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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 post-transcriptional regulation by microRNAs. In a global microRNA gain-of-function screen performed in the Dicer-deficient HCT116 variant, several microRNAs 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'UTR, 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 yielded increased microvascular densities and vessel sizes. While the overall contribution of miR-194 to neoplastic growth is context-dependent, p53-induced activation of this GI tract-specific microRNA during ischemia could promote angiogenesis and facilitate tissue repair.

Keywords

microRNA; thrombospondin-1; angiogenesis; colorectal cancer; p53

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INTRODUCTION

Thrombospondin-1 (TSP-1) encoded by the *THBS1* gene is a major negative regulator of angiogenesis compromising endothelial cells survival (1, 2), migration (3, 4), and responses to the vascular endothelial growth factor (VEGF) (5). TSP-1 parlayes its anti-angiogenic activity into inhibition of tumor growth and metastases in many different tumor types (6, 7). Thus, the overall trend is that while tumor suppressors increase its expression, oncoproteins exert the opposite effects. Namely, p53 increases TSP-1 expression by up-regulating the *THBS1* promoter in fibroblasts from patients with Li-Fraumeni syndrome (8) and the loss of p53 leads to rapid down-regulation of TSP-1 and an increase in VEGF levels (9). PTEN (10) and Smad4 (11) positively regulate TSP-1 in glioma and pancreatic adenocarcinoma, respectively. Conversely, TSP-1 is consistently down-regulated by oncogenes 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 (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 the p53 status (25). Similarly, HCMV infection inhibits TSP-1 transcription, both in the presence of absence of p53 (26).

Additionally, post-transcriptional microRNA (miRNA)-based mechanisms of TSP-1 regulation have recently come to the fore. Our laboratory first reported down-regulation of TSP-1 by members of the miR-17-92 cluster, namely miR-18a and miR-19 (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). In order to identify all major miRNA regulators of TSP-1, we conducted a global screen for miRNAs that acutely down-regulate TSP-1 mRNA in colon adenocarcinoma cell lines. Here we report that in addition to miR-18 and -19, several other microRNAs do so, including miR-194. This microRNA was of particular interest because its expression is dependent on the presence of wild-type 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^{ex5}, DLD-1Dicer^{ex5}, and HCT116p53^{-/-} (all provided by Dr. Bert Vogelstein, Johns Hopkins University). Dicer^{ex5} cell lines were hypomorphic for Dicer function (31). HCT116 cell lines stably expressing the retroviral pQXIP/*THBS1* ORF with or without the 3'UTR were generated using retroviral transduction. p53-null Ki-Ras-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 DMSO and added to 5×10⁵ cells at the final concentration of 10 μM. Cells were harvested for protein analysis 24 h later. Where indicated, cells were pre-treated for 24h with TGFβ at the final concentration of 0.5 ng/ml. The identity of all cell lines with hypomorphic Dicer (Dicer^{ex5}) was validated by miRNA profiling using qPCR.

miRNA gain-of-function screen

miRNA mimics (Dharmacon) were introduced into Dicer^{ex5} cell lines at the final concentration of 25nM. 10 h post-transfection RNAs were extracted, and microarray analysis was performed as described in (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, 25nM of individual mimics were transfected into the HCT116 Dicer^{ex5} cell lines and RNA was harvested and analyzed for mRNA and miRNA levels 10h after transfection. In inhibitor experiments, 50nM of MHI was transfected into HCT116 cells, and 48 h 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 3.5kb *THBS1* ORF was sub-cloned into the retroviral vector pQCXIP (Clontech) as two fragments – a XbaI/ClaI fragment and a ClaI/BclI 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 sub-cloned into pQCXIP/*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 performed 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 mRNA 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 performed using “THBS1 qPCR primers” and “GAPDH qPCR primers” obtained from the Harvard Primer Bank. Additionally, we used three pairs of “THBS1 hnRNA primers” designed to amplify the primary transcript. For miRNA expression levels, reverse-transcription reaction and real-time PCR was performed using Taqman miRNA assays (ABI). miR-194 levels were normalized to RNU48 controls. Both SYBR® Green and Taqman qPCRs were performed using ABI 7900-HT detection system and the data were analyzed using the RQ manager software v1.2.

