Multiple microRNAs Derived from Chemically Synthesized Precursors Regulate Thrombospondin 1 Expression

Afzal M. Dogar,^{*} Giuseppe Semplicio,^{*,#} Boris Guennewig,[†] and Jonathan Hall

Thrombospondin 1 (THBS1) is a secreted protein with a variety of biological functions, including a potent anti-angiogenic activity and activation of latent transforming growth factor beta (TGF- β). In many human cancers it is expressed at low levels, although mutations in the *THBS1* gene have been rarely reported. Instead, the loss of THBS1 expression has been proposed to be due to transcriptional and post-transcriptional deregulations. In a systematic screen of predicted microRNA (miRNA) binding sites in the THBS1 3' untranslated region (UTR) we employed chemically synthesized pre-miRNAs—a new class of pre-miRNA mimics—to show that several miRNAs (let-7a, miR-18a, miR-29b, miR-194, and miR-221) can modulate THBS1 expression at the post-transcriptional level. Sequence-specific downregulation of THBS1 by let-7a, miR-18a or by a small interfering RNA induced TGF- β 1 and SMAD4 transcript levels. Ectopic expression of latent TGF- β 1 reduced THBS1 protein expression and was associated with increased expression of let-7a, let-7-b, and miR-18a in cells. These data suggest an inverse correlation of THBS1 and latent TGF- β 1 expression levels possibly involving miRNAs.

Introduction

THROMBOSPONDINS ARE SECRETED PROTEINS that act as regulators of cell interactions (Adams and Lawler, 2011). Thrombospondin 1 (THBS1) is predominantly secreted from platelet α -granules and is incorporated into fibrin clots (Murphy-Ullrich and Mosher, 1985). It is also temporally expressed by other cell types that participate in wound-healing responses (Agah et al., 2002). One of the principal functions of THBS1 is its potent anti-angiogenic activity (Good et al., 1990; Jimenez et al., 2000; Bornstein, 2009). A second main function is its binding to and activation of latent TGF- β 1 (Schultz-Cherry et al., 1994) in multiple cell types, including endothelial cells, mesangial cells, fibroblasts, T cells, and macrophages (Sweetwyne and Murphy-Ullrich, 2012).

An understanding of the mechanisms regulating THBS1 biogenesis and function is imperative because of its important roles in diseases. A variety of epigenetic, transcriptional, and post-transcriptional mechanisms have been described. For example, hypoxia leads to hypermethylation of the THBS1 promoter region and reduces its expression in certain cancers (Oue et al., 2003; Chen et al., 2011). Glucose can also regulate THBS1 secretion in adipocytes from obese rats,

possibly at a post-transcriptional level (Garcia-Diaz et al., 2011). Regulation of THBS1 expression by TP53, PTEN, and SMAD4 has also been reported (Henkin and Volpert, 2011). Furthermore, the 3' untranslated region (UTR) of THBS1 contains conserved AU-rich elements, mutations of which have been shown to increase its mRNA stability leading to increased THBS1 expression (McGray et al., 2011).

MicroRNAs (miRNAs) are ~22-nucleotides (nt)-long noncoding RNAs which regulate the expression of a large fraction of genes (Bartel, 2009). They bind with partial sequence complementarity to sites, usually in the 3'UTR of mRNAs, and inhibit protein translation or induce mRNA decay. An increasingly recognized feature of miRNA function is the parallel targeting of regulatory pathway members by multiple miRNAs (Bartel, 2009). Recent studies have revealed the importance of miRNAs in the regulation of THBS1. In particular, some members of the miR-17-92 cluster, which are transcriptionally induced by MYC, have been shown to suppress THBS1 during angiogenesis (Dews et al., 2006, 2010). In age-related heart failure, increased levels of miR-18 and miR-19 are reported to decrease THBS1 (van Almen et al., 2011). We could demonstrate that suppression of miR-18a partially restored TGF- β 1 maturation by de-repressing THBS1. We concluded that TGF- β 1

Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland.

[#]Present affiliation: Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany.

[†]Present affiliation: Garvan Institute of Medical Research, Darlinghurst, Australia.

^{*}These authors contributed equally to this work.

maturation controls and is controlled by miRNAs targeting two of its maturation factors, THBS1 and FURIN, as part of a regulatory loop (Dogar et al., 2011). Recently miR-194 was also shown to target the 3'UTR of THBS1 (Sundaram et al., 2011). Taken together these studies underscore the importance of THBS1 regulation by the miR-17-92 cluster (Dews et al., 2006) and miR-194 (Sundaram et al., 2011); however, there are no reports of other miRNAs affecting THBS1 expression.

A systematic investigation of the THBS1 3'UTR revealed that several miRNAs (let-7a, miR-18a, miR-29b, miR-194, miR-221) modulate THBS1 expression. For this study we used chemically synthesized pre-miRNAs (syn-pre-miRs), which we recently introduced as a new class of pre-miRNA mimics that are processed in cells into mature miRNAs (Guennewig et al., 2013). The downregulation of THBS1 by syn-pre-let-7a, syn-pre-18a, or a small interfering RNA (siRNA) specific to THBS1 mRNA induced mRNA levels of TGF- β 1 and SMAD4. Conversely, ectopic expression of latent TGF- β 1 reduced THBS1 protein expression by increasing the expression of let-7a, let-7b, and miR-18a. These data suggest an inverse correlation between THBS1 and latent TGF- β 1 expression, at least partially mediated by miRNAs in some cancer cells.

Materials and Methods

Cell culture and transfections

HeLa (ATCC, CCL-2) and LN-18 (ATCC, CRL-2610) cells purchased from LGC Standards were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Thrombospondin 1 siRNA (sc-36665) was purchased from Santa Cruz Biotechnology. *Renilla* siRNA (GAGCGAAGAGGGCGAGAAAUU) was from Dharmacon, and the negative control siRNA (siCON: Silencer[®] Negative Control No. 5 AM4640) was from Ambion. RNAs were transfected using Oligofectamine (No. 12252-011, Invitrogen) according to manufacturer's instructions.

