EXPERT OPINION

Effects of anti-miR-182 on TSP-1 expression in human colon cancer cells: there is a sense in antisense?

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Objective: miRNAs are attractive molecules for cancer treatment, including colon rectal cancer (CRC). We investigate on the molecular mechanism by which miR-182 could regulate thrombospondin-1 (TSP-1) expression, a protein downregulated in CRC and inversely correlated with tumor vascularity and metastasis.

Background: MicroRNAs are small non-coding RNAs that regulate the expression of different genes, involved in cancer progression, angiogenesis and metastasis. miR-182, over-expressed in colorectal cancer (CRC), has like predictive target thrombospondin-1 (TSP-1), a protein inversely correlated with tumor vascularity and metastasis that results downregulated in different types of cancer including CRC.

Results: We found that TSP-1 increased after transfection with anti-miR-182 and we showed that miR-182 targets TSP-1 3'UTR-mRNA in both cells. Moreover, we observed that anti-miR-182 did not induce significant variation of Egr-1 expression, but affected the nuclear translocation and its binding on *tsp-1* promoter in HCT-116. Equally, Sp-1 was slightly increased as total protein, rather we found a nuclear accumulation and its loading on the TSP-1 promoter in HT-29 transfected with anti-miR-182.

Conclusion: Our data suggest that miR-182 targets the anti-angiogenic factor TSP-1 and that anti-miR-182 determines an upregulation of TSP-1 expression in colon cancer cells. Moreover, anti-miR-182 exerts a transcriptional regulatory mechanism of *tsp-1* modulating Egr-1 and Sp-1 function. Anti-miR-182 could be used to restore TSP-1 expression in order to contrast angiogenic and invasive events in CRC.

Keywords: anti-miR-182, colon cancer, Egr-1, Sp-1, thrombospondin-1

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1. Introduction

Colorectal cancer (CRC) is the second most frequent malignant disease [1] and the second most common cause of cancer-related mortality in Europe [2]; 20 – 25% of patients have already metastasis after diagnosis [3]. Newest chemotherapeutics agents, the so-called 'targeted' or 'biological' therapies, have improved survival in patients with metastatic CRC, but having a relatively small effect on overall survival [4-6]. For this reason, new therapeutic approaches are necessary to control angiogenic spread. Angiogenesis is an important determinant of tumor progression. Dysregulated expression of pro-angiogenic factors and angiogenic inhibitors negatively influences the balance between capillary formation and regression, resulting in new vessels formation from existing blood vessels and subsequent remodeling.

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MicroRNAs (miRNAs) are small non-coding RNAs of ~ 20 – 22 nucleotides in length that negatively regulate gene expression in a variety of eukaryotic organisms [7]. miRNAs are known to regulate different cellular processes such as proliferation, differentiation, apoptosis, cell metabolism [8,9]. Recently, several miRNAs were found to regulate angiogenic processes [10] including pro-angiogenic, as VEGF [11,12], and anti-angiogenic factors [13], as TSP-1, the first described endogenous angiogenesis inhibitor. TSP-1 is a member of calcium-binding extracellular proteins family [14]; it is a platelet- and cell-derived homotrimeric glycoprotein, secreted in a wide range of tissues where it is bound to the extracellular matrix [15]. Numerous *in vitro* and *in vivo* experiments have been carried out in order to identify multiple mechanisms by which TSP-1 inhibits angiogenesis. Three copies of the thrombospondin type 1 repeat (TSR) [16] are essential to inhibit tumor angiogenesis and growth.

An important mechanism through which TSP-1 regulates angiogenesis is through interactions with the receptor CD47, universally expressed by vascular and circulating blood cells of land-dwelling vertebrates. High-affinity binding of TSP-1 to CD47 occurs at two peptide motifs on the carboxy-terminal domain [17]. TSP-1 at picomolar concentrations activates CD47, inhibiting the pro-angiogenic nitric oxide (NO)-stimulated activation of soluble guanylyl cyclase (sGC) in endothelial cells, vascular smooth muscle cell (VSMC), platelets and T cells [18-21]. This activity is lost in CD47 null but not in CD36 null cells and is mimicked by certain ligands of CD47 [22]. Ligation of CD36 also inhibits sGC activation, but only at higher concentrations of TSP-1 and only in cells that express CD47. TSP-1 also enhanced VSMC proliferation by ligation of CD47 [23,24].

Pre-existing levels of TSP-1 in the arterial wall or blood constantly limit NO-mediated vasodilation and blood flow via CD47 [25]. In fact, healthy TSP-1 null mice showed a greater increase in skeletal muscle blood flow in response to NO within 5 min [26]. The direct inhibition of VSMC NO signaling and indirect inhibition via limiting endothelial NO synthesis are CD47-dependent targets that can control vascular tone. Additional TSP-1 receptors are present on vascular cells, some of which can oppose these CD47-dependent signals if TSP-1 levels are sufficiently elevated [27].

VEGFA is an important driver of NO signaling via stimulation of endothelial nitric oxide synthase (eNOS) [28] and TSP-1 inhibits VEGF signaling via several mechanisms. TSP-1 binds to VEGF and prevents VEGFR2 signaling either by competing with extracellular VEGF for binding to cell surface proteoglycans or by promoting VEGF clearance via low-density lipoprotein receptor-related protein-1 (LRP1/CD91) [29,30]. Second, TSP-1 binding to its receptor CD36 modulates VEGFR2 via a complex involving CD36, the TSP-1 binding integrin $\alpha 6\beta 1$ and VEGFR2 [31]. Third, CD47 is a proximal binding partner of VEGFR2, and TSP-1 binding to CD47 disrupts this complex and inhibits VEGFR2 activation [32].

Through TSRs, TSP-1 binds CD36, $\beta 1$ integrins and TGF-β. Several lines of evidence indicate that anti-angiogenic activity of TSP-1 is mostly mediated by the inhibition of endothelial cell migration and by the induction of apoptosis via interaction of TSP-1 with CD36 [33,34]. The interaction of TSRs and $\beta 1$ integrins inhibits the migration of endothelial cells in a CD36- independent manner [35].

TSP-1 also indirectly influences angiogenesis through the activation of TGF-β. In fact, TSP-1 is the only member of the thrombospondin family that can activate TGF-β [36]. Even if the precise mechanism underlying the activation of TGF-β by TSP-1 is not fully understood, it seems that the amino acid sequence RFK between the first and second TSRs of TSP-1 is essential [37]. Of considerable importance is the transcriptional regulation of *tsp-1*, Donoviel *et al.* found that the *tsp-1* promoter region and the 5′-flanking region between -234 and + 750 are important for basal transcriptional activity [38]. In this region are localized several putative binding sites for known transcriptional factors, including early growth response 1 (Egr-1) [39] and specificity protein 1 (Sp-1) [40] sites. Huang *et al.* demonstrated that Egr-1 expression increases the Sp-1 activation of non-overlapping Sp-1 + Egr-1 sites, but inhibits the Sp-1 activity when the sites are overlapping by competing with Sp-1 for the binding site. In addition, Sp-1 is a strong inducer of Egr-1 suggesting a mechanism by which Sp-1 facilitates the inhibition of its own transactivating potential by induction of Egr-1. This 'facilitated inhibition' of Sp-1 transactivation activity by Egr-1 could be a common mechanism for the regulation of a wide range of growth-related genes [41].