Dual-Luciferase (DLR) 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 RIPA buffer supplemented with phenylmethylsulfonyl fluoride (Sigma) and a cocktail of protease inhibitors (Pierce). Conditioned media were treated as described in Supplementary data. Samples were separated in 7.5% SDS-PAGE (Lonza) gels, transferred on to a PVDF membrane and blocked in 5% milk. The membrane was probed

first with primary antibodies to TSP-1 (Ab-11 from Labvision) at 1:400 dilution, and p53 (sc-6243 from Santa Cruz) at 1:1000 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 performed as described previously [see (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. $2 \div 5 \times 10^6$ cells were subcutaneously injected into NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Sug}/Jic* Tac mice obtained from the in-house breeding facility (CHOP Research Institute), with 5 mice per cell type. 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 post-transcriptional 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 wild type 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 ~1.3 fold (Figure 1A, top panel). Surprisingly, the *THBS1* message was not decreased; in fact there was a slight trend for higher expression levels in adenocarcinoma samples (Figure 1A, bottom panel). Furthermore, *THBS1* mRNA levels remained unchanged across various histological 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 (Figure 1B, left panels). The difference was also minimal when p53 expression was induced with nutlin-3, which blocks p53 degradation by Mdm-2 (36) (Figure 1B, right panels). To determine whether *THBS1* message is induced by p53, we performed qPCR on the same cells and again observed no difference in *THBS1* steady-state mRNA levels (Figure 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 (Figure 1D). These data suggested that p53 indeed induces *THBS1* promoter activity, but then its transcript undergoes post-transcriptional regulation, potentially by microRNAs.

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

Previous data from our laboratory had shown that TSP-1 indeed is regulated by a microRNA-based post-transcriptional mechanism. Specifically, miR-18a and miR-19, members of the miR-17-92 cluster, negatively regulate TSP-1 levels during 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^{ex5} and DLD-1Dicer^{ex5} (31). Western blot analysis for endogenous TSP-1 revealed that its expression was higher in both HCT116Dicer^{ex5} and DLD-1Dicer^{ex5} variants compared to their Dicer wild-type counterparts (Figure 2A), consistent with findings by (28) and (37). To test whether these effects were mediated by *THBS1* 3'UTR, we generated pQCXIP retroviral constructs carrying *THBS1* ORF with and without the 3'UTR. These viruses were used to transduce Dicer-deficient and -sufficient HCT116 cells. Wild-type HCT116 cells expressing *THBS1* ORF fused to the cognate 3'UTR had diminished levels of TSP-1 compared to the same cell line expressing *THBS1* ORF alone (Figure 2B, three left lanes). However, the difference in TSP-1 expression was much less pronounced in the Dicer-deficient variant (Fig 2B, three right lanes). This result suggested a likely mechanism whereby microRNA-dependent TSP-1 regulation is mediated by its own 3'UTR.

To determine what miRNAs might regulate TSP-1 in colon cancer, we conducted a global miRNA gain-of-function screen. HCT116Dicer^{ex5} cells were transfected with 25nm of all known miRNA mimics, as described earlier (32). 10 h after transfection, RNAs were harvested and analyzed for changes in TSP-1 mRNA levels using Affymetrix microarrays. The heatmap in Figure 2C shows microRNAs that had negative effects on TSP-1 mRNA levels and possessed seed homology sequences in the *THBS1* 3'UTR. Additionally, we stipulated that they must be predicted to target TSP-1 by either microT (38) or Target Scan (39). These filters limited the candidate list to 10 individual miRNAs or miRNA families (red 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 positively in this assay.

To validate the results of the miRNA gain-of-function screen, Taqman real-time RT-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. miR19a, miR-19b, miR-194, miR-218, miR-1/206, and miR-144 significantly down-regulated TSP-1 mRNA levels (asterisks in Figure 2D), confirming the microarray results. To determine if these miRNAs were endogenously expressed in colon adenocarcinoma cells, qPCR analysis was performed on total RNA from HCT116 and DLD-1 cells, wild-type and hypomorphic for Dicer. Of the 10 miRNAs tested, miR-1 [known to be muscle-specific (41)], miR-206 and miR-144 were not detectable in either HCT116 or DLD-1 cell lines. All other microRNAs were readily detectable in both cell lines, with consistently higher expression levels in DLD-1 cells (Figure 2E). Conversely, Dicer hypomorph variants had reduced miRNA levels, attesting to the fidelity of detection assays.

