p53-Responsive miR-194 Inhibits Thrombospondin-1 and Promotes Angiogenesis in Colon Cancers

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Abstract

Thrombospondin-1 (TSP-1) is an endogenous inhibitor of angiogenesis encoded by the THBS1 gene, whose promoter is activated by p53. In advanced colorectal cancers (CRC), its expression is sustained or even slightly increased despite frequent loss of p53. Here, we determined that in HCT116 CRC cells, p53 activates the THBS1 primary transcript, but fails to boost THBS1 mRNA or protein levels, implying posttranscriptional regulation by microRNAs (miRNA). In a global miRNA gain-of-function screen done in the Dicer-deficient HCT116 variant, several miRNAs negatively regulated THBS1 mRNA and protein levels, one of them being miR-194. Notably, in agreement with published data, p53 upregulated miR-194 expression in THBS1 retrovirus-transduced HCT116 cells, leading to decreased TSP-1 levels. This negative effect was mediated by a single miR-194 complementary site in the THBS1 3′-untranslated region, and its elimination resulted in TSP-1 reactivation, impaired angiogenesis in Matrigel plugs, and reduced growth of HCT116 xenografts. Conversely, transient overexpression of miR-194 in HCT116/THBS1 cells boosted Matrigel angiogenesis, and its stable overexpression in Ras-induced murine colon carcinomas increased microvascular densities and vessel sizes. Although the overall contribution of miR-194 to neoplastic growth is context dependent, p53-induced activation of this GI tract–specific miRNA during ischemia could promote angiogenesis and facilitate tissue repair.

Introduction

Thrombospondin-1 (TSP-1) encoded by the THBS1 gene is a major negative regulator of angiogenesis compromising endothelial cell survival (1, 2), migration (3, 4), and responses to the VEGF (5). TSP-1 paralyzes its antiangiogenic activity into inhibition of tumor growth and metastases in many different tumor types (6, 7). Thus, the overall trend is that whereas tumor suppressors increase its expression, oncoproteins exert the opposite effects. Namely, p53 increases TSP-1 expression by upregulating the THBS1 promoter in fibroblasts from patients with Li–Fraumeni syndrome (8), and the loss of p53 leads to rapid downregulation of TSP-1 and an increase in VEGF levels (9). PTEN (10) and Smad4 (11) positively regulate TSP-1 in gioma and pancreatic adenocarcinoma, respectively. Conversely, TSP-1 is consistently downregulated by oncproteins such as Myc (12, 13), Ras (14), Src (15), and Jun (16).

Regulation of TSP-1 by p53 is of particular interest, yet it is a complex and controversial phenomenon. A correlation between p53 loss and TSP-1 silencing has been reported in several tumor types: ovarian carcinoma, bladder cancer, glioma, prostate cancer, and renal cell carcinoma [reviewed in Teodoro and colleagues (17)]. In a Myc-induced hematopoietic tumor model, restoration of p53 was absolutely necessary for TSP-1 expression and sustained tumor regression (18). However, other studies revealed no correlation between TSP-1 and p53 expression in breast cancer (19, 20), stage T3 prostate cancer (21), and cholangiocarcinoma (22). In colon cancer, such studies have proven largely inconclusive because of their reliance on small sample sizes and immunohistochemical detection of p53 (23, 24). In some cases, tumor microenvironment might override the effects of p53. For instance, hypoxia can reduce TSP-1 levels and induce VEGF expression, irrespective of p53 status (25). Similarly, HCMV infection inhibits TSP-1 transcription, both in the presence and absence of p53 (26).

In addition, posttranscriptional microRNA (miRNA)-based mechanisms of TSP-1 regulation have recently come to the fore. Our laboratory first reported downregulation of TSP-1 by members of the miR-17-92 cluster, namely miR-18a and miR-19...
miR-194 Regulates Thrombospondin-1 and Angiogenesis

(27). Subsequently, silencing of Dicer and Drosha, key components of the miRNA biogenesis machinery, was reported to elevate TSP-1 levels even more robustly, suggesting that multiple miRNAs regulate TSP-1 (28). To identify all major miRNA regulators of TSP-1, we conducted a global screen for miRNAs that acutely downregulate TSP-1 mRNA in colon adenocarcinoma cell lines. Here, we report that in addition to miR-18 and miR-19, several other miRNAs do so, including miR-194. This miRNA was of particular interest because its expression is dependent on the presence of wild-type (WT) p53 (29, 30) and could represent a novel component of the p53–TSP-1 axis.