In addition, TSP-1 expression seems to be regulated also by miRNAs. In fact, a downregulation of *c-myc* increases the stability of TSP-1 mRNA through the decreased expression of *miR-17-92* cluster after treatment with 5-fluorouracil (5-FU) [42]. MiR-17 ~ 92 cluster members, namely miR-18a and miR-19, are involved in downregulating TSP-1 mRNA and protein levels [43]. A direct regulator of TSP-1 expression in colon epithelium-derived cell lines is miR-194 [44].

As a consequence, a widely employed approach in miRNA loss-of-function studies is to use chemically modified antisense oligonucleotides, termed antagomiRs, which sequester the mature miRNA in competition with cellular target mRNAs leading to functional inhibition of the miRNA and derepression of the direct targets [45]. A deregulated expression of miR-9, miR-31 and miR-182 during carcinogenesis plays a significant role in the development of colon cancer by promoting proliferation and tumor cell survival [46]. Interestingly, the *miR-182* gene, a component of the miRNA cluster miR-183-96-182 located in the 7q32 genomic region, was found amplified in 26% of primary tumors and 30% of liver metastases, suggesting that miR-182 stimulates CRC progression by increasing the cell survival. Given that the liver is the most common target organ for metastases of CRC and the small oligonucleotides tend to accumulate in the liver for clearance [47], anti-miR-182 therapy may have a potential in the treatment of metastatic colon cancer.

Since bio-informatic analyses reveal that TSP-1 mRNA is a predictive target of miR-182 and that, in colon tumor samples, miR-182 is highly expressed with respect to normal specimens [48], the aim of this study is to understand if the silencing of miR-182 affects TSP-1 expression regulation in human colon cancer cell lines. Because it is known that miRNAs have pleiotropic effects, we focused our attention on Egr-1 and Sp-1, predictive targets of *miR-96/182/183* cluster, to establish if the effects of anti-miR-182 are mediated by these transcriptional factors.

2. Materials and methods

2.1 Cell cultures

Three human colon cancer cell lines have been used: HT-29 (ATCC, Catalog No. HTB-38), Caco-2 (ECACC, Catalog No. 86010202) and HCT-116 (ECACC, Catalog No. 91091005). HT-29 and HCT-116 were routinely grown in Gibco Mc-Coy's (Invitrogen, Carlsbad, CA, USA). Caco-2 cell line were grown in Gibco DMEM:F-12 (L-glutamine (+), 15 mM HEPES (+) NEAA (+), Invitrogen).

All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 50 mg/ml streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.2 miRNA target prediction

The miRNA targets predicted by computer-aided algorithms were obtained from DIANA micro-T (http://diana.cslab.ece.ntua.gr/), miRBD (http://mirdb.org/miRDB/) and microRNA.org (http://www.microrna.org/microrna/home.do).

2.3 Luciferase activity assay

The human *tsp-1* 3′-UTR (untranslated region) target site was amplified by polymerase chain reaction (PCR) and cloned downstream of the luciferase gene in pMIR-REPORT luciferase vector (Ambion, Austin, TX, USA). This construct, named *pMIR-TSP-1*, was used for transfection in HT-29 and HCT-116 cell lines. Colon cancer cells were cultured in 24-well plates and each transfected with 0.1 μg of either pMIR-TSP-1 or pMIR-REPORT together with 0.01 μg of pRL-TK vector (Promega) containing Renilla luciferase and 30 pmol of miR-182 (pre-miR-182) or negative control (Ambion) according to manufacturer's protocol. Transfection was done using LipofectAMINE 2000 and Opti-MEM I reduced serum medium (Life Technologies, Carlsbad, CA, USA) in a final volume of 0.6 ml. Twenty-four hours after transfection, firefly and Renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate. Normalized data were calculated as the quotient of Renilla/firefly luciferase activities.

2.4 Anti-miRNAs transfections

Transfections were performed with anti-miR-182 (Ambion). HT-29 and HCT-116 cells were seeded at 5×10^5 in 60 mm culture dish. After 24 h (40 – 50% confluence), the cells were transfected with anti-miR-182 (100 nM) using siPort Neo Fx transfection agent (Ambion), according to manufacturer's instructions. The mixture was transfected into all colon cancer cell lines for 24 h in conditioned medium. Non-specific anti-miR (Ambion) was used as negative control together with mock control. The success of transfection was confirmed by quantitative real-time PCR (qRT-PCR).

2.5 Quantitative real-time PCR to determine the expression of miRNAs and TSP-1

Total cellular RNA and miRNAs were isolated using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and quantified through RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA), and, using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), we evaluated the integrity. For TSP-1 mRNA detection, 2 μg of total RNA were reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's instructions and 5 μl of the RT products were used to amplify TSP-1 mRNA sequence using the Hs00962914-m1 TSP-1 TaqMan gene expression assay (Applied Biosystems). Ten nanograms of total RNA were reverse transcribed using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). The obtained cDNA was amplified using Taqman hsa-miR-182 MicroRNA assay (Applied Biosystems). The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantitative PCR was performed on an Applied Biosystems 7900HT fast RT-PCR system, and data were collected and analyzed using ABI SDS version 2.3. To normalize qRT-PCR reactions, parallel reactions were run on each sample for RNU6B snRNA or cyclophilin A. Changes in the target mRNA content were determined using the comparative CT method to calculate changes in CT, and ultimately fold and percent change. An average CT value for each RNA was obtained for replicate reactions.

2.6 Immunofluorescence

A total of 5×10^4 HT-29 and HCT-116 were plated in 4-well Labtek II chamber slides (Nunc, Rockester, NY, USA). After 24 h, the cells were transfected with anti-miR-182 (100 nM) for 48 h. Then, the cells were extensively washed with phosphate buffered saline (PBS) and fixed for 20 min at 4°C in 4% paraformaldehyde. Next, the cells were permeabilized with 0.2% Triton X-100 for 20 min, and unspecific binding was blocked in 7.5% bovine serum albumin (BSA) fraction V for 1 h at room temperature. Egr-1 expression was detected using anti-Egr-1 (S-25) mouse monoclonal IgG (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-SP-1 (H-225) rabbit polyclonal IgG (1:100; Santa Cruz Biotechnology) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:1000; Santa Cruz Biotechnology) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000,

Molecular Probes; Invitrogen). In control experiments, primary antibodies (Abs) were replaced by non-immune serum. The slides were covered with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) to allow visualization of cell nuclei. The abundance of nuclear Egr-1 and Sp-1 was assessed using Axioscope-40 microscope and Zeiss axiovision LE software (Carl Zeiss, Jena, Germany).

