditions and particular EFT cell line. For this reason, we adopted a strategy of high-efficiency lentiviral shRNA transduction followed by a comparatively short 48-h incubation time. In this way, we were able to assess changes in gene expression before signs of cellular toxicity. Third, in some model systems, transduction of siRNA has resulted in nonspecific induction of IFN-related genes. However, microarray expression analysis of our shRNA-transduced EFT cell lines failed to show consistent significant increase in expression of 18 candidate IFN response genes (data not shown). Taken as a whole, this would indicate that up-regulation of TSP1 is a result of down-regulation of EWS/FLI1.

Forced expression of TSP1 inhibits in vivo growth of EFT xenografts. The fact that this effect is seen in both TC71 and TTC6647 cell lines suggests that it is not simply idiosyncratic. Taken as a whole, our data suggest that EWS/FLI1 actively represses TSP1 mRNA expression and in doing so promotes EFT tumor growth. The effects of thrombospondins on malignant cell growth vary across different cancers and with the systems by which they are studied (for review, see ref. 33). In certain human cancer models, there seems to be an inverse correlation between thrombospondin expression and malignant growth characteristics. In similar fashion to our EFT results, overexpression of TSP1 has been shown to inhibit in vivo growth of glioblastoma, fibrosarcoma, and breast carcinoma cell lines (26, 39–43). Finally, genetically knocking out TSP1 or TSP2 has been shown to enhance tumorigenesis in several murine model systems (for review, see ref. 4).
TSP1 and TSP2 are thought to antagonize tumor growth primarily by inhibiting angiogenesis. The data from our EFT model system is consistent with this notion. Immunohistochemical analysis of TC71 tumors showed an approximate 2.5-fold decrease in neovascularization in xenografts expressing TSP1. Two main pathways have been identified through which thrombospondins exert their antiangiogenic effects: (a) inhibition of activation of MMP9 sequestered in the ECM and (b) promotion of apoptosis of vascular epithelial cells by binding to the CD36 cell surface receptor. Both immunohistochemical and immunoblot analyses from TC71 xenografts failed to show a difference in MMP9 expression in explants with or without TSP1 expression (data not shown). The molecular mechanisms underlying thrombospondin inhibition of EFT tumor growth require further investigation.

The physiologic effects of angiogenesis inhibitors on tumors are complex and not well understood. Tumor blood vessels are highly disorganized and lack the vascular hierarchy that is seen in normal tissues. Several studies have shown that the excessive leakiness of tumor vessels results in increased interstitial pressure and decreased efficiency of delivery oxygen and nutrients to the tumor cells (44, 45). Angiogenesis inhibitors, specifically agents that block VEGF, normalize this phenotype by pruning the growth factor-dependent tumor vessels, leaving behind a more structured vasculature that supports a higher efficiency of delivery (42, 46–49). Thrombospondins have been shown to decrease VEGF availability in the ECM by regulating the activity of matrix remodeling enzymes (10, 50). This suggests that antiangiogenic compounds, such as thrombospondins, may be able to remodel the tumor vasculature to deliver antitumor drugs more efficiently. From this perspective, multimodal regimens that combine cytotoxic therapy with antiangiogenic compounds provide a rational approach toward treating EFTs. Multicenter pilot studies using this strategy are currently under way.

Acknowledgments

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References
3. Fungam J. Angiogenesis in cancer, vascular, rheuma-
6. Jimenez B, Volpert OV, Crawford SE, Febraro M, Silverstein BL, Bouck N. Signals leading to apotosis-
dependent inhibition of neovascularization by thrombospod-
7. Murphy-Ullrich JE, Schultz-Cherry S, Hook M. Transforming growth factor-β complexes with thrombospon-
8. Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N. Peptides derived from two separate domains of the matrix protein thrombospondin-
1 have anti-angiogenic activity. J Cell Biol 1993;122:
497–511.
9. Qian X, Wang TX, Rothman VL, Nicollia RF, Tuzynski GP. Thrombospondin-1 modulates angio-
genesis in vitro by up-regulation of matrix metallo-

Research.

Tumor Angiogenesis in Ewing’s Sarcoma


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Holmgren L, O'Reilly MS, Folkman J. Dormancy of

Pharmacogenetics and Proceeding of Ewing's sarcoma chromatin.


497–511.


References
2. Ashinuma K, Taguchi K, Sugimachi K, et al. The role of thymidine phosphorylase and thrombospondin-1 in angiogenesis and progression of intrahepatic cholangi-
3. Fungam J. Angiogenesis in cancer, vascular, rheuma-
6. Jimenez B, Volpert OV, Crawford SE, Febraro M, Silverstein BL, Bouck N. Signals leading to apoptosis-
dependent inhibition of neovascularization by thrombospod-
7. Murphy-Ullrich JE, Schultz-Cherry S, Hook M. Transforming growth factor-β complexes with thrombospon-
8. Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N. Peptides derived from two separate domains of the matrix protein thrombospondin-
1 have anti-angiogenic activity. J Cell Biol 1993;122:
497–511.
9. Qian X, Wang TX, Rothman VL, Nicollia RF, Tuzynski GP. Thrombospondin-1 modulates angio-
genesis in vitro by up-regulation of matrix metallo-
10. Rodriguez-Manzaneque JC, Lane TE, Ortega MA, Hynes RO, Lawler J, Iruela-Arispe ML. Splice donor-
and 18 U.S.C. Section 1734 solely to indicate this fact.

Research.

Tumor Angiogenesis in Ewing’s Sarcoma


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Gary Potikyan, Rupert O.V. Savene, Julie M. Gaulden, et al.


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