Materials and Methods

Cell lines and drug treatment

Colon adenocarcinoma cell lines used in our study included HCT116, DLD-1, and their derivatives, namely HCT116Dicer<sup>-</sup>, DLD-1Dicer<sup>-</sup>, and HCT116p53<sup>-/-</sup> (all provided by Dr. Bert Vogelstein, Johns Hopkins University). Dicer<sup>ex5</sup> cell lines were hypomorphic for Dicer function (31). HCT116 cell lines stably expressing the retroviral pQXIP/THBS1 ORF (open reading frame) with or without the 3′-UTR (untranslated region) were generated using retroviral transduction. p53-null Ki-Ras<sup>-</sup>transformed murine colonocytes (27) were stably transduced with the pLU-GFP-miR-215-194-1 lentivirus using standard techniques. The small molecule Mdm-2 inhibitor Nutlin-3 was dissolved in dimethyl sulfoxide (DMSO) and added to 5 × 10<sup>5</sup> cells at a final concentration of 10 μmol/L. Cultures were harvested for protein analysis 24 hours later. Where indicated, cells were pretreated for 24 hours with TGF-β at a final concentration of 0.5 ng/mL. The identity of all cell lines with hypomorphic Dicer (Dicer<sup>ex5</sup>) was validated by miRNA profiling using qPCR.

miRNA gain-of-function screen

miRNA mimics (Dharmacon) were introduced into Dicer<sup>ex5</sup> cell lines at the final concentration of 25 nmol/L. Ten hours posttransfection, RNAs were extracted and microarray analysis was done as described in Linsley and colleagues (32).

Transient transfection of miRNA mimics and inhibitors

miRNA mimics and miRNA hairpin inhibitors (MHI) were obtained from Dharmacon. Both were transfected into cells using Lipofectamine 2000 (Invitrogen) as previously described (33). In mimic experiments, 25 nmol/L of individual mimics were transfected into the HCT116 Dicer<sup>ex5</sup> cell lines, and RNA was harvested and analyzed for miRNA and mRNA levels 10 hours after transfection. In inhibitor experiments, 50 nmol/L of MHI was transfected into HCT116 cells, and 48 hours after transfection, both RNA and protein were harvested to analyze TSP-1 expression.

Generation of THBS1 constructs and site-directed mutagenesis

The source of THBS1 ORF and 3′-UTR was pGEM4/TSP4.4, obtained from Dr. Dean Mosher, University of Wisconsin. The 3.5-kb THBS1 ORF was subcloned into the retroviral vector pQXIP (Clontech) as 2 fragments—a XbaI/Clal fragment and a ClaI/BglII fragment. Different fragments of the 3′-UTR were PCR amplified from pGEM4/TSP4.4 using the "THBS1 3′-UTR" primers (see Supplementary Data for nucleotide sequences of all primers) and subcloned into pQXIP/THBS1 plasmid. The pGL3 reporter construct with the full-length THBS1 3′-UTR used in the DLR assay was obtained from Dr. Olga Stenina, Cleveland Clinic. We additionally inserted into this plasmid the last 100 bp from THBS1 ORF flanked by the "THBS1 ORF" primers. Site-directed mutagenesis was carried out to create a 4-nt substitution in the miR-194 seed sequence of the THBS1 3′-UTR using the Stratagene site-directed mutagenesis-XL Kit and "miR-194 mutagenesis primers."

Quantitative real-time PCR analyses

Total RNA for both mRNA and miRNA analysis was extracted from cells using the TRIzol reagent (Sigma) and contaminating DNA was removed using the Turbo-DNA-free kit (Ambion). For miRNA analyses, the first strand of cDNA was synthesized using random primers and Superscript III (Invitrogen) and reverse transcription product was amplified using SYBR Green real-time PCR (ABI). Quantitations were done using "THBS1 qPCR primers" and "GAPDH qPCR primers" obtained from the Harvard Primer Bank. In addition, we used 3 pairs of "THBS1 InRNA primers" designed to amplify the primary transcript. For mRNA expression levels, reverse-transcription reaction and real-time PCR was done using TaqMan miRNA assays (ABI). miR-194 levels were normalized to RNU48 controls. Both SYBR Green and TaqMan qPCRs were done using ABI 7900-HT detection system and the data were analyzed using the RQ manager software v1.2.

Dual-luciferase sensor assay

The full-length THBS1 3′-UTR with either WT or mutated miR-194 seed sequence was cloned downstream of the luciferase reporter in the pGL3 plasmid (Promega). These sensor constructs were used as described previously [(33) and Supplementary Data].