2.7 TSP-1 detection by ELISA

A total of 5×10^5 HT-29 and HCT-116 cells were transfected with anti-miR-182, as described before. After treatment, conditioned medium was collected to measure secreted TSP-1 expression levels using HumanQuantikine ELISA Kits (R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. All points were done in triplicate and the experiments were repeated three times. All TSP-1 concentrations were within the range of standard curve. Linear regression analysis was performed to create the standard curve. The range of curve standards was 7.81, 15.6, 31.3, 62.5, 125, 250, 500 pg/ml.

2.8 Western blotting

The cells, after transfection with anti-miR-182, were lysed to obtain total proteins using Complete Lysis-M Reagent (Roche, Mannheim, Germany) or cytoplasmic and nuclear protein fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology Inc., Rockford, IL, USA). The protein expression was analyzed in 120 µg of total protein lysates and 80 – 50 µg of cytoplasmic and nuclear protein fractions. The following Abs were used for western blotting (WB): anti-TSP-1 (3F357) mouse monoclonal IgG_1 (1:1000; Santa Cruz Biotechnology), anti-Egr-1 (S-25) mouse monoclonal IgG (1:500; Santa Cruz Biotechnology), anti-SP-1 (H-225) rabbit polyclonal IgG (1:1000; Santa Cruz Biotechnology), anti-GAPDH (6C5) mouse monoclonal Ab (1:1000; Santa Cruz Biotechnology), anti-C23 (MS-3) mouse monoclonal Ab (1:1000; Santa Cruz Biotechnology). The proteins were separated on an 8% polyacrylamide gel under denaturing conditions and transferred by electrophoresis to a nitrocellulose membrane. Non-specific binding was blocked by soaking membranes in 1X TBS and 5% powered milk for at least 30 min at room temperature. After the membrane was washed three times with TBS and incubated with the following peroxidase (HRP)-conjugated secondary Ab: goat anti-mouse IgG (sc-2005) and goat anti-rabbit IgG (sc-2004) (Santa Cruz Biotechnology) diluted 1:1000. The specific signal was detected with ECL-WB substrate (Pierce Biotechnology Inc.).

2.9 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Chromatin Immunoprecipitation Assay kit (Upstate, Temecula, CA, USA), according to manufacturer's instructions. HT-29 and HCT-116 cells were transfected with 100 nM anti-miR-182 for 48 h, or left untreated. Next, the cells were cross-linked with 1% formaldehyde and chromatin was collected and sonicated. Soluble chromatin was immunoprecipitated with the following Abs: 5 µg anti-Egr-1 (S-25) mouse monoclonal Ab (Santa Cruz Biotechnology) or 5 µg anti-SP-1 (H-225) rabbit polyclonal IgG Ab (Santa Cruz Biotechnology). DNA–protein immune complexes were eluted, reverse cross-linked and DNA was extracted with phenol/chloroform and precipitated. The presence of the TSP-1 promoter domain containing consensus sequence for Egr-1 in immunoprecipitated DNA was identified first by conventional PCR using the following primers: TSP-1 (region -482 to +162) forward 5′-AACGAATGGC TCTCTTGGTG-3′, reverse 5′-GGGCGACTTACCTGTG TGTA-3′ then by qRT-PCR.

qRT-PCR of DNA obtained from ChIP was performed using SYBR Green I (Applied Biosystems). The same primers amplify the promoter region containing Sp-1 consensus sequences. The PCR conditions for the *tsp-1* promoter region were: 40 cycles at 1 min, 30 s at 95°C, 30 s at 60°C, 1 min at 72°C. In control samples, the primary Abs were replaced with non-immune rabbit IgG. To normalize qRT-PCR reactions, chromatin inputs were used as control and were run on each sample. Changes in the content relative to input were determined using a comparative CT method (ABI User Bulletin no. 2). An average CT value for each sample was obtained for triplicate reactions.

2.10 Statistical analysis

The correlations were studied by Student's *t*-test. p-Values < 0.05 were considered statistically significant.

3. Results

3.1 miR-182 overexpression and TSP-1 downregulation in HT-29 and HCT-116 colon cancer cell lines

The *miR-182* gene, a component of the miRNA cluster miR-96/182/183 located in the 7q32 genomic region, was found amplified in primary tumors and liver metastases, suggesting that miR-182 stimulates CRC progression [48]. Given that the liver is the most common target organ for metastases of CRC [49], anti-miR-182 therapy may have a potential in the treatment of metastatic colon cancer. Recently, the efficient downregulation of miR-182 has been demonstrated to decrease melanoma liver metastasis and tumor burden in mice [50]. Preliminary evaluations were performed to assess miR-182 and TSP-1 mRNA expression levels in HCT and HT-29 cells, using Caco-2 as control, by qRT-PCR. We decided to use Caco-2 cells, because it has been shown that these cells spontaneously differentiate in culture and form a polarized monolayer similar to that of the small intestine [51]. We observed that miR-182 was overexpressed by threefold in HT-29 and by 2.1-fold in HCT-116 cells in respect to Caco-2 cell line (**Figure 1A**). Then, we evaluated TSP-1 mRNA