Cloning of THBS1 3'UTR

THBS1 3'UTR was cloned into the dual luciferase plasmid psiCHECK2 (No. C8021, Promega) by digestion with *Not*I and *Xho*I restriction enzymes. The inserts were cloned into the multiple cloning sites after the *Renilla* gene, such that the reporter gene expression was regulated by the THBS1 3'UTR. Given that the selected miRNAs are predicted to bind in the first 2 kb of the 3'UTR (Fig. 1A), we divided the 3'UTR

FIG. 1. Screening for Thrombospondin 1 (THBS1) regulatory microRNAs (miRNAs). **(A)** Schematic representation of the conserved binding sites of miRNAs in the THBS1 3' untranslated region (UTR) predicted by TargetScan. The 3'UTR was divided into two parts of approximately equal length, which were introduced into the luciferase reporter plasmids. Thirteen miRNAs conserved in vertebrates were chosen for investigation. **(B)** Selected miRNAs suppressed the THBS1 3'UTR in dual-luciferase reporter assays. Transfections of negative control small interfering (si)RNA (siCON), chemically synthesized pre-miRNAs (syn-pre-miRs), and siRen into HeLa cells were performed at increasing concentrations (0, 2.25, 9, and 36 nM); 24 hours later, THBS1 3'UTR plasmids were transfected at 20 ng per well in 96-well plates. Luciferase activity was measured 72 hours after si/miRNA transfection. Each transfection was performed in triplicates and assays were repeated three times. *Renilla* luciferase values were normalized against firefly luciferase, and then to the value of the 0 nM transfection for each treatment. Error bars are standard deviation (SD) of three transfections. Asterisks highlight significant suppression of luciferase activity between the samples of highest concentration to the 0 nM dose. Significance was calculated using two tailed Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

into two fragments that were amplified by polymerase chain reaction (PCR) from HeLa genomic DNA, and the vectors were transformed into DH5 α -*Escherichia coli* cells. The list of primers used for cloning is given in Table 1. To confirm that the miRNAs directly interact with the 3'UTR of THBS1, we mutated predicted individual target sites in the 3'UTR of THBS1 in chemically synthesized 170-nt DNA fragments (Table 2). The wild-type and seed mutated target sites containing fragments were cloned into a psiCHECK2 vector as described above.

Pre-miRNA synthesis

We used miRNA sequences from miRBase (www.mirbase.org) and chemically synthesized pre-miRNA hairpins (syn-pre-miRs) containing a 2-nt overhang at the 3' end under standard conditions on a MerMade 12 synthesizer (Bioautomation Corporation) using UnySupport controlled-pore glass (CPG) (Glen Research) and phosphoramidites from Thermo Fisher Scientific (Table 3) (Guennewig et al., 2013). After completion of synthesis cycles, the CPG was treated with gaseous methylamine at 65°C and 1 bar for 2 hours. RNAs were eluted with ethanol/water (1:1). Desilylation was carried out on dry RNAs using freshly prepared 1-methyl-2-pyrrolidone, triethylamine (TEA), and TEA.3HF (6:3:4) for 90 minutes at 70°C. Isopropoxytrimethylsilane was added and samples were lyophilized. The crude RNA was purified by reversed phase high performance liquid chromatography (HPLC) (Agilent 1200 Series; Agilent Technologies). Dimethoxytrityl groups were cleaved using 40% aqueous acetic acid for 30 minutes at room temperature. RNAs were again purified by HPLC using a C18 column (XBridge OST, particle size 2.5 mm; Waters). Purified samples were analyzed on an Agilent 6130 Series Quadrupole LC/MS (Agilent Technologies) with electron spray ionization. Their identity was confirmed by HPLC (LC-MS) (Table 3).

TABLE 1. PRIMERS USED FOR CLONING LONG 3' UNTRANSLATED REGIONS OF THBS1

Primer name	Sequence (5' > 3')
thbs1-F	CATCAAATTGTTGATTGAAAGACTG
thbs1-R	CATGACATAAAGCCAGTAGAGAACA
thbs1-1 F	AAActcgagCATCAAATTGTTGATTG AAAGACTG
thbs1-1 R	GTCgggccgcTCCATGAATAATCCT TCAGC
thbs1-2 F	GTCctcgagAATTGCAAAGAAAGCCAT GAGGT
thbs1-2 R	TTTgcgccgcCATGACATAAAGCCAG
PsiCheck-F	CCTCCACTTCAGCCAGGA
PsiCheck-R	GCGAGGTCCGAAGACTCA

Fully complementary primers [Thrombospondin (thbs)1-F, thbs1-R] flanking the 3' untranslated region (UTR) of THBS1 were designed using primer3 (www.bioinformatics.nl/cgi-bin/primer3-plus/primer3plus.cgi) to amplify the sequences from HeLa genomic DNA. In addition, restriction sites *Xho*I to forward primer and *Not*I to reverse primer were added to allow incorporation of the extracted sequence into the multiple cloning sites of the *Renilla* luciferase of the psiCHECK2 plasmid.

F, forward; R, reverse.

Luciferase assays

HeLa (ATCC, No. CCL-2) cells were seeded in white 96-well plates and RNAs were transfected after 6 hours. After 24 hours, reporter plasmids (20 ng per well) were transfected using jetPEI (No. 101-10, Polyplus) according to the manufacturer's protocol. After 48 hours, supernatants were removed and firefly substrate (15 μ L; Dual-Glo[®] Luciferase Assay System, Promega), diluted with 15 μ L H₂O, was added. All transfections were performed in triplicates. Luminescence was measured on a microtiter plate reader (Mithras LB940, Berthold Technologies, Bad Wildbad, DE). After 30 minutes 15 μ L of *Renilla* substrate was added per well and the measurement was repeated. Values were normalized against firefly luciferase.