Western blotting analysis of cell lysates and conditioned media

Cells were lysed with radioimmunoprecipitation assay buffer supplemented with phenylmethylsulfonyl fluoride (Sigma) and a cocktail of protease inhibitors (Pierce). Conditioned media (CM) were treated as described in Supplementary Data. Samples were separated in 7.5% SDS-PAGE (Lonza) gels, transferred on to a polyvinylidene difluoride membrane, and blocked in 5% milk. The membrane was probed first with primary antibodies to TSP-1 (Ab-11 from Lab Vision) at 1:400 dilution and primary antibodies to TSP-1 (Ab-11 from Lab Vision) at 1:400 dilution, and primary antibodies to TSP-1 (Ab-11 from Lab Vision) at 1:400 dilution. This was followed by incubation with the respective secondary antibodies conjugated to horseradish peroxidase (HRP) from Amersham and the resulting chemiluminescence was detected. Monoclonal anti-actin antibody conjugated with HRP (A3854 from Sigma) was used at 1:500,000 dilution.

Matrigel assay and microvessel quantitation

The Matrigel neovascularization assay was done as described previously (see ref. 34 and Supplementary Data).
Tumor xenograft studies and vessel quantitation

Tumor xenografts were produced from retrovirally transduced HCT116 cell lines overexpressing pQCXIP/THBS1 ORF with No 3'-UTR, WT, and Mut 3'-UTR as well as lentivirally transduced mouse Ras colonocytes overexpressing pLU-GFP-miR194. A total of $2.5 \times 10^6$ cells were subcutaneously injected into NSG mice, which were obtained from the in-house breeding facility. Five mice per cell type were injected. Tumor were excised on day 14 and analyzed for microvascular densities as described in Supplementary Data.

Results

In colon cancers, TSP-1 is regulated by TP53 by a posttranscriptional mechanism

To elucidate the connection between p53 and TSP-1 in colon cancers, we first analyzed microarray data from the Bittner Colon study (Gene Expression Omnibus record GSE2109) using the Oncomine interface (35). Specifically, we compared adenoma–adenocarcinoma transition samples (presumably WT for p53) with frank adenocarcinoma samples, many of which bear deletions and loss-of-function mutations in the TP53 gene. Indeed, in the latter the TP53 transcript levels were decreased by approximately 1.3-fold (Fig. 1A, top panel). Surprisingly, the THBS1 message was not decreased; in fact, there was a slight trend for higher expression levels in adenocarcinoma samples (Fig. 1A, bottom panel). Furthermore, THBS1 mRNA levels remained unchanged across various histologic grades and Dukes stages (data not shown).

To validate this finding in a more controlled setting, we compared endogenous TSP-1 levels in a pair of isogenic HCT116 colorectal carcinoma cell lines that were either p53 sufficient or p53 null. We observed that in untreated cultures there was little difference in TSP-1 levels (Fig. 1B, left panels). The difference was also minimal when p53 expression was induced with nutlin-3, which blocks p53 degradation by Mdm-2 (ref. 36; Fig. 1B, right panels). To determine whether THBS1 message is induced by p53, we carried out qPCR on the same cells and again observed no difference in THBS1 steady-state mRNA levels (Fig. 1C). However, when we measured the levels of unspliced THBS1 primary transcript (heterogeneous nuclear RNA or hnRNA) using pairs of primers spanning exon–intron junctions, we discovered that THBS1 hnRNA expression was consistently elevated in HCT116 p53+/+ cells upon treatment with nutlin-3 (Fig. 1D). These data suggested that p53 indeed induces THBS1 promoter activity but then its transcript undergoes posttranscriptional regulation, potentially by miRNAs.

TSP-1 expression is regulated by multiple miRNAs in colon cancer cell lines

Previous data from our laboratory had shown that TSP-1 indeed is regulated by a miRNA-based posttranscriptional mechanism. Specifically, miR-18a and miR-19, members of the miR-17~92 cluster, negatively regulate TSP-1 levels during...