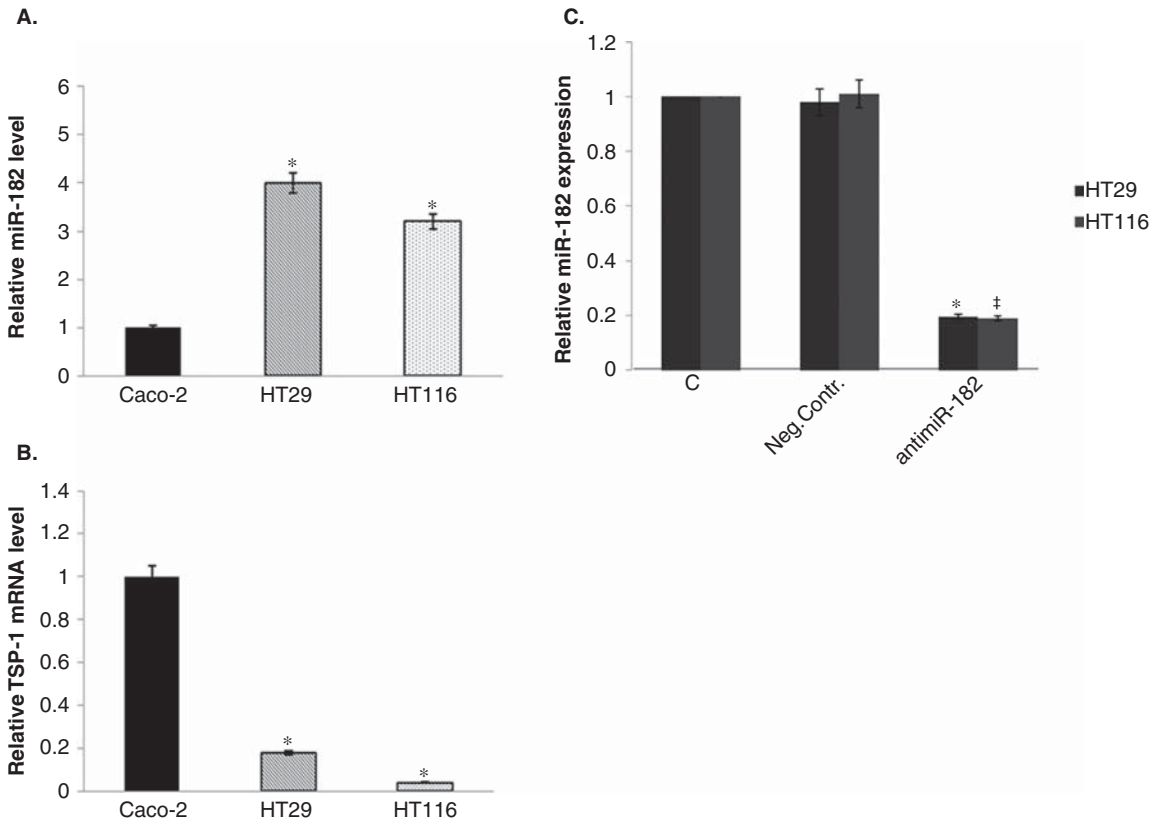

Figure 1. miR-182 and TSP-1 expression in colon cancer cell lines. A. miR-182 is upregulated in HT-29 and HCT-116 cancer cell lines. The miR-182 expression level was studied with qRT-PCR. We used RUN6B as endogenous control. The graphs represent respectively the fold abundance of miR-182 relative to Caco-2 colon cancer cell line – SD. **B.** TSP-1 mRNA is downregulated in HT-29 and HCT-116 cancer cell lines. The abundance of TSP-1 mRNA was studied with qRT-PCR. To normalize TSP-1 qRT-PCR reactions, parallel reactions were run on each sample for cyclophilin A (PPIA). The graphs represent respectively the reduction of TSP-1 mRNA relative to Caco-2 colon cancer cell line – SD. **C.** miR-182 is downregulated by 20% in HT-29 and 18% HCT-116 after transient transfection with anti-miR-182. The graphs represent respectively the fold abundance of miR-182 relative to control and negative control; – SD.
A. *$p < 0.05$. **B.** *$p < 0.05$. **C.** *$p < 0.05$, ‡$p < 0.01$.

expression levels in the same cells. Data showed that TSP-1 mRNA levels were downregulated by 80% and by 95% in HT-29 and HCT-116 cells, respectively, with respect to Caco-2 (**Figure 1B**). These first results indicate the inverse correlation of the expression levels of TSP-1 and miR-182 in the *in vitro* model that we proposed as in literature reported [52,48]. Then, hypothesizing the possible role of miR-182 in TSP-1 regulation, we investigated the putative angiogenetic role of miR-182 evaluating the consequence of transient silencing of this miRNA on TSP-1 expression in colorectal cancer cells. Relative expression data obtained after transfection of anti-miR-182 in HT-29 and HCT-116 indicated an efficiency of silencing of miR-182 by 80 and 82%, respectively (**Figure 1C**).

3.2 Effects of anti-miR-182 on TSP-1 expression

TSP-1 expression levels are inversely correlated with tumor vascularity; in fact, microvessel density is significantly higher in TSP-1-negative colorectal tumors. Furthermore, it has been shown that patients with TSP-1-negative tumors have a significantly worse prognosis than those with TSP-1-positive tumors.

TSP-1 may be useful for predicting recurrence in patients with colon cancer because the frequency of hepatic recurrence is significantly higher in patients with VEGF-positive and TSP-1-negative colorectal tumors [53] in comparison with those TSP-1-positives [52].

The fact that TSP-1 is a potent endogenous inhibitor of angiogenesis and is often downregulated in tumor tissue lays the basis to explore therapeutic applications of TSP-1. These efforts fall into two basic approaches: the identification of strategies to upregulate endogenous TSP-1, and the delivery of recombinant TSRs or synthetic peptides that contain sequences from the TSRs [54], though in theory a reasonable approach must be balanced by the possible expected effects of systemic loss of pro-flow signals from NO and VEGF.

Here, to investigate the effect of miR-182 on the expression levels of TSP-1, we evaluated the effect of transient silencing miR-182 to restore TSP-1 expression in colon cancer cell lines. Anti-miR-182 is a synthetic single-stranded oligonucleotide chemically modified designed to specifically bind and to inhibit the endogenous miR-182. Following miR-182 silencing, we observed that TSP-1 mRNA increased by 2.4- and 3.1-fold in HT-29 and HCT-116, respectively, relative to untransfected and negative control cells (**Figure 2A**). We also evaluated intracellular and secreted TSP-1 protein levels. WB results, obtained from total cellular lysates, indicated that intracellular TSP-1 protein levels increased in transfected HT-29 and in HCT-116 cells by 1.8- and 3.6-fold, respectively (**Figure 2B**).

The effects of anti-miR-182 on the secreted TSP-1 protein mirrored the above findings. As expected, after transfection, we analyzed the amount of secreted TSP-1 that showed fold changes of 2.2 and 2.8 in HT-29 and HCT-116 cells, respectively (**Figure 2C**). According to these results, miR-182 affects TSP-1 protein levels but could have influence on transcriptional regulation of *tsp-1* expression.

3.3 miR-182 directly targets TSP-1

Identification of miRNA-regulated gene targets is a necessary step to understand miRNA functions. The online target prediction databases indicate one highly conserved putative target site in the 3′UTR of *tsp-1* for the seed sequence of miR-182 located at 1959–1965 nt (**Figure 3A**). To investigate whether a direct interaction is involved between miR-182 and its target TSP-1, we performed luciferase reporter assays. We generated a luciferase construct that contains the potential binding sequence of TSP-1 3′UTR to miR-182, then HT-29 and HCT-116 cells were transfected with this construct and luciferase activity was measured 24 h after transfection.

Luciferase activity was significantly decreased by 46 and 39% fold in HT-29 and HCT-116 transfected with *pMIR-TSP-1* compared with cells transfected with pMIR-REPORT, the empty vector, suggesting translational repression of TSP-1 mRNA by overexpressed endogenous miR-182. In addition, miR-182 precursor significantly decreased luciferase reporter by 75 and 65% in HT-29 and HCT-116, respectively. By contrast, anti-miR-182 markedly increased TSP-1 3′UTR-associated luciferase reporter translation by 1.89- and 1.92-fold in HT-29 and HCT-116 cells with respect to co-transfected cells with negative control (**Figure 3B**).

3.4 Silencing of miR-182 slightly affects Egr-1 and Sp-1 protein levels in HCT-116 and HT-29 cells

The 5′-flanking region between -234 and + 750 on *tsp-1* promoter region presents binding sites for Egr-1 and Sp-1 transcriptional factor. These transcriptional factors can compete to bind the same GC-rich sites.

Since Egr-1 and Sp-1 are predictive targets of miR-96/182/183 cluster [55], we investigated the molecular mechanism by which anti-miR-182 could determine TSP-1 upregulated expression in colon cancer cells, as reported previously. For this reason, we evaluated if anti-miR-182 influences Egr-1 and Sp-1 expression in the *in vitro* model that we decided to study. WB analysis did not reveal significant variation of Egr-1 total protein levels, band analysis in fact indicated just an upregulation of 22% after transfection with anti-miR-182 in HCT-116 cell lines. In parallel, Sp-1 was slightly increased, only 20%, in HT-29 cells transfected with anti-miR-182 with respect to untransfected cells. Moreover, we did not find any variation of Egr-1 and Sp-1 in HT-29 and HCT-116, respectively with anti-miR-182 compared with control and negative control (**Figure 4**). These results reveal that the silencing of miR-182 not affects significantly the protein expression of these transcriptional factors involved in activation of *tsp-1* expression.