Western blot analysis

Cells were transiently transfected with syn-pre-miRs or siRNAs in four concentrations in triplicates and samples were harvested 48 hours post transfection. Aliquots from the supernatants of each triplicate were pooled and analyzed for THBS1. HeLa cells were transfected with 60 ng complementary DNA (cDNA) of TGF- β 1 (SC119746) from OriGene using jetPEI in 24-well plates. For intracellular proteins, cells were lysed with radioimmunoprecipitation assay buffer (R 0278; Sigma) and supernatants from the same transfection were used for THBS1 protein analysis. Protein concentrations were determined using a bicinchoninic acid assay (Thermo Fisher Scientific 23225): 10–20 μ g of protein was mixed with SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue). Samples were heated at 99°C for 5 minutes, separated on SDS gels, and transferred to polyvinylidene difluoride membranes. Non-specific membrane binding was blocked for 1 hour at room temperature with 5% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20. Membranes were incubated overnight at 4°C with primary antibodies in blocking buffer; anti-TGF- β 1 (No. 3711) from Cell Signaling and anti-THBS1 (sc-81755) from Santa Cruz Biotechnology. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1–2 hours at room temperature in blocking buffer. Signals generated by the chemiluminescent substrate [ECL (+); Amersham Biosciences] were captured by a cooled charge coupled device (CCD) camera (Bio-Rad). Protein bands were quantified by densitometry using the analysis software ImageJ (rsbweb.nih.gov/ij/). For THBS1, we occasionally observed the appearance of a double band. This might have occurred during the processing of samples for western blotting (i.e., degradation of protein during sample collection or during heating).

RNA and miRNA quantitative real-time PCR

Total RNA was extracted using mirVana[™] miRNA Isolation kit (AM1560; Ambion). For mRNA analysis, 1 μ g of total RNA was reverse transcribed using the M-MLV reverse transcriptase kit (28025-013, Invitrogen) according to the manufacturer's instructions. Expression levels were assayed using Power SYBR[®] Green PCR Master Mix (4367659; Applied Biosystems). Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) cycling

TABLE 2. CHEMICALLY SYNTHESIZED DNAs USED FOR TARGET SITE CLONING

Name	Sequence (5' > 3')
sc-miR-18a	GCCTAActcgagTCATCAAATTGTTGATTGAAAGACTGATCATAAACCAATGCTGGTATT GCACCTTCTGGAACATATGGGCTTGAGAAAACCCCCAGGATCACTTCTCCTTGGCTTCCTT CTTTTCTGTGCTTGCATCAGTGTGGACTCCTAGAACGTGCGAgcgccgcGCCTAA
sc-miR-18a mut	GCCTAActcgagTCATCAAATTGTTGATTGAAAGACTGATCATAAACCAATGCTGGTATT GAAACGTCTGGAACATATGGGCTTGAGAAAACCCCCAGGATCACTTCTCCTTGGCTTCCTT CTTTTCTGTGCTTGCATCAGTGTGGACTCCTAGAACGTGCGAgcgccgcGCCTAA
sc-miR-1	CCTCCACTTCAGCCAGGActcgagATTGAGCCTCCAATGAATAAGACATCTTCCAAGCAT ATAAACAAATTGCTTTGGTTTCTTTTGAAAAAGCATCTACTTGCTTCAGTTGGGAAGGTG CCCATTCCACTCTGCCTTTGTACAgcgccgcGAGTCTTCGGACCTCGC
sc-miR-1 mut	CCTCCACTTCAGCCAGGActcgagATTGAGCCTCCAATGAATAAGACATCTTCCAAGCAT ATAAACAAATTGCTTTGGTTTCTTTTGAAAAAGCATCTACTTGCTTCAGTTGGGAAGGTG CCCCTGCAACTCTGCCTTTGTACAgcgccgcGAGTCTTCGGACCTCGC
sc-miR-29b	CCTCCACTTCAGCCAGGActcgagGCCTTTGTACAGAGCAGGGTGCTATTGTGAGGCCA TCTCTGAGCAGTGGACTCAAAAGCATTTTCAGGCATGTCAGAGAAGGGAGGACTCACTAG AATTAGCAAACAAAACCCCTGACgcgccgcGAGTCTTCGGACCTCGC
sc-miR-29b mut	CCTCCACTTCAGCCAGGActcgagGCCTTTGTACAGAGCAGGGTTCTGATTGTGAGGCCA TCTCTGAGCAGTGGACTCAAAAGCATTTTCAGGCATGTCAGAGAAGGGAGGACTCACTAG AATTAGCAAACAAAACCCCTGACgcgccgcGAGTCTTCGGACCTCGC
sc-miR-194	CCTCCACTTCAGCCAGGActcgagAAGCACTAAGGGGAGGGCGCATACCCGAGACGATTG TATGAAGAAAATATGGAGGAACTGTTACATGTTTCGGTACTAAGTCATTTTCAGGGGATTG AAAGACTATTGCTGGATTTCATGATgcgccgcGAGTCTTCGGACCTCGC
sc-miR-194 mut	CCTCCACTTCAGCCAGGActcgagAAGCACTAAGGGGAGGGCGCATACCCGAGACGATTG TATGAAGAAAATATGGAGGAACTGTTTCGGTACTAAGTCATTTTCAGGGGATTG AAAGACTATTGCTGGATTTCATGATgcgccgcGAGTCTTCGGACCTCGC
sc-let-7a	CCTCCACTTCAGCCAGGActcgagTCTTTCATTTTCCAAAAGAGAAAAAATGACAAAA GGTGAAGAACTTACATACAAATATTACCTCATTTGTTGTGTGACTGAGTAAAGAATTTTGG ATCAAGCGGAAAGAGTTTAAGTGTCgcgccgcGAGTCTTCGGACCTCGC
sc-let-7a mut	CCTCCACTTCAGCCAGGActcgagTCTTTCATTTTCCAAAAGAGAAAAAATGACAAAA GGTGAAGAACTTACATACAAATATTCCATAATTTGTTGTGTGACTGAGTAAAGAATTTTGG ATCAAGCGGAAAGAGTTTAAGTGTCgcgccgcGAGTCTTCGGACCTCGC
sc-miR-101/200a	CCTCCACTTCAGCCAGGActcgagGAGTAAAGAATTTTGGATCAAGCGGAAAGAGTTTA AGTGTCTAACAACTTAAAGCTACTGTAGTACCTAAAAAGTCAGTGTGTACATAGCATA AAAACCTCTGCAGAGAAGTATTTCCAgcgccgcGAGTCTTCGGACCTCGC
sc-miR-101/200a mut	CCTCCACTTCAGCCAGGActcgagGAGTAAAGAATTTTGGATCAAGCGGAAAGAGTTTA AGTGTCTAACAACTTAAAGCTCCGGGAGTACCTAAAAAGTCCGGGGTGTACATAGCATA AAAACCTCTGCAGAGAAGTATTTCCAgcgccgcGAGTCTTCGGACCTCGC
sc-miR-221	CCTCCACTTCAGCCAGGActcgagGTGCTGGCTGCCATTGCCTGGTCACATTGAAATTGG TGGCTTCATTCTAGATGTAGCTTGTGCAGATGTAGCAGGAAAATAGGAAAACCTACCATC TCAGTGAGCACCAGCTGCCTCCCAAgcgccgcGAGTCTTCGGACCTCGC
sc-miR-221 mut	CCTCCACTTCAGCCAGGActcgagGTGCTGGCTGCCATTGCCTGGTCACATTGAAATTGG TGGCTTCATTCTAGAGGGATCTTGTGCAGAGGGATCAGGAAAATAGGAAAACCTACCATC TCAGTGAGCACCAGCTGCCTCCCAAgcgccgcGAGTCTTCGGACCTCGC