Figure 1. TSP-1 primary transcript but not the mature mRNA is regulated by TP53 in colon cancers. A, average fold change in TP53 (probe 201746_at) and THBS1 (probe 201107_s_at) expression in colon adenoma versus adenocarcinoma. Data are from the Bittner Colon dataset. B, immunoblotting showing expression levels of TSP-1 (left and right) and p53 (right) in HCT116 cells, either WT (+/+) or null (−/−) for p53. When indicated, cells were pretreated with nutlin-3 to stabilize p53. C, expression levels of THBS1 mRNA in cells depicted in B. D, expression levels of THBS1 primary transcript in cells from B. Error bars in C and D represent SDs from 3 biological replicates. P values were calculated using Student’s t test.
Figure 2. TSP-1 is regulated by multiple miRNAs in colon cancer cell lines. A, TSP-1 protein levels in Dicer<sup>−/−</sup> CRC cell lines HCT116 and DLD-1. B, TSP-1 protein levels in HCT116 cells transduced with the full-length THBS1 ORF incorporated into the pQCXIP retroviral vector. The 3′-UTR is absent in the “NO-3′-UTR” retroviral construct, whereas the “3′-UTR” construct contains the first 800 bp of the THBS1 3′-UTR cloned downstream of THBS1 ORF. C, microarray profiling of gene expression in HCT116 Dicer<sup>−/−</sup> cells following transfection of miRNA mimics. The column on the heat map corresponding to the THBS1 gene encoding TSP-1 is framed in a vertical box. Rectangles denote THBS1-targeting miRNAs identified in the screen. D, qPCR analysis of TSP-1 mRNA levels in miRNA-transfected HCT116 Dicer<sup>−/−</sup> cells. Two nontargeting miRNAs (a C. elegans miRNA and miR-16) were used as controls. Asterisks denote P values below 0.05. E, real-time PCR showing endogenous expression of TSP-1–targeting miRNAs in HCT116/DLD-1 WT versus Dicer<sup>−/−</sup> cells. F, relative expression levels of mature miRNAs in HCT116 cells following transfection with cognate antisense hairpin inhibitors (MHI), as determined by qPCR. Error bars represent SDs from 2 biological replicates. P values were calculated using Student's t test. G, immunoblotting of TSP-1 produced in the same cells.
Myc-induced angiogenesis (27, 33). To measure the extent of miRNA-based regulation of TSP-1, we examined the endogenous levels of TSP-1 in HCT116 and DLD-1 colon adenocarcinoma cell lines rendered hypomorphic for Dicer: HCT116Dicer<sup>ex5</sup> and DLD-1Dicer<sup>ex5</sup> (31). Western blot analysis of endogenous TSP-1 revealed that its expression was higher in both HCT116Dicer<sup>ex5</sup> and DLD-1Dicer<sup>ex5</sup> variants compared with their Dicer WT counterparts (Fig. 2A), consistent with findings by Kuehbacher and colleagues (28) and Suarez and colleagues (37). To test whether these effects were mediated by T<sub>H</sub>B<sub>S</sub>I 3′-UTR, we generated pQCXIP retroviral constructs carrying T<sub>H</sub>B<sub>S</sub>I ORF with and without the 3′-UTR. These viruses were used to transduce Dicer-deficient and Dicer-sufficient HCT116 cells. WT HCT116 cells expressing T<sub>H</sub>B<sub>S</sub>I ORF fused to the cognate 3′-UTR had diminished levels of TSP-1 compared with the same cell line expressing T<sub>H</sub>B<sub>S</sub>I ORF alone (Fig. 2B, 3 left lanes). However, the difference in TSP-1 expression was much less pronounced in the Dicer-deficient variant (Fig. 2B, 3 right lanes). This result suggested a likely mechanism whereby miRNA-dependent TSP-1 regulation is mediated by T<sub>H</sub>B<sub>S</sub>I 3′-UTR.

To determine what miRNAs might regulate TSP-1 in colon cancer, we conducted a global miRNA gain-of-function screen. HCT116Dicer<sup>ex5</sup> cells were transfected with 25 nm of all known miRNA mimics, as described earlier (32). Ten hours after transfection, RNAs were harvested and analyzed for changes in TSP-1 mRNA levels using Affymetrix microarrays. The heat map in Fig. 2C shows miRNAs that had negative effects on TSP-1 mRNA levels and possessed seed homology sequences in the T<sub>H</sub>B<sub>S</sub>I 3′-UTR. In addition, we stipulated that they must be predicted to target TSP-1 by either microT (38) or TargetScan (39). These filters limited the candidate list to 10 individual miRNAs or miRNA families (boxes): miR-194, miR-199a*, miR-144, miR-1, miR-206, miR-19a, miR-19b, miR-218, miR-18a, and let-7g (a representative of the let-7 family). As expected, miR-18a and miR-19a/b, previously implicated in the regulation of TSP-1 (27, 40), scored positive in this assay.

