Journal of Thrombosis and Haemostasis, **9**: 1985–1992 DOI: 10.1111/j.1538-7836.2011.04451.x

ORIGINAL ARTICLE

Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome

R. TERUEL,* C. PÉREZ-SÁNCHEZ,† J. CORRAL,* M. T. HERRANZ,‡ V. PÉREZ-ANDREU,* E. SAIZ,§ N. GARCÍA-BARBERÁ,* I. MARTÍNEZ-MARTÍNEZ,* V. ROLDÁN,* V. VICENTE,* C. LÓPEZ-PEDRERA† and C. MARTÍNEZ*

**Centro Regional de Hemodonación, University of Murcia, Murcia; †Unidad de Investigación/Instituto de Investigación Biomédica de Córdoba (IMIBIC), Hospital Universitario Reina Sofía, Córdoba; ‡Internal Medicine Unit; and §Section of Rheumatology, Hospital Universitario Morales Meseguer, Murcia, Spain*

To cite this article: Teruel R, Pérez-Sánchez C, Corral J, Herranz MT, Pérez-Andreu V, Saiz E, García-Barberá N, Martínez-Martínez I, Roldán V, Vicente V, López-Pedrera C, Martínez C. Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. *J Thromb Haemost* 2011; **9**: 1985–92.

Summary. *Background:* Tissue factor (TF) is the main initiator of the coagulation cascade and elements that may upregulate its expression might provoke thrombotic events. Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are autoimmune diseases characterized by a high TF expression in monocytes. *Objectives:* To examine the role of microRNAs (miRNAs) in TF expression and to evaluate their levels in SLE and APS patients. *Methods:* An *in silico* search was performed to find potential putative binding sites of miRNAs in TF mRNA. *In vitro* validation was performed transfecting cells expressing TF (THP-1 and MDA-MB-231) with oligonucleotide miRNA precursors and inhibitors. Additionally, reporter assays were performed to test for the binding of miR-20a to TF mRNA. Levels of miRNAs and TF were measured by quantitative (qRT-PCR) in patients with APS and SLE. *Results:* Overexpression of miRNA precursors, but not inhibitors, of two of the members of cluster miR-17∼92, for example miR-19b and miR-20a, in cells expressing TF decreased TF mRNA, protein levels, and procoagulant activity between 30% and 60%. Reporter assays showed that miR-20a binds to TF mRNA. Finally, we measured levels of miR-19b and miR-20a in monocytes from patients with APS and SLE and observed significantly lower miRNAs levels in comparison with healthy subjects inversely correlated with the levels of TF. *Conclusions:* Down-regulation of miR-19b and miR-20a observed in patients with SLE and APS could contribute to increased TF expression and thus provoke the hypercoagulable state characteristic of these patients.

Correspondence: Constantino Martínez, Centro Regional de Hemodonación, C/ Ronda de Garay S/N, 30003 Murcia, Spain.
Tel: +34 968341990; fax: +34 968261914.
E-mail: constant@um.es

Received 13 January 2011, accepted 21 June 2011

Keywords: antiphospholipid syndrome, miRNAs, systemic lupus erythematosus, thrombosis, tissue factor.

Introduction

Recently, a myriad of papers described a family of non-coding RNA as extremely relevant in many biological functions [1,2]. MicroRNAs (miRNAs) are approximately 21 to 24 nucleotide-long RNA derived from hairpin transcripts that have become a major focus of research in molecular biology where at least a thousand are predicted to operate in humans. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated and that changes in their expression are observed in human pathologies, including cancer and cardiovascular diseases [3–6]. It is accepted that miRNAs post-transcriptionally control gene expression by regulating mRNA translation or stability in the cytoplasm by forming hetero-duplexes. What is certain is that the discovery of miRNAs has unraveled a new step towards decrypting the complexity of post-transcriptional regulation of a large number of eukaryotic genes. Interestingly, very recently a growing body of evidence shows that miRNAs may play a role in hemostasis [7–9]. The hemostatic system is a highly efficient and rapid system to avoid hemorrhage and thus to overcome the effects that are produced during vascular damage [10,11]. The hemostatic system responds through a series of proteolytic processes with a high efficacy. However, this system must be finely tuned and be spatially and temporally restricted to the site of the vessel injury. Thus, the hemostatic system is an equilibrium perfectly regulated between procoagulant and anticoagulant elements that regulate blood flowing under normal conditions but respond quickly and strongly to form the hemostatic plug to the vascular damage. Tissue factor (TF) is the primary physiological initiator of blood coagulation. TF is constitutively expressed in different cell types such as smooth muscle

cells or fibroblasts, cells that come in contact with blood only during vascular damage. In contrast, TF expression in blood cells and endothelium is regulated through a number of signaling pathways and occurs after cell activation with a variety of agonists [12]. Indeed, a number of pathological conditions show a dysregulation of the basal hemostatic balance towards a hypercoagulable status related in certain cases to a higher expression of TF and a higher level of circulating TF-bearing microparticles [12,13]. Among these pathologies, autoimmune diseases such as antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) display increased TF expression in monocytes [14–16]. However, the exact pathogenic mechanisms responsible for the increased thrombosis risk in patients with APS and SLE are still unclear. Indeed, TF expression in endothelial cells and monocytes, the main producers of TF-bearing microparticles, is certainly fine-tuned and alterations in its modulation may play a transcendental role in the development of thrombotic events in APS and SLE patients. Very recently, miR-19a has been shown to inhibit TF expression in cancer cell lines [17]. In the present study, we additionally showed that TF expression may be regulated by miR-19b and miR-20a in THP-1 cells and that down-regulation of these miRNAs in monocytes from patients with APS and SLE may be a relevant event in the observed hypercoagulable state of these patients.

Methods

In silico *studies*

Several web databases and algorithms of miRNA target prediction were used for the search of miRNA targeting TF. We essentially used TargetScan [18] (release 5.1: http://www.targetscan.org), which provides the prediction results computed by the TargetScanS algorithm, PicTar (http://pictar.mdc-berlin.de) [19] and miRanda (http://www.microrna.org/microrna/home.do) [20].

Cell culture and transfection

The following cell lines were used: MDA-MB-231 (breast cancer) and THP-