Synthetic short constructs (sc) containing *Xho*I and *Nor*I cloning sites shown in lowercase letters.
miR, microRNA.

conditions were 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C; 1 minute at 60°C). Values were normalized using glyceraldehyde 3-phosphate dehydrogenase or average of all measured mRNAs. RT-qPCR primers sequences are shown in Table 4. For miRNA analyses, the TaqMan[®] Human MicroRNA Assay set v1.0 (4383443; Applied Biosystems) was used. Total RNA was reverse transcribed with miRNA-specific RT primers and amplified with TaqMan miRNA-specific primers. Complementary DNA was synthesized from 10–20 ng of total RNA in a 5 µL volume using TaqMan MicroRNA Reverse Transcription kit (4366597; Applied Biosystems), according to the manufacturer's instructions. All samples were measured in triplicates; the threshold cycle values were transformed to relative quantities. Levels of small nucleolar RNAs (snoRNAs) were not

consistent in our control and treated samples, therefore values were normalized to the averages of all measured miRNAs (Wotschovsky et al., 2011).

Results

Screen of miRNAs targeting the *THBS1* 3'UTR

The *THBS1* gene has a long 3'UTR with conserved predicted binding sites for multiple miRNAs. Among them miR-18a and miR-19 negatively regulate *THBS1* levels during MYC-induced angiogenesis (Dews et al., 2006; Dews et al., 2010). A recent report from the same group showed that several miRNAs can regulate *THBS1* in colon cancer but only p53-regulated miR-194 was a major modulator of *THBS1* at the post-transcriptional level (Sundaram et al.,

TABLE 3. CHEMICALLY SYNTHESIZED miRNA HAIRPIN SEQUENCES

<i>Name</i>	<i>Sequence</i>	<i>Length (nt)</i>	<i>Mass calculated</i>	<i>Mass found</i>
let-7a	UGAGGUAGUAGGUUGUAUAGUUUUAGGGUCACACCCACC ACUGGGAGAUAAACUAUACAAUCUACUGUCUUUCC	73	23,336	23,323
mir-1	ACAUACUUCUUUAUAUGCCCAUAUGGACCUGCUAA GCUAUGGAAUGUAAAGAAGUAU	57	18,208	18,208
mir-18a	UAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGAUUAGC AUCUACUGCCCUAAGUGCUCCUUCUG	63	20,168	20,166
mir-19a	AGUUUUGCAUAGUUGCACUACAAGAAGAAUGUAGUUGU GCAAUUCUAUGCAAAACUGA	58	18,640	18,639
mir-29b	GCUGGUUUCUAUUGGUGGUUUAGAUUUAAAUAUGUGA UUGUCUAGCACCAUUUGAAAUCAGUGU	63	20,174	20,172
mir-101	CAGUUAUCACAGUGCUGAUGCUGUCUAUUCUAAAGGUA CAGUACUGUGAUAAACUGAA	57	18,240	18,237
mir-106a	AAAAGUGCUUACAGUGCAGGUAGCUUUUUGAGAUCUA CUGCAAUGUAAGCACUUCUUC	59	18,851	18,893
miR-139	UCUACAGUGCAGUGUCUCCAGUGGUCGAGGCU GGAGACGCGGCCUGUUGGAGU	59	19,030	19,029
mir-194	UGUAAACAGCAACUCCAUGUGGACUGUGUACCAAUUU CCAGUGGAGAUGCUGUUACUUU	58	18,514	18,513
mir-221	ACCUGGCAUACAAUGUAGAUUUCUGUGUUCGUUAGGC AACAGCUACAUUGUCUGCUGGGUUUC	63	20,099	20,099
mir-182	UUUGGCAAUGGUAGAACUCACACUGGUGAGGUAACAGG AUCCGGUGGUUCUAGACUUGCCAACUA	65	20,921	20,920
mir-181b	AACAUUCAUUGCUGUCGGUGGGUUGAACUGUGUGGAC AAGCUCACUGAACA AUGAAUGCAAC	62	19,948	19,950
mir-200a	CAUCUUACCGGACAGUGCUGGAUUUCCCAGCUUGACU CUAACACUGUCUGGUAACGAUGUU	61	19,406	19,407