To validate the results of the miRNA gain-of-function screen, TaqMan real-time reverse transcriptase PCR was used. For this, transfections of the 10 TSP-1–targeting miRNA mimics were repeated and TSP-1 mRNA levels were quantitated as described in Materials and Methods. C. elegans control miRNA mimic and non-TSP-1–targeting miR-16a were used as internal controls. miR-19a, miR-19b, miR-194, miR-218, miR-1/206, and miR-144 significantly downregulated TSP-1 mRNA levels (asterisks in Fig. 2D), confirming the microarray results. To determine whether these miRNAs were endogenously expressed in colon adenocarcinoma cells, qPCR analysis was done on total RNA from HCT116 and DLD-1 cells, WT and hypomorphic for Dicer. Of the 10 miRNAs tested, miR-1 (known to be muscle specific; ref. 41), miR-206 and miR-144 were not detectable in either HCT116 or DLD-1 cell lines. All other miRNAs were readily detectable in both cell lines, with consistently higher expression levels in DLD-1 cells (Fig. 2E). Conversely, Dicer hypomorph variants had reduced miRNA levels, attesting to the fidelity of detection assays.

To determine whether the remaining 7 miRNAs control TSP-1 protein expression when endogenously expressed, we transfected HCT116 cells with 50 nmol/L of MHIs. All miRNAs were targeted individually with the respective MHI except let-7, for which we used a mix of oligonucleotides targeting all 11 let-7 family members. Forty-eight hours later, RNA and protein were harvested and RNA samples were tested by qPCR for miRNA expression levels. The levels of miR-18a, miR-19b, and miR-218 showed a significant reduction upon respective MHI treatment, whereas the others were inhibited more modestly (let-7g) or not at all (miR-19a; Fig. 2F). A similar pattern of miRNA inhibition was observed in DLD-1 cells (Supplementary Fig. A). Then protein lysates were analyzed for TSP-1 expression using Western blotting (Fig. 2G). HCT116 cells treated with the miR-19a and miR-1999 MHI showed a mix of let-7 MHIs showed no changes in TSP-1 protein levels, consistent with the ineffective inhibition of the respective miRNAs. Modest elevation of TSP-1 levels was seen with MHIs targeting miR-18 and miR-218. We observed the most robust and consistent increases in TSP-1 levels in cells treated with MHI against miR-19b and miR-194. Of note, in DLD-1 cells, where basal levels of miR-194 were much higher (Fig. 2E), only anti-miR-19b MHI was effective in restoring TSP-1 expression (Supplementary Fig. B). However, because the role of miR-19 in TSP-1 regulation has already been established, we focused in subsequent experiments on miR-194.

miR-194 is a direct regulator of T<sub>H</sub>B<sub>S</sub>I 3′-UTR

To determine whether miR-194 is a direct regulator of T<sub>H</sub>B<sub>S</sub>I 3′-UTR, we employed the dual-luciferase (DLR) sensor assay. To this end, we cloned the full-length 3′-UTR of TSP-1 downstream of the firefly luciferase reporter gene. Two versions of this T<sub>H</sub>B<sub>S</sub>I 3′-UTR sensor construct were created: one containing the WT miR-194 seed homology sequence and one carrying a 4-nt substitution (Fig. 3A). The sensor constructs were then transfected into DLD-1Dicer<sup>ex5</sup> cell line in the presence of either control or miR-194 mimics. Renilla luciferase construct was also cotransfected as an internal control. Forty-eight hours after transfection, cells were lysed and the firefly and Renilla luminescence levels were measured independently. An approximately 2.3-fold reduction in the firefly-to-Renilla ratio was observed in cells cotransfected with the WT miR-194 sensor construct and miR-194 mimic (Fig. 3B). Mutating the seed homology sequence fully restored firefly luciferase output, as did omitting the miR-194 mimic. These results validated the predicted miR-194 seed sequence in the T<sub>H</sub>B<sub>S</sub>I 3′-UTR.

To extend this finding to the full-length TSP-1 gene, we also tested the effects of mutating the miR-194 seed homology sequence using a retrovirus-based TSP-1 expression system. To this end, the T<sub>H</sub>B<sub>S</sub>I 3′-UTR segment mapping to nt 425 to 847 was cloned into a pQCXIP retroviral construct already expressing the T<sub>H</sub>B<sub>S</sub>I ORF (Fig. 3C). This 423-bp region has no other miR binding sites that were predicted to affect TSP-1 levels. Western blots for TSP-1 expression revealed that HCT116 Dicer-sufficient cells expressing WT 3′-UTR had significantly lower TSP-1 levels than cells expressing the Mut 3′-UTR, whereas their expression levels were similar in Dicer hypomorph cells (Fig. 3D). These results suggested a significant role for miR-194 in TSP-1 regulation.
miR-194 expression is regulated by TP53 in colon cancer