3.5 Anti-miR-182 modulates Egr-1 and Sp-1 expression and nuclear translocation in colon cancer cells

At the light of the data described above, we hypothesized that miR-182 could affect cytoplasmatic and nuclear localization of Egr-1 and Sp-1, for this reason we studied the effect of anti-miR-182 on Egr-1 and Sp-1 expression on fractionated lysates after transfection with anti-miR-182. We found both cytosolic and nuclear accumulation of Egr-1 in transfected HCT-116 with respect to untransfected cells. Moreover, we observed that anti-miR-182 affects nuclear accumulation of Egr-1 in HT-29 cells. In fact, WB data evidenced a reduction of cytosolic fraction and an increase of nuclear translocation of Egr-1, at the same time, in HT-29 transfected with anti-miR-182. Probably, miR-182 has a regulatory role in this event that here we did not investigate (**Figure 5A**). Furthermore, WB analysis on fractionated protein extracts revealed that nuclear abundance of Sp-1 slightly increased in HT-29 cells transfected with anti-miR-182 with respect to untransfected and negative control cells, whereas anti-miR-182 seems to not affect Sp-1 nuclear accumulation in HCT-116 cell line (**Figure 5A**).

Changes in nuclear and cytoplasmic accumulation were confirmed by immunofluorescence analysis, which revealed that Egr-1 levels were considerably upregulated in both cytoplasm and nucleus of HCT-116 transfected with anti-miR-182 compared with untransfected cells (**Figure 5B**). Moreover, immunofluorescence analysis shows that transfection with anti-miR-182 determined increased levels of nuclear Sp-1 in HT-29 and not causes any variation on Sp-1 levels in HCT-116 (**Figure 5C**).

3.6 Egr-1 and Sp-1 binding to TSP-1 promoter is influenced by anti-miR-182 in HT-29 and HCT-116 cells

Next, we assessed whether these factors bind specific consensus sequences into *tsp-1* promoter (from -482 to + 162) after

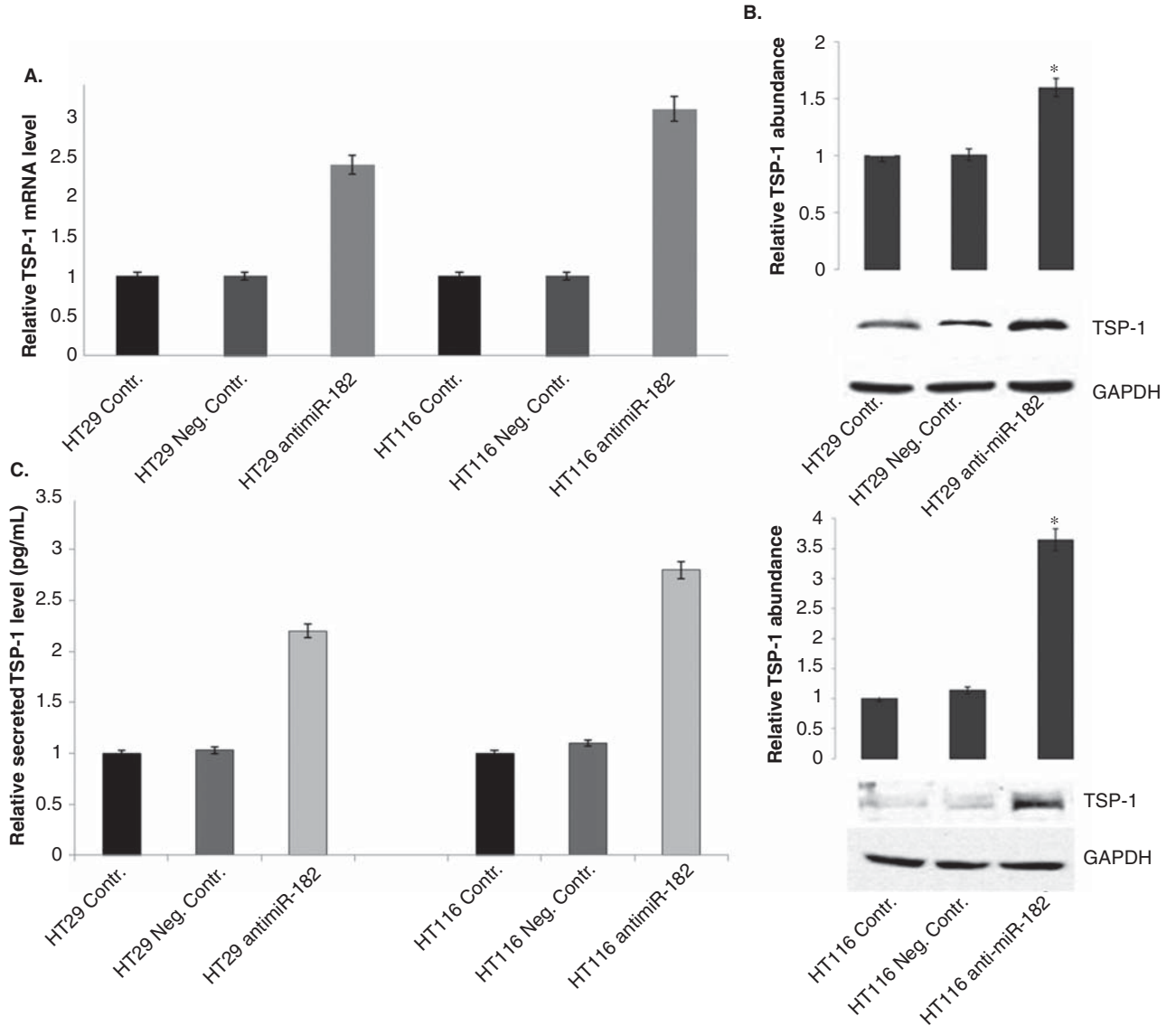

Figure 2. Anti-miR-182 influences TSP-1 expression. **A.** The expression of TSP-1 mRNA was studied after HT-29 and HCT-116 transfection with synthetic oligonucleotides targeting miR-182 (anti-miR-182) or with negative control anti-miR molecules, as described in Section 2. The graph indicates that TSP-1 mRNA levels increase in transfected cells relative to negative control. $p < 0.05$ control vs anti-miR-182. **B.** The abundance of TSP-1 total protein was determined by WB in 120 μg of total proteins, as described in Section 2. The images indicate the increase of TSP-1 protein levels relative to untransfected cells. **C.** The concentration of secreted TSP-1 was measured by ELISA in cells transfected with anti-miR-182 or with negative control anti-miR. The concentrations represent pg TSP-1/ml conditioned medium from 5×10^5 cells.
*$p < 0.05$

transfection with anti-miR-182. Using ChIP assay, we found that the Egr-1 binding to *tsp-1* promoter significantly increased by 3.5-fold in HCT-116 cells transfected with anti-miR-182 compared with untransfected cells. Although our data showed that anti-miR-182 induces the nuclear accumulation of Egr-1 in HT-29 cells, as before describe, we did not observe the Egr-1 recruitment on the consensus sequence into *tsp-1* promoter in this cell line (**Figure 6A**). Since Sp-1 binds to two GC boxes lying between -267 and -71 at the 5′-flanking region of the *tsp-1* gene [40], we assessed the recruitment of Sp-1 on *tsp-1* promoter sequence after transfection with anti-miR-182. Data indicated that Sp-1 bound by 1.7-fold to *tsp-1* promoter sequence in transfected HT-29 with respect to control cells. However, Sp-1 did not show significant binding to *tsp-1* consensus region both in transfected with anti-miR-182 and untransfected HCT-116 cells (**Figure 6A**). In addition, we validated these results by RT-PCR and our findings showed that Egr-1 is