To determine whether the remaining 7 microRNAs control TSP-1 protein expression when endogenously expressed, we transfected HCT116 cells with 50nM of miRNA hairpin inhibitors (MHI). 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. 48 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, miR-194 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) (Figure 2F). A similar pattern of microRNA inhibition was observed in DLD-1 cells (Supplementary Figure A). Then protein lysates were analyzed for TSP-1 expression using Western blotting (Fig 2G). HCT116 cells treated with the miR-19a and miR-199* MHIs and a mix of let-7 MHIs showed no changes in

TSP-1 protein levels, consistent with the ineffective inhibition of the respective microRNAs. 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 (Figure 2E) only anti-miR-19b MHI was effective in restoring TSP-1 expression (Supplementary Figure B). However, since 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 *THBS1* 3'UTR

To determine whether miR-194 is a direct regulator of *THBS1* 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 *THBS1* 3'UTR sensor construct were created: one retaining the wild-type miR-194 seed homology sequence and one carrying a 4-nucleotide substitution (Figure 3A). The sensor constructs were then transfected into DLD-1 Dicer^{ex5} cell line, in the presence of either control or miR-194 mimics. Renilla luciferase construct was also co-transfected as an internal control. 48 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 co-transfected with the wild-type miR-194 sensor construct and miR-194 mimic (Figure 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 *THBS1* 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 *THBS1* 3'UTR segment mapping to nucleotides 425 – 847 was cloned into a pQCXIP retroviral construct already expressing the *THBS1* ORF (Figure 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 (Figure 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 Figure 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 (Figure 4A). We also used as a model p53-sufficient and -deficient HCT116 isogenic variants. We found that loss of p53 resulted in lower miR-194 levels (Figure 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 microRNAs were induced by nutlin-3 in a manner dependent on the presence of p53 (Figure 4C). To determine if 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 -deficient cells. As shown in Figure 4D, HCT116 p53^{+/+} cells stably expressing pQCXIP/TSP-1 with WT 3'UTR had reduced levels of TSP-1 when compared to 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 appreciable higher than those in WT 3'UTR p53^{+/+} cells (Figure 4D). These results supported the idea that loss of p53 reduces miR-194 levels strongly enough for TSP-1 to escape regulation by this microRNA.

miR-194 alleviates TSP-1-mediated anti-angiogenesis

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/*THBS1* ORF/3'UTR viruses as in Figure 3D. Western blot analysis of cell lysates and serum-free conditioned media (CM) (Figures 5A and B, respectively) confirmed that the presence of the 3'UTR greatly diminished the expression of TSP-1 compared to that driven by the 3'UTR-less construct. However, mutating the miR-194 seed homology sequence restored TSP-1 expression levels.

To determine if these differences in TSP-1 expression levels are sufficient to control the angiogenic switch, we first performed Matrigel neovascularization assay. CM from cells depicted in panel 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. CD-31 positive areas were counted using the MetaMorph software and the vessel counts were compared among samples. As expected, TSP-1^{high} CM from cells expressing pQCXIP/*THBS1* ORF with NO 3'UTR and MUT 3'UTR showed strong inhibition of angiogenesis compared to TSP-1^{low} CM from Wt 3'UTR cells (Figures 5C and D).

To test the effects of varying TSP-1 levels on tumor growth, we performed 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 Figure 5E, tumors derived from all three cell lines displayed similar growth kinetics at early time points. However, by day 11, tumors expressing pQCXIP/*THBS1* ORF with Wt 3'UTR (TSP-1^{low}) started to outgrow their Mut 3'UTR and especially No 3'UTR TSP-1^{high} counterparts. On day 14, this increase in growth rates yielded larger tumors as measured by volume and weight (Figures 5F and G). Thus, regulation of TSP-1 by miR-194 alleviates anti-angiogenesis.