Having established the direct involvement of miR-194 in TSP-1 regulation, we examined the relevance of this regulation in the context of p53 activation. Given that miR-194 expression is known to be GI tract specific (41–43) and also activated by p53 (29, 30), we asked how it impacts the p53–TSP-1 axis in colon tumors. We first analyzed the effects of p53 loss on miR-194 expression using the same comparison between late adenomas (presumably p53+/+) and frank adenocarcinoma (frequently p53−/−; as in Fig. 1A). Both BAX, a well-known p53 target (top panel) and miR-194-1 primary transcript (bottom panel) exhibited decreases in RNA levels in advanced cancers (Fig. 4A). We also used as a model p53-sufficient and p53-deficient HCT116 isogenic variants. We found that loss of p53 resulted in lower miR-194 levels (Fig. 4B). To verify that miR-194 is directly induced by p53, we compared its levels in HCT116 variants left untreated or treated with nutlin-3; p53-activated miR-34a (44) was used for comparison. As anticipated, both miRNAs were induced by nutlin-3 in a manner dependent on the presence of p53 (Fig. 4C). To determine whether this decrease was sufficient for TSP-1 regulation, we compared the expression levels of WT 3′-UTR and Mut 3′-UTR retroviral constructs in p53-sufficient and p53-deficient cells. As shown in Fig. 4D, HCT116 p53+/− cells stably expressing pQCXIP/TBS1 3′-UTR with WT 3′-UTR had reduced levels of TSP-1 when compared with their counterparts with Mut 3′-UTR. However, in HCT116 p53−/− cells, there was little difference in expression levels between WT and Mut 3′-UTR constructs and both were appreciably higher than those in WT 3′-UTR p53+/+ cells (Fig. 4D). These results supported the idea that loss of p53 reduces miR-194 levels strongly enough for TSP-1 to escape regulation by this miRNA.

miR-194 alleviates TSP-1–mediated antiangiogenesis

Having established miR-194 as a new link between p53 and TSP-1, we examined the relevance of this link in angiogenesis and tumor growth. For this purpose, we used the same pQCXIP/TBS1 ORF/3′-UTR viruses as in Fig. 3D. Western blot analysis of cell lysates and serum-free CM (Figs. 5A and B, respectively) confirmed that the presence of the 3′-UTR greatly diminished the expression of TSP-1 compared with that driven by the 3′-UTR less construct. However, mutating the miR-194 seed homology sequence restored TSP-1 expression levels. To determine whether these differences in TSP-1 expression levels are sufficient to control the angiogenic switch, we first carried out Matrigel neovascularization assay. CM from cells depicted in Fig. 5B were mixed with Matrigel and injected into mice as described in Materials and Methods. Upon conclusion of the experiment, snap-frozen Matrigels were sectioned and stained for blood vessels using an anti-CD31 antibody reactive with endothelial cells. CD31−positive areas were counted using the MetaMorph software and the vessel counts were compared among samples. As expected, TSP-1high CM from cells expressing pQCXIP/TBS1 ORF with NO 3′-UTR and Mut 3′-UTR
showed strong inhibition of angiogenesis compared with TSP-1low CM from WT 30-UTR cells (Fig. 5C and D).

To test the effects of varying TSP-1 levels on tumor growth, we carried out xenograft studies in NOG mice using the same cell lines. Tumor sizes were measured daily from day 7 until day 14 after injection, at which point mice were sacrificed and tumors were measured and weighed. As shown in Fig. 5E, tumors derived from all 3 cell lines displayed similar growth kinetics at early time points. However, by day 11, tumors expressing pQCXIP/THBS1 ORF with WT 30-UTR (TSP-1low) started to outgrow their Mut 30-UTR and, especially, NO 30-UTR TSP-1high counterparts. On day 14, this increase in growth rates yielded larger tumors as measured by volume and weight (Fig. 5F and G). Thus, regulation of TSP-1 by miR-194 alleviates antiangiogenesis.

In prior experiments, we were measuring angiogenesis following the loss of the miR-194–THBS1 3’-UTR interaction. To determine whether miR-194 promotes angiogenesis in a TSP-1–dependent manner, we used the HCT116 Dicer−/− cells stably expressing pQCXIP/TSP-1 with WT or Mut 3’-UTR, which initially had similar TSP-1 expression levels (see Fig. 3D, lanes 4–6). As expected, following transfection with the miR-194 mimic, TSP-1 levels were reduced only in cells expressing THBS1 ORF with WT 3’-UTR (Fig. 5H). To determine whether this decrease in TSP-1 levels in miR-194–transfected cells affected angiogenesis, we harvested CM from these cells, verified TSP-1 downregulation by Western blotting (Fig. 5I), and carried out the Matrigel neovascularization assay as described above. We observed a significant increase in angiogenesis (vessel area count) in miR-194 mimic-treated cells expressing TSP-1 WT 3’-UTR compared with control mimic-treated cells (Fig. 5J and K). We also observed a slight but statistically significant increase in angiogenesis in miR-194 mimic-treated cells expressing TSP-1 Mut 3’-UTR, suggesting the existence of additional non-TSP-1–mediated effects of miR-194 on angiogenesis. However, these effects seem to be less significant than those mediated by TSP-1 downregulation.