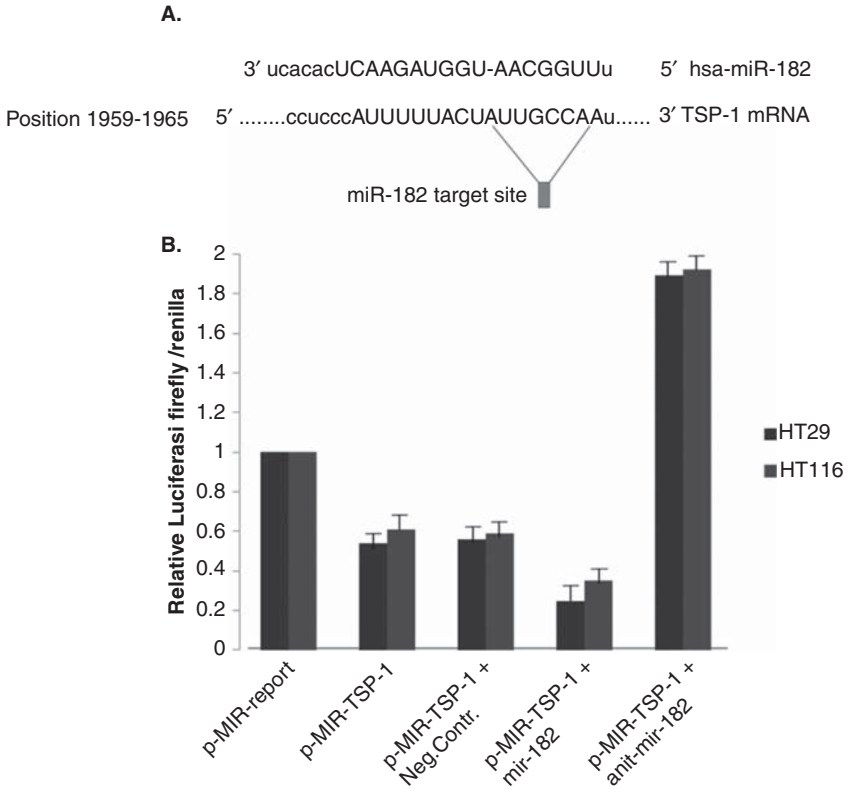

Figure 3. miR-182 directly regulates TSP-1 by luciferase reporter assay. **A.** Putative *miR-182* binding site in the 3′UTR of *TSP-1* mRNA was identified with the microRNA.org database. **B.** Luciferase reporter assay was performed using the vector encoding partial sequences of 3′UTR which contained the putative *miR-182* target site. The vector and pre-miR-182 (50 nM), anti-miR-182 (100 nM) or negative control were co-transfected into HT-29 and HCT-116 cell lines. Renila luciferase activity was measured after 24 h transfection. The results were normalized by firefly luciferase values.
*$p < 0.05$.

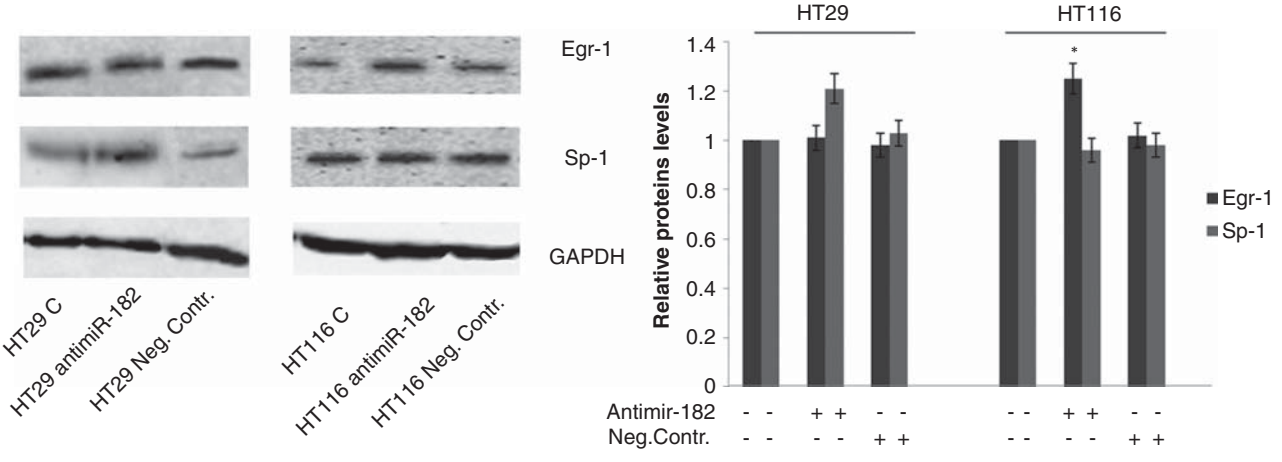

Figure 4. Effects of anti-miR-182 on Egr-1 and Sp-1 protein expression. HCT-116 and HT-29 cells were transfected with anti-miR-182 as previously described. Egr-1 and Sp-1 levels were assessed by WB using specific Abs, as described in Section 2.
*$p < 0.05$