2011). We have previously shown that THBS1 was regulated by miR-18a during growth inhibitory TGF- β 1 signaling (Dogar et al., 2011). In the present study we cloned the THBS1 3'UTR in two pieces into psiCHECK2 dual luciferase vectors (Fig. 1A). The TargetScan prediction tool (Lewis et al., 2005) was used to identify candidate miRNAs. It showed twenty-six conserved and poorly conserved potential binding sites for miRNAs (Table 5). We selected eight out of

the nine miRNAs with at least one conserved predicted target site. Mir-144 was not included because it shares its seed with that of miR-101. We also randomly selected five miRNAs with poorly conserved target sites (Fig. 1A). We obtained the miRNA sequences from miRBase and chemically synthesized them as full-length pre-miRNAs containing a 2-nt overhang at the 3' end (Table 3). All of the syn-pre-miRs were successfully synthesized in yields sufficient for

TABLE 4. REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION PRIMERS

<i>Ref_Seq.</i>	<i>Name</i>	<i>Primer sequence</i>
NM_000660.3	TGFB1_F	GCAGGGATAACACACTGCAA
	TGFB1_R	GGCCATGAGAAGCAGGAA
NM_006132	BMP1_F	GACGGGTCCATTAACAAAGC
	BMP1_R	TGTAGCTGCCAGGGTGT
NM_003246	THBS1_F	AGCATGGTCCTGGAACCTCAG
	THBS1_R	CAGCTCATTTGGCCAACTCTT
NM_138473.2	SP1_F	TATAGCAAATGCCCCAGGTG
	SP1_R	GGGCTGTTTTCTCCTTCCTC
NM_000314	PTEN_F	TTGAAGGCGTATACAGGAACAA
	PTEN_R	GCAATTAAATTTGGCGGTGT
NM_003998	NFKB1_F	TGGAGTCTGGGAAGGATTTG
	NFKB1_R	CGAAGCTGGACAAACACAGA
NM_002467	MYC_F	CTGGTGTCTCCATGAGGAGA
	MYC_R	CTCTGACCTTTTGCCAGGAG
NM_005359.4	SMAD4_F	GATACGTGGACCCTTCTGGA
	SMAD4_R	ACCTTTGCTATGTGCAACC
NM_002046.3	GAPDH_F	GAAGGTGAAGGTCGGAGTCA
	GAPDH_R	AATGAAGGGGTCATTGATGG

Ref_Seq, the reference sequence of National Center for Biotechnology Information (NCBI) data base collection.

TABLE 5. TARGETSCAN-PREDICTED MICRORNAS
TARGETING THBS1 3'UTR BROADLY
CONSERVED AMONG VERTEBRATES

<i>miRNA</i>	<i>Conserved sites</i>	<i>Poorly conserved sites</i>
let-7/98	1	0
miR-101	1	0
miR-139-5p	1	1
miR-144	1	1
miR-18ab	1	0
miR-194	1	0
miR-19ab	1	0
miR-1ab	1	1
miR-221/222	1	1
miR-132	0	1
miR-135ab	0	1
miR-138	0	1
miR-141/200a	0	1
miR-143	0	1
miR-181	0	1
miR-182	0	1
miR-204	0	1
miR-205	0	1
miR-21	0	1
miR-217	0	1
miR-218	0	1
miR-29abcd	0	1
miR-338	0	2
miR-383	0	1
miR-7/7ab	0	1
miR-93/106a	0	1

transfections. Their identities were confirmed by HPLC-MS. We have recently demonstrated with several examples how they are processed into functionally active mature miRNAs in cells (Guennewig et al., 2013). The syn-pre-miRNAs showed a high level of selectivity when tested against several matched and mismatched luciferase reporters and their potency was similar to that of commercially available mimics. We transiently transfected HeLa cells with the syn-pre-miRs, and 24 hours post transfection, the reporter plasmids were added to the cells. Luciferase activity on its specific substrate was measured 72 hours after miRNA transfection. We used a small interfering RNA (siRNA) against the mRNA of the *Renilla* protein (siRen) as a positive control. The negative control (siCON) is a commercially available nontargeting double-stranded RNA from Ambion. SiCON was used to confirm that the effects from the transfected siRNAs and miRNAs were sequence-specific. From the thirteen selected syn-pre-miRs, five (syn-pre-let-7a, syn-pre-1, syn-pre-18a, syn-pre-194, syn-pre-221) significantly decreased the luciferase activity of the 3'UTR reporters in a concentration-dependent manner (Fig. 1B). Whereas syn-pre-19a, syn-pre-106a, syn-pre-139-5p, syn-pre-181b, syn-pre-182, and syn-pre-200a showed no effects, two syn-pre-miRs (syn-pre-101 and syn-pre-29b) gave inconclusive results (Fig. 1B).

Target site mutagenesis and analysis of THBS1 protein

The syn-pre-miRs that showed significant suppression of luciferase activity on the reporter constructs were further