miR-194 counteracts endogenous TSP-1

All angiogenesis data obtained thus far were derived from HCT116 cells with retrovirally expressed TSP-1. To rule out the possibility that miR-194 is only angiogenic in the context of TSP-1 overexpression, we utilized Ras-transformed murine p33-null colonocytes (27). They were transduced with pLU-GFP lentivirus and its derivative expressing the entire miR-194/215 cluster (29). Fluorescence-activated cell sorting analysis
Figure 5. miR-194 alleviates TSP-1–mediated antiangiogenesis. A, TSP-1 expression levels in HCT116 cells stably infected with the retrovirus pQCXIP expressing THBS1 ORF with or without THBS1 3′-UTR. Western blotting was done on whole-cell lysates. B, immunoblotting done on CM from cells in A. C, microvascular densities of Matrigel plugs containing CM from B. VEGF was used as the inducer of angiogenesis. Frozen sections of Matrigel plugs were stained for the endothelial marker CD31 and vessels were quantitated using the MetaMorph software. Three Matrigel sections with 5 fields per section were analyzed per mouse. D, representative images of Matrigel sections from C. CD31-positive vessels appear in red. Blue 4′, 6-diamidino-2-phenylindole staining corresponds to nuclei of Matrigel-invading cells. E–G, quantitative analysis of tumor xenografts formed by the same cells in NOG mice. Parameters assessed were kinetics of tumor growth (E), tumor volume on day 14 (F), and tumor weight on day 14 (G), with n = 5 mice per group. Statistical significance was determined using 2-tailed Student t test. H, TSP-1 expression levels in HCT116 Dicerex5 cells stably infected with the retrovirus pQCXIP expressing THBS1 ORF with WT or Mut 3′-UTR. Western blotting was done on whole-cell lysates. I, Western blotting done on CM from cells in A. J, microvascular densities of Matrigel plugs containing CM from B. Analysis was done as in C. K, representative images of Matrigel sections from C. Staining was done as in D.
miR-194 counteracts endogenous TSP-1. A, flow cytometric analysis of Ras colonocytes transduced with pLU-GFP (empty vector) and pLU-GFP-miR-215-194 lentiviruses. B, fluorescent images of cell cultures from A. C, miR-194 expression levels in the same cultures as determined by qPCR. Error bars represent SDs from 2 biological replicates. D, immunoblot showing the effect of miR-194 overexpression on TSP-1 levels. Where indicated, cells were treated with TGF-β for 24 hours. E, miR-194 expression levels in 2 representative Ras tumors of each type. F, immunoblotting of TSP-1 done on lysates of the same tumors. G and H, microvascular densities and mean lumen areas, respectively, of Ras tumor sections. For vessel quantitation, 2 adjacent sections from 2 nonadjacent pieces of tumor were stained for the endothelial marker CD34. In total, 4 tumors per group were included in the analysis. Statistical significance was calculated using 2-tailed Student t test. I, representative vessels images corresponding to tumors in G and H. J, the current model for bimodal regulation of TSP-1 by p53. See Discussion for more explanations.
miR-194 Regulates Thrombospondin-1 and Angiogenesis

was used to isolate the brightest 10% of green fluorescent protein (GFP)-positive cells ensuring robust expression of miR-194 (Fig. 6A). Representative images of cells thus obtained are shown in Fig. 6B. Despite strong GFP fluorescence, miR-194 overexpression was only approximately 10-fold, as determined by qPCR (Fig. 6C). We tested, using immunoblotting, whether this mild miR-194 overexpression affects TSP-1 level. To elicit robust TSP-1 expression, we pretreated cells with TGF-β, which we had found to be a major positive regulator of THBS1 expression (33, 45). As shown in Fig. 6D, in miR-194–transduced cells TSP-1 levels were markedly reduced. We then used these cells to generate tumor xenografts in NOG mice. Resultant neoplasms were harvested and sustained overexpression of miR-194 was confirmed by qPCR (Fig. 6E) and sustained downregulation of TSP-1 in the same tumors was confirmed by immunoblotting (Fig. 6F). The tumors were also weighed, sectioned, stained for vessels using CD34 as the endothelial marker, and vessels were enumerated as described in Materials and Methods. We did not observe a statistically significant difference in tumor sizes. However, compared with control GFP Ras tumors, miR-194–transduced neoplasms (with reduced TSP-1 levels) showed slightly but significantly increased microvessel densities (Fig. 6G). Most notably, the vessels in these tumors were of very significantly larger calibers (Fig. 6H), resulting in much better vessel coverage (see representative images in Fig. 6I). Thus, miR-194 is intrinsically angiogenic even in the context of endogenously expressed TSP-1.