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^{ex5} cells stably expressing pQCXIP/TSP-1 with Wt or Mut 3'UTR, which initially had similar TSP-1 expression levels (see Figure 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 (Figure 5H). To determine if this decrease in TSP-1 levels in miR-194 transfected cells affected angiogenesis, we harvested CM from these cells, verified TSP-1 down-regulation by Western blotting (Figure 5I) and performed 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 to control mimic-treated cells (Figures 5J and 5K). 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 appear to be less significant than those mediated by TSP-1 down-regulation.

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 p53-null colonocytes (27). They were transduced with pLU-GFP lentivirus and its derivative expressing the entire miR-194/215 cluster (29). FACS analysis was used to isolate the brightest 10% of GFP-positive cells ensuring robust expression of miR-194 (Figure 6A). Representative images of cells thus obtained are shown in Figure 6B. Despite strong GFP fluorescence, miR-194 overexpression was only ~10-fold, as determined by qPCR (Figure 6C). We tested, using immunoblotting, whether this mild miR-194 over-expression affects TSP-1 level. To elicit robust TSP-1 expression, we pre-treated cells with TGF β , which we had found to be a major positive regulator of *THBS1* expression (33, 45). As shown in Figure 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 (Figure 6E) and sustained down-regulation of TSP-1 in the same tumors was confirmed by immunoblotting (Figure 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 to control GFP Ras tumors, miR-194-transduced neoplasms (with reduced TSP-1 levels) showed slightly but significantly increased microvessel densities (Figure 6G). Most notably, the vessels in these tumors were of very significantly larger calibers (Figure 6H) resulting in much better vessel coverage (see representative images in Figure 6I). Thus, miR-194 is intrinsically angiogenic even in the context of endogenously expressed thrombospondin-1.

DISCUSSION

The positive regulation of TSP-1 expression by the p53 tumor suppressor has been demonstrated multiple times in the literature [reviewed in (17)]. However, the paradox remains as to why the tight correlation between p53 and TSP-1 appears to be disrupted in colon cancers. One possible answer is that in colon tissues it is maintained at the level of transcription but is reversed at the post-transcriptional 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 down-regulating TSP-1 mRNA and protein levels (27, 33). In this paper, we demonstrate that at least 10 other microRNAs 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 -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 system, we demonstrated 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 CRC cells the non-angiogenic 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 microRNA in GI cancers. Additionally, this microRNA 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 to 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 anti-angiogenic (17). However, in the intestine, which is highly sensitive to ischemia-reperfusion (I/R) [(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 appear to involve p53 activation (48), the ensuing up-regulation of miR-194 and down-regulation of TSP-1 could provide just such a mechanism. Furthermore, for p53-retaining colon cancers, the miR-194 -| thrombospondin-1 axis could provide an additional pro-angiogenic 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 (Figure 6J). The overall effect of miR-194 on tumor growth has proven to be more complex. While disruption of the miR-194 -| thrombospondin-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 anti-tumorigenic in hepatocytes through decreasing the EMT transition and ensuing metastasis (49). Additionally, miR-215, which is co-expressed 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 microRNAs (50).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Thrombospondin-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 vs adenocarcinoma. Data are from the Bittner Colon dataset. (B) Immunoblotting showing expression levels of TSP-1 (left and right panels) and p53 (right panel) in HCT116 cells, either wild-type (+/+) 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 panel B. (D) Expression levels of *THBS1* primary transcript in cells from panel B. Error bars in C and D represent standard deviations from three biological replicates. p-values were calculated using Student's t-test.

Figure 2. Thrombospondin-1 is regulated by multiple miRNAs in colon cancer cell lines (A) TSP-1 protein levels in Dicer^{ex5} colorectal cancer 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 800bp of the *THBS1* 3'UTR cloned downstream of *THBS1* ORF. (C) Microarray profiling of gene expression in HCT116 Dicer^{ex5} 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^{ex5} cells. Two non-targeting miRNAs (a *C. elegans* miRNA and miR-16) were used as controls. Asterisks denote p-values below 0.05. (E) Real-time PCR demonstrating endogenous expression of TSP-1-targeting miRNAs in HCT116/DLD-1 wt vs. Dicer^{ex5} 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 standard deviations from two biological replicates. p-values were calculated using Student's t-test. (G) Immunoblotting of TSP-1 produced in the same cells.