investigated. We also included syn-pre-101 and syn-pre-29b, which had given inconclusive results in the first screen. In order to verify the predicted binding sites as direct sites of interaction between the miRNAs and the 3'UTR, we introduced mutations at specific positions within the seed sequence of the THBS1 3'UTR. We chemically synthesized the target sites as 170-nt-long DNA fragments containing natural up- and down-stream flanking sequences (Table 2). For each miRNA target site, two versions were synthesized: a wild-type and a mutated form. Three bases complementary to the seed region of the miRNA at nonterminal positions were mutated by applying the base permutation rules ($A > C$, $C > A$, $U > G$, or $G > U$). These short constructs were cloned downstream of the *Renilla* luciferase gene in the psiCHECK2 vector. Assays were performed as described above. The data showed that syn-pre-miRs (let-7a, miR-18a, miR-29b, miR-101, miR-194, and miR-221) were able to suppress wild-type THBS1 3'UTR fragments. Only syn-pre-1 did not show any activity on its single site-containing reporter construct (Fig. 2A). The effect from miR-18a was rather weak but reproducible. By mutating the seed sites in each of these shorter constructs, we were able to abolish the inhibitory activity of the respective miRNAs except for miR-29b, which still showed residual activity against the mutated reporter at the highest treatment concentration (Fig. 2A). Overall, these reporter assays showed that most of the short constructs designed for selected miRNAs performed similarly to the longer reporter gene. The results suggested that most of the selected miRNAs use the predicted conserved target sites for direct interaction and that they have the potential to suppress THBS1 expression *in vivo*. Next we examined whether the aforementioned miRNAs were able to regulate endogenous THBS1 protein expression in cells. To address this question we used the human malignant glioma cell line LN-18. We have previously shown that LN-18 cells express levels of THBS1, which can be detected without media concentration (Dogar et al., 2011). We transiently transfected these cells with the syn-pre-miRs of let-7a, miR-1, miR-18a, miR-29b, miR-101, miR-194, miR-221, and an siRNA-targeting endogenous THBS1 (siTHBS1). Cells were transfected with four concentrations in triplicates and samples were collected 48 hours post transfection, supernatants of each triplicate were pooled and analyzed by western blotting. Figure 2B shows the western blots of all tested syn-pre-miRs. A concentration-dependent suppression of THBS1 protein in LN-18 cells was observed after 48 hours by transfection of syn-pre-194, syn-pre-let-7a, syn-pre-221, or syn-pre-18a. Syn-pre-29b reduced THBS1 protein levels moderately, however syn-pre-1 and syn-pre-101 showed no effects. Similar trends were also found in HeLa cells (data not shown). These data demonstrate that at least five of the thirteen conserved sites predicted by TargetScan were *bona fide* miRNA binding sites both in 3'UTR reporters and in the endogenous transcript of THBS1.

Induction of TGF- β 1 and SMAD4 by THBS1 regulatory miRNAs

To determine whether the aforementioned miRNAs also suppress THBS1 mRNA levels, we performed RT-qPCR after transfection with syn-pre-miRs. We selected the four miRNAs, which showed the strongest effects on the protein levels,

The data showed that the mRNAs of TGF- β 1 and SMAD4 were strongly induced by syn-pre-let-7a, syn-pre-18a, and siTHBS1. Such strong effects from these miRNAs were not observed on levels of BMP1, SP1, PTEN, NFKB1, or MYC, although the unrelated control siCON showed some effects that were mostly concentration-independent (Fig. 3F). Syn-pre-194 did not increase TGF- β 1 but induced SMAD4 mRNA, whereas syn-pre-221 did not show any effects on TGF- β 1 and SMAD4 transcripts. These data imply that some of the THBS1 targeting miRNAs may also play a role in regulating TGF- β 1 signaling components.

FIG. 3. THBS1, TGF- β 1, and SMAD4 mRNA analysis. (A–F) TGF- β 1, BMP1, THBS1, SP1, PTEN, MYC, and SMAD4 mRNA levels after transfection of HeLa cells with syn-pre-18a, syn-pre-194, syn-pre-let-7a, syn-pre-221, siTHBS1, or siCON at increasing concentrations (0, 5, 15, and 45 nM). Relative expression of mRNA is displayed [means of real-time quantitative reverse transcription-polymerase chain reactions (RT-qPCR) triplicates; single RNA samples $\pm$ SD]. Asterisks highlight significantly down or upregulated mRNAs from the technical triplicates of single RNA samples of 0 and 45 nM transfections. Significance was calculated using two tailed Student's *t*-test (* p < 0.05, ** p < 0.01, *** p < 0.0001).

Inverse functional correlation between THBS1 and latent TGF- β 1

THBS1 is in some cell types required for the activation of latent TGF- β 1 (Sweetwyne and Murphy-Ullrich, 2012). In our previous study, the suppression of latent TGF- β 1 by RNAi resulted in a strong induction of THBS1 protein that was accompanied by a modest reduction of miR-18a (Dogar et al., 2011). Furthermore, the levels of latent TGF- β 1 were rescued through simultaneous transfection of miR-18a in cells undergoing TGF- β 1 RNAi. An important unresolved question in that study was whether the overexpression of TGF- β 1 has effects on THBS1 and miR-18a, miR-24 or on other miRNAs. Latent TGF- β 1 was overexpressed in HeLa cells by transfecting TGF- β 1 cDNA. Western blot analysis confirmed a strong overexpression of intracellular latent TGF- β 1 (Fig. 4A). Secreted THBS1 expression levels were modestly (35%) reduced in the supernatants of the same transfection (Fig. 4A), consistent with the activity of miRNAs. Hence we examined the effects of latent TGF- β 1 overexpression on miRNAs using stem-loop RT-qPCR. HeLa cells express abundantly most of the aforementioned miRNAs, with the exception of miR-194 whose expression is low. Most of the miRNAs tested were not significantly affected by TGF- β 1 overexpression. However, there were small increases in the levels of let-7a, let-7b, and miR-18a (Fig. 4B). No effects were observed on the levels of miR-221, whereas the levels of miR-19a, miR-20a, and miR-93 were slightly downregulated upon

latent TGF- β 1 overexpression. Taken together, overexpression of latent TGF- β 1 may not only suppress THBS1 through activation of miR-18a, but also through let-7. This suggests that an inverse correlation between latent TGF- β 1 and THBS1 likely involves multiple miRNAs.

Discussion

The loss of THBS1 expression is a common event in several cancer types and also in noncancerous pathologies (Henkin and Volpert, 2011). The expression of THBS1 is reported to be tightly controlled at both transcriptional and post-transcriptional levels. As THBS1 is an important inhibitor of angiogenesis knowledge of its regulation may contribute to a fuller understanding of selected cancers as well as some angiogenesis-driven diseases. It is clear today that miRNAs play pivotal roles in fine-tuning and in some cases even switching on and off critical cellular pathways (Flynt and Lai, 2008). A single miRNA may target multiple transcripts or one transcript may be targeted by multiple miRNAs indicating that cellular context of a particular miRNA might determine its function in specific cell types.