Discussion

The positive regulation of TSP-1 expression by the p53 tumor suppressor has been shown multiple times in the literature (reviewed in [17]). However, the paradox remains as to why the tight correlation between p53 and TSP-1 seems to be disrupted in colon cancers. One possible answer is that in colon tissues, TSP activation is maintained at the level of transcription but is reversed at the posttranscriptional level, possibly by miRNAs. Previous data from our laboratory has shown the involvement of the miR-17–92 cluster members, namely miR-18a and miR-19, in downregulating TSP-1 mRNA and protein levels (27, 33). In this article, we show that at least 10 other miRNAs affect TSP-1 mRNA levels, consistent with the length of THBS1 3′-UTR (>2 kb). Some of them are regulated by c-Myc, most notably miR-18 and miR-19 (27) and the let-7 family members (46). However, one of the most potent direct regulators of TSP-1 expression in colon epithelium-derived cell lines turned out to be miR-194. Using retrovirus transduction, we showed that mutating the miR-194 binding site in the 3′-UTR of TSP-1 sharply increases TSP-1 levels and confers upon originally angiogenic HCT116 human colorectal cancer (CRC) cells a nonangiogenic phenotype. It also limits their growth as xenografts in immunocompromised mice.

The in vivo relevance of these findings stems from the fact that expression of miR-194 is largely limited to the GI tract, suggesting a potential role for this miRNA in GI cancers. In addition, this miRNA is known to be induced by p53 (29, 30). Indeed, the loss of p53 in HCT116 cells significantly reduces its steady-state levels. Consequently, compared with their p53-sufficient counterparts, p53 null HCT116 cells have elevated levels of TSP-1 encoded by a stably integrated retrovirus (where the 3′-UTR is the only TSP-1–derived regulatory element) and comparable levels of endogenous TSP-1 (driven by p53-responsive THBS1 promoter). Similarly, in the largest to-date mRNA profiling study comparing adenomas (presumably p53+/−) and adenocarcinomas (largely p53−/) no differences in TSP-1 mRNA expression levels were apparent (Bittner Colon study).

Why should two opposing mechanisms linking p53 and TSP-1 exist in colon cancer? One could reason that in most tissues, p53 needs to sustain TSP-1 levels and renders them antiangiogenic (17). However, in the intestine, which is highly sensitive to ischemia reperfusion (I/R ref. 47 and references therein), there might be a need to temporarily “suspend” TSP-1 expression and allow angiogenesis to occur as a prelude to tissue repair. Given that I/R seems to involve p53 activation (48), the ensuing upregulation of miR-194 and downregulation of TSP-1 could provide just such a mechanism. Furthermore, for p53 retaining colon cancers, the miR-194–TSP-1 axis could provide an additional proangiogenic stimulus.

Indeed, our study has revealed that miR-194 is intrinsically angiogenic in both murine and human colon cancer cell lines. The underlying mechanism is mainly TSP-1 dependent, but we observed less pronounced TSP-1–independent effects on angiogenesis as well (Fig. 6J). The overall effect of miR-194 on tumor growth has proven to be more complex. Whereas disruption of the miR-194–TSP-1 axis suppressed tumor growth in HCT116 xenografts, miR-194 overexpression in murine Ras-induced carcinomas did not appreciably promote neoplastic growth. This observation is consistent with the idea that miR-194 undoubtedly has many other targets pertaining to tumor growth. For example, in a recent study miR-194 was found to be antitumorigenic in hepatocytes through decreasing the EMT transition and ensuing metastasis (49). In addition, miR-215, which is coexpressed with miR-194, has cell-intrinsic inhibitory effects on cell cycle (29). Thus, the overall effects of miR-194 on neoplastic growth are highly context dependent—as is the case for most known miRNAs (50).

Disclosure of Potential Conflicts of Interest

MA. Clary is an employee of Merck & Co. Inc. The other authors disclosed no potential conflicts of interest.

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