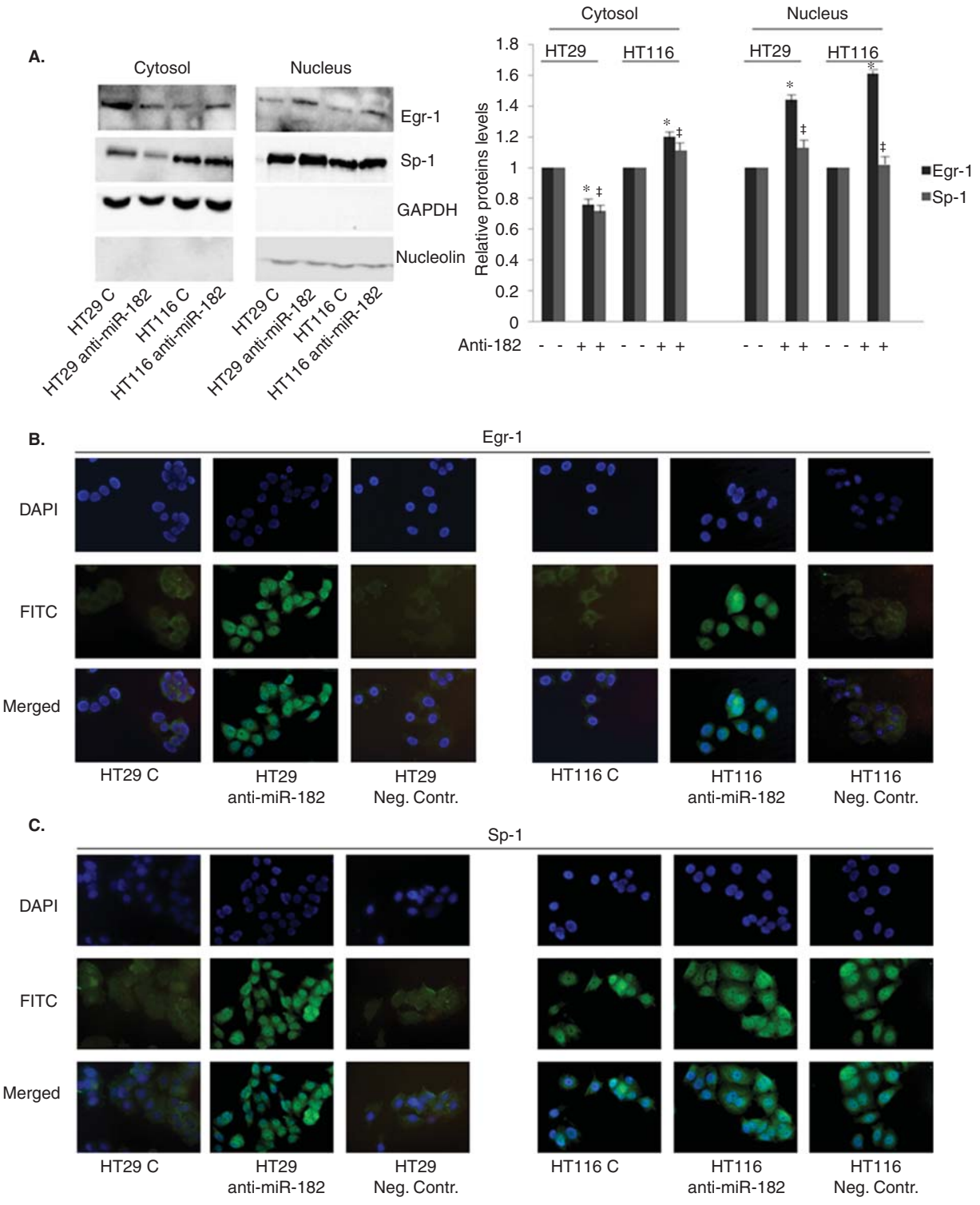

Figure 5. Anti-miR-182 modulates Egr-1 and Sp-1 nuclear accumulation. A. Egr-1 and Sp-1 cytosolic and nuclear protein levels were studied by WB, as described in Section 2. HT-29 and HCT-116 cells were transfected with anti-miR-182 for 48 h or left untreated. Protein loading was controlled by re-probing WB filters for the expression of a nuclear marker (nucleolin) or cytosolic protein (GAPDH). **B** and **C.** The expression of Egr-1 and Sp-1 (green fluorescence) was assessed by immunostaining with specific Abs and fluorescence microscopy, as detailed in Section 2. Cell nuclei were stained with DAPI (blue fluorescence). Scale bar, 10 mm.