It seems possible that levels of THBS1 transcription are maintained but protein output is determined at the post-transcriptional level through RNA binding proteins and miRNAs. In the present study, a systematic investigation of the 3'UTR of THBS1 provided evidence that a handful of miRNAs plays a role in its regulation. Several miRNAs have

FIG. 4. Inverse correlation between THBS1 and TGF-β1 through miRNAs. TGF-β1 overexpression by cDNA reduced THBS1 and upregulated let-7a, let-7b, and miR-18a. **(A)** HeLa cells were transfected with TGF-β1 complementary DNA (0 and 60 ng) in 24-well plates. Forty-eight hours later, the supernatants were used for analysis of THBS1, and the lysates for analysis of latent TGF-β1 protein by western blotting. **(B)** Total RNA was isolated 48 hours post transfection and subjected to miRNA stem-loop RT-qPCR. Data were normalized to total average of expressed miRNAs. Relative miRNA expression levels are displayed (means of RT-qPCR triplicates; single RNA samples ± SD). Asterisks highlight significantly upregulated miRNAs from the technical triplicates of single RNA samples of 0 and 60 ng transfections. Significance was calculated using two tailed Student's *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.0001).

highly conserved binding sites in the 3'UTR of THBS1. As a source of miRNAs we used chemically synthesized pre-miRNAs. These are RNA hairpins with terminal hydroxyl groups, which are of identical sequence to pre-miRNAs produced endogenously. We have demonstrated with many examples how they fully recapitulate the properties of pre-miRNAs, including their processing into mature miRNAs. These miRNAs suppressed the luciferase reporter when fused to the 3'UTR of THBS1 (Fig. 1A), as well as to reporter genes for the individual target sites (Fig. 2A). When those predicted binding sites were mutated, the repression of those reporters was lost (Fig. 2A). Further, they reduced THBS1 protein levels in cells (Fig. 2B). Let-7a, miR-18a, and miR-221 are well known angiogenesis regulatory miRNAs and are also regulated by various angiogenesis stimuli (Caporali and Emanueli, 2011). Recently miR-194 was shown to be intrinsically angiogenic in murine and human colon cancer cell lines (Sundaram et al., 2011). Our data suggests that these miRNAs might modulate angiogenesis by directly targeting the THBS1 3'UTR.

A second important function of THBS1 is the activation of latent TGF-β1 (Schultz-Cherry et al., 1994). In cells TGF-β1 is produced as a pro-protein consisting of latency-associated peptide (LAP) and mature TGF-β1 (Annes et al., 2003). After

nicking by furin-like proteases, it is secreted and becomes part of the extra-cellular matrix. Upon certain stimuli TGF-β1 is activated by THBS1 and other proteins depending upon the cellular context. In a previous study we showed that in some cancer cells (cervical carcinoma and glioblastoma), suppression of intracellular latent TGF-β1 by RNAi enhanced the activation of latent TGF-β1 present in the extra-cellular matrix through post-transcriptional upregulation of THBS1 and concomitant downregulation of miR-18a (Dogar et al., 2011). Transient overexpression of miR-18a enhanced latent TGF-β1 mRNA levels, suggesting that reduced expression levels of THBS1 help maintain a high level of latent TGF-β1, partially through increased transcription. In this study we examined whether suppression of THBS1 by miRNAs or an siRNA has any effect on TGF-β1 mRNA abundance. Indeed miR-18a (Dogar et al., 2011) and let-7a enhanced TGF-β1 mRNA two- to six-fold, respectively. We confirmed this observation by using a THBS1-specific siRNA, which also increased TGF-β1 mRNA up to five-fold. It is interesting to note that miR-18a, let-7a, miR-194, and siTHBS1 also increase the mRNA levels of SMAD4. It has been shown in human pancreatic carcinoma cells, where *SMAD4* is frequently deleted, that its restoration increased levels of the THBS1 causing the cells to switch from potently

angiogenic to anti-angiogenic *in vitro* and *in vivo* despite the fact that the THBS1 promoter is devoid of known SMAD-binding elements (Schwarte-Waldhoff et al., 2000). At the time of this study, post-transcriptional regulation of mRNAs by miRNAs was not well understood and the authors suggested that indirect mechanisms were responsible for the phenomenon. Our study points to a regulatory loop involving THBS1, latent TGF- β 1, SMAD4, and miRNAs. Consistent with such a mechanism, overexpression of latent TGF- β 1 by a cDNA in HeLa cells reduced THBS1 expression and was accompanied by a modest increase in the levels of miR-18a and let-7. Altogether these data suggest that some cancer cells might sustain their transformed state by using miRNAs to suppress THBS1 and increase latent TGF- β 1 levels.

Acknowledgments

We thank M. Zimmermann for help with RNA synthesis. We are grateful to H. Towbin for helpful suggestions. This work was supported in part by a grant from the Swiss National Science Foundation to JH (205321_124720/1).

Author Disclosure Statement

No competing financial interests exist.