Figure 3. miR-194 is a direct regulator of THBS1 3'UTR

(A) The predicted miR-194 binding site in the *THBS1* 3'UTR. The 8-mer seed is highlighted in bold. A 4-nt substitution in the miR-194 seed homology sequence was introduced by site-directed mutagenesis, with the mutated bases underlined. (B) Protein output of firefly luciferase encoded by the pGL3-*THBS1* 3'UTR carrying wild-type or mutated miR-194 seed homology sequences. Constitutively expressed Renilla luciferase was used as an internal control in the Dual-Luciferase Assay. Error bars represent standard deviations from two biological replicates. p-values were calculated using Student's t-test. (C) The location of the miR-194 response element in relation to other putative miR-binding sites. The composition of the pQCXIP/*THBS1* 3'UTR retrovirus used in subsequent experiments is shown below. (D) Immunoblotting analysis of TSP-1 expression in HCT116 cells (Dicer wt or Dicer^{ex5}) expressing pQCXIP/*THBS1* ORF/3' UTR in either wt or mutant conformation.

Figure 4. p53 regulates miR-194 in colon cancer

(A) Average fold-change of BAX mRNA, a p53 target gene (top) and miR-194 primary transcript (bottom) in adenoma vs. adenocarcinoma, as determined in the Bittner Colon study (see also Figure 1A). (B) miR-194 expression levels in HCT116 cells wt (+/+) and null (-/-) for p53, as measured by qPCR. (C) miR-194 and miR-34a expression levels in HCT116 cells WT and null (-/-) for p53, as measured by qPCR. When indicated, cells were pretreated with nutlin-3 to stabilize p53. Error bars represent standard deviations from two biological replicates. p-values were calculated using Student's t-test. (D) Immunoblotting of TSP-1 in HCT116 cells expressing retrovirally encoded *THBS1* ORF with wt or mut 3'UTR. Two isogenic HCT116 variants, with wild-type and p53-null background, were tested.

Figure 5. miR-194 alleviates TSP-1-mediated anti-angiogenesis

(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 performed on whole cell lysates. (B) Immunoblotting performed on conditioned media from cells in panel A. (C) Microvascular densities of Matrigel plugs containing conditioned media from panel 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. 3 matrigel sections with 5 fields/section were analyzed per mouse. (D) Representative images of Matrigel sections from panel C. CD31-positive vessels appear in red. Blue DAPI 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/group. Statistical significance was determined using two-tailed Student's t-test. **(H)** TSP-1 expression levels in HCT116 Dicer^{ex5} cells stably infected with the retrovirus pQCXIP expressing *THBS1* ORF with Wt or Mut 3'UTR. Western blotting was performed on whole cell lysates. **(I)** Western blotting performed on conditioned media from cells in panel A. **(J)** Microvascular densities of Matrigel plugs containing conditioned media from panel B. Analysis was performed as in panel 5C. **(K)** Representative images of Matrigel sections from panel C. Staining was performed as in panel 5D.

Figure 6. 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) Microscopic images of cell cultures from panel A. (C) miR-194 expression levels in the same cultures as determined by qPCR. Error bars represent standard deviations from two biological replicates. (D) Immunoblot showing the effect of miR-194 over-expression on TSP-1 levels. Where indicated, cells were treated with TGF β for 24h. (E) miR-194 expression levels in two representative Ras tumors of each type. (F) Immunoblotting of TSP-1 performed on lysates of the same tumors. (G,H) Microvascular densities and mean lumen areas, respectively, of Ras tumor sections. For vessel quantitation, two adjacent sections from two non-adjacent pieces of tumor were stained for the endothelial marker CD34. In total, 4 tumors/groups were included in the analysis. Statistical significance was calculated using two-tailed Student's t test. (I). Representative vessels images corresponding to tumors in panels G and H. (J) The current model for bimodal regulation of TSP-1 by p53. See Discussion for more explanations.