*$p < 0.05$; ‡$p < 0.01$

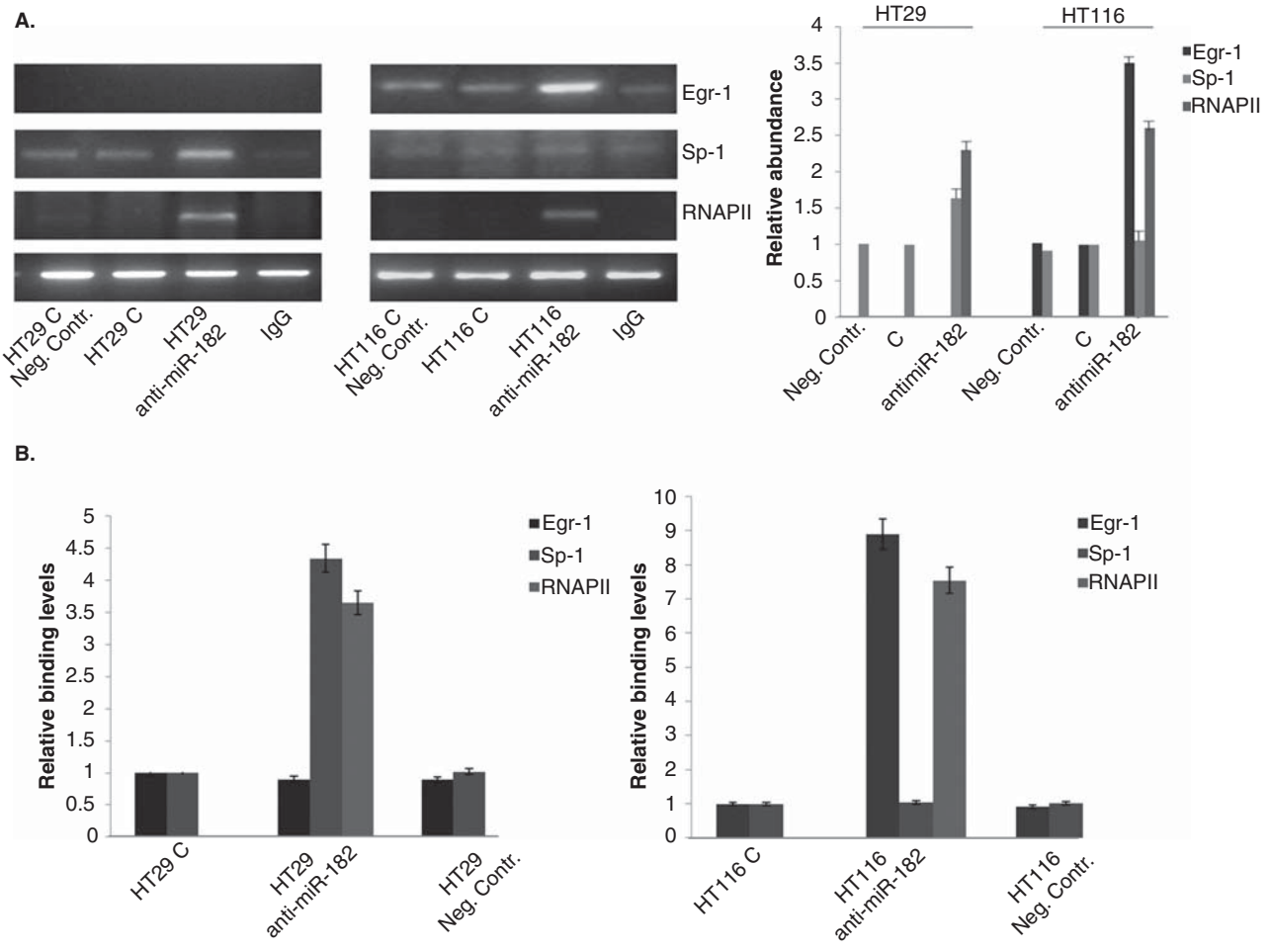

Figure 6. Egr-1 and Sp-1 binding to TSP-1 promoter is modulated by miR-182 in HT-29 and HCT-116 cells. The binding of Egr-1 and Sp-1 to the proximal TSP-1 promoter region (-482/+ 162) was tested by ChIP (**A**) and qRT-PCR (**B**) in HT-29 and HCT-116 cells transfected with anti-miR-182. Increased Egr-1 binding on TSP-1 promoter sequence in transfected HCT-116 cells; Egr-1 is not recruited after transfection with anti-miR-182 in HT-29 cells. Sp-1 increased binding on its consensus motif in HT-29 cells induced by anti-miR-182; Sp-1 is not recruited in HCT-116 cells. ChIP experiments were performed as described in Section 2.

recruited on *tsp-1* promoter in HCT-116 transfected cells and Sp-1 in HT-29 with anti-miR-182 promoting assembly of a functional transcription complex in both cases (**Figure 6B**).

4. Discussion

The anti-angiogenic effects of TSP-1 is mediated also by the ligation of CD47, inhibiting VEGFR2 activation [56,32], decreasing migration of endothelial cells [35] and adenylate cyclase activation [57].

Given the importance of TSP-mediated effects on angiogenesis, apoptosis and extracellular matrix composition, considerable interest has been shown in using this protein as a clinical therapeutic. Generally, two approaches to increasing TSP-mediated inhibition of angiogenesis have been explored in the context of neoplasia: administration of chemotherapies based on recombinant TSP or a TSP-derived peptide, including the TSRs of TSP-1 and TSP-2 and upregulation or potentiation of the effects of endogenous TSPs. However, in contexts in which tissue ischemia limits recovery, it may be beneficial to suppress TSP expression in order to promote angiogenesis, in fact interrupting the TSP-1–CD47 interaction is highly beneficial in ischemia, ischemia-reperfusion injury, hypoxia and high-dose radiation settings suggesting a priority signal in the cardiovascular system [58].

Aberrant expression of miRNAs is correlated with the development and progression of tumors, and the reversal of their expression has been shown to modulate the cancer phenotype, suggesting the potential of miRNAs as targets for anti-cancer drugs. Considering that several miRNAs have been found to regulate the process of cancer metastasis independent of primary tumorigenesis [59], in this study, we

describe the effects of anti-miR-182 in the expression of TSP-1 in colon cancer cell lines. Our data indicated an inverse correlation in expression that induced us to investigate whether miR-182 could regulate TSP-1 expression. For this purpose, we silenced miR-182 using a synthetic oligonucleotide, anti-miR-182, that targets the mature forms of miR-182 and thereby contrasts their expression and function in HT-29 and HCT-116 cells. We found that the transfection with anti-miR-182 increased TSP-1 intracellular and secreted protein expression levels. By luciferase assay, we validated that TSP-1 is a target of miR-182, in fact, this miRNA directly interacts with 3′-UTR of TSP-1 and blocks TSP-1 translation.

Moreover, we found that anti-miR-182 determined increased expression levels of mRNA TSP-1, supposing that anti-miR-182 could affect TSP-1 expression by affecting transcriptional factors expression that bind *tsp-1* gene promoter, we focused our attention on Egr-1 and Sp-1. Both are Cys_2-His_2-type zinc-finger transcription factors and binds to GC-rich, *cis*-acting promoter elements, in fact, overlapping Sp-1 and Egr-1 sites are frequent [41].

Our results, obtained after transfection, indicated that anti-miR-182 influences nuclear accumulation both Egr-1 and Sp-1 in HT-29 cells, whereas anti-miR-182 affects nuclear translocation of Egr-1 only in HCT-116 cells. Subsequent ChIP analyses revealed an increased Sp-1 binding into proximal promoter region of *tsp-1* gene as a consequence of its increased nuclear accumulation in HT-29. Conversely, we did not observe Egr-1 recruitment despite its major nuclear amount. Moreover, we found an Egr-1 increased loading on *tsp-1* promoter in HCT-116 cells transfected with anti-miR-182 and we did not observe the recruitment of Sp-1, which not shows any variation between cytosolic and nuclear levels. Sp-1 increases the amount of Egr-1, which then becomes a potent competitor of Sp-1 for binding to consensus sequence [41]. Since Egr-1 inhibits the transactivation of Sp-1 on overlapping Sp-1/Egr-1 binding sites and Sp-1 activity could be augmented by Egr-1 at non-overlapping sites in the *Egr-1* gene promoter, we hypothesized a possible regulatory mechanism in which anti-miR-182 increased the nuclear amount of both Egr-1 and Sp-1 in HT-29 cells. In this context, probably there is not a competition between Egr-1 and Sp-1 to load consensus sequence because Sp-1 binds non-overlapping sites. Reduced levels of miR-182 make Sp-1 the major transcriptional factor implicated in the TSP-1 expression without the involvement of Egr-1 in HT-29 cells. On the contrary, in HCT-116 cell line, the molecular mechanism by which anti-miR-182 could indirectly modulate TSP-1 is based on the physical displacement of Sp-1 on DNA by Egr-1. In this work, we showed that anti-miR-182 mediates suppression of angiogenic activity through the induction of the TSP-1 angiogenesis inhibitor by direct targeting, regulation transcriptional events on *tsp-1* promoter. The fact that TSP-1 is a potent endogenous inhibitor of angiogenesis prompted several groups to explore therapeutic applications of TSP-1, endeavoring to identify strategies to increase endogenous TSP-1 and the delivery of recombinant TSP-1 repeats (TSRs) or synthetic peptides that contain sequences from TSRs.

Research undertaken to identify and optimize short bioactive peptides derived from the two TSP-1 anti-angiogenic domains, which bind CD47 and CD36 cell surface receptors [60].

Future investigations are expected to reveal the molecular basis for the different effects of TSP-1 on tumorigenesis in several tumor types and to describe the molecular pathways for the regulation of TSP-1 by multiple tumor suppressor genes and oncogenes. In particular, we focused our attention on miRNAs, small regulatory non-coding RNAs, which regulate mRNA function and which play a crucial role in cancer, acting as oncogenes or tumor suppressor genes. miRNA therapeutics appears as a novel field in which miRNA activity is the major target of the intervention.

5. Conclusion

In this work, we showed the potential regulatory role of anti-miR-182 on TSP-1 expression in colon cancer. In the light of these results that remark the involvement of miRNAs in cancer pathogenesis, miRNAs could become new targets for the development of novel therapeutic strategies in order to restore physiological gene expression levels and to modulate indirectly the expression of their targets. Moreover, the discovery of more miRNA targets will be useful to better define the cancer cells signaling and to identify new and more effective drugs.

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Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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