References

- ADAMS, J.C., and LAWLER, J. (2011). The thrombospondins. *Cold Spring Harb. Perspect. Biol.* **3**, a009712.
- AGAH, A., KYRIAKIDES, T.R., LAWLER, J., and BORNSTEIN, P. (2002). The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. *Am. J. Pathol.* **161**, 831–839.
- ANNES, J.P., MUNGER, J.S., and RIFKIN, D.B. (2003). Making sense of latent TGF β activation. *J. Cell Sci.* **116**, 217–224.
- BARTEL, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233.
- BORNSTEIN, P. (2009). Thrombospondins function as regulators of angiogenesis. *J. Cell Commun. Signal.* **3**, 189–200.
- CAPORALLI, A., and EMANUELI, C. (2011). MicroRNA regulation in angiogenesis. *Vascul. Pharmacol.* **55**, 79–86.
- CHEN, J.K., ZHAN, Y.J., YANG, C.S., and TZENG, S.F. (2011). Oxidative stress-induced attenuation of thrombospondin-1 expression in primary rat astrocytes. *J. Cell Biochem.* **112**, 59–70.
- DEWS, M., FOX, J.L., HULTINE, S., SUNDARAM, P., WANG, W., LIU, Y.Y., FURTH, E., ENDERS, G.H., ELDEIRY, W., SCHELTER, J.M., CLEARY, M.A., and THOMAS-TIKHONENKO, A. (2010). The myc-miR-17~92 axis blunts TGF β signaling and production of multiple TGF β -dependent antiangiogenic factors. *Cancer Res.* **70**, 8233–8246.
- DEWS, M., HOMAYOUNI, A., YU, D., MURPHY, D., SEVIGNANI, C., WENTZEL, E., FURTH, E.E., LEE, W.M., ENDERS, G.H., MENDELL, J.T., and THOMAS-TIKHONENKO, A. (2006). Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat. Genet.* **38**, 1060–1065.
- DOGAR, A.M., TOWBIN, H., and HALL, J. (2011). Suppression of latent transforming growth factor (TGF)- β 1 restores growth inhibitory TGF- β signaling through microRNAs. *J. Biol. Chem.* **286**, 16447–16458.
- FLYNT, A.S., and LAI, E.C. (2008). Biological principles of microRNA-mediated regulation: shared themes amid diversity. *Nat. Rev. Genet.* **9**, 831–842.
- GARCIA-DIAZ, D.F., ARELLANO, A.V., MILAGRO, F.I., MORENO-ALIAGA, M.J., PORTILLO, M.P., MARTINEZ, J.A., and CAMPION, J. (2011). Glucose and insulin modify thrombospondin 1 expression and secretion in primary adipocytes from diet-induced obese rats. *J. Physiol. Biochem.* **67**, 453–461.
- GOOD, D.J., POLVERINI, P.J., F. RASTINEJAD, M. LE BEAU, M., LEMONS, R.S., FRAZIER, W.A., and BOUCK, N.P. (1990). A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6624–6628.
- GUENNEWIG, B., ROOS, M., DOGAR, A.M., GEBERT, L.F., ZAGALAK, J.A., VONGRAD, V., METZNER, K.J., and HALL, J. (2013). Synthetic pre-microRNAs reveal dual-strand activity of miR-34a on TNF- α . *RNA* **20**: 61–75.
- HENKIN, J., and VOLPERT, O.V. (2011). Therapies using anti-angiogenic peptide mimetics of thrombospondin-1. *Expert Opin. Ther. Targets* **15**, 1369–1386.
- JIMENEZ, B., VOLPERT, O.V., CRAWFORD, S.E., FEBBRAIO, M., SILVERSTEIN, R.L., and BOUCK, N. (2000). Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat. Med.* **6**, 41–48.
- LEWIS, B.P., BURGE, C.B., and BARTEL, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15–20.
- MCGRAY, A.J., GINGERICH, T., PETRIK, J.J., and LAMARRE, J. (2011). Regulation of thrombospondin-1 expression through AU-rich elements in the 3'UTR of the mRNA. *Cell Mol. Biol. Lett.* **16**, 55–68.
- MURPHY-ULLRICH, J.E., and MOSHER, D.F. (1985). Localization of thrombospondin in clots formed *in situ*. *Blood* **66**, 1098–1104.
- OUE, N., MATSUMURA, S., NAKAYAMA, H., KITADAI, Y., TANIYAMA, K., MATSUSAKI, K., and YASUI, W. (2003). Reduced expression of the TSP1 gene and its association with promoter hypermethylation in gastric carcinoma. *Oncology* **64**, 423–429.
- SCHULTZ-CHERRY, S., RIBEIRO, S., GENTRY, L., and MURPHY-ULLRICH, J.E. (1994). Thrombospondin binds and activates the small and large forms of latent transforming growth factor- β in a chemically defined system. *J. Biol. Chem.* **269**, 26775–26782.
- SCHWARTE-WALDHOFF, I., VOLPERT, O.V., BOUCK, N.P., SIPOS, B., HAHN, S.A., KLEIN-SCORY, S., LUTTGES, J., KLOPPPEL, G., GRAEVEN, U., EILERT-MICUS, C., HINTELMANN, A., and SCHMIEGEL, W. (2000). Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9624–9629.
- SUNDARAM, P., HULTINE, S., SMITH, L.M., DEWS, M., FOX, J.L., BIYASHEV, D., SCHELTER, J.M., HUANG, Q., CLEARY, M.A., VOLPERT, O.V., and THOMAS-TIKHONENKO, A. (2011). p53-responsive miR-194 inhibits thrombospondin-1 and promotes angiogenesis in colon cancers. *Cancer Res.* **71**, 7490–7501.

- SWEETWYNE, M.T., and MURPHY-ULLRICH, J.E. (2012). Thrombospondin1 in tissue repair and fibrosis: TGF-beta-dependent and independent mechanisms. *Matrix Biol.* **31**, 178–186.
- VAN ALMEN, G.C., VERHESEN, W., VAN LEEUWEN, R.E., VAN DE VRIE, M., EURLINGS, C., SCHELLINGS, M.W., SWINNEN, M., CLEUTJENS, J.P., VAN ZANDVOORT, M.A., HEYMANS, S., and SCHROEN, B. (2011). MicroRNA-18 and microRNA-19 regulate CTGF and TSP-1 expression in age-related heart failure. *Aging Cell* **10**, 769–779.
- WOTSCHOFSKY, Z., MEYER, H.A., JUNG, M., FENDLER, A., WAGNER, I., STEPHAN, C., BUSCH, J., ERBERSDOBLER, A., DISCH, A.C., MOLLENKOPF, H.J., and JUNG, K. (2011). Reference genes for the relative quantification of microRNAs in renal cell carcinomas and their metastases. *Anal. Biochem.* **417**, 233–241.

Address correspondence to:

Jonathan Hall, PhD

Swiss Federal Institute of Technology Zurich

Institute for Pharmaceutical Sciences HCI H437

Vladimir-Prelog-Weg 4

8093 Zurich

Switzerland

E-mail: jonathan.hall@pharma.ethz.ch

Received for publication October 13, 2013; accepted after revision December 18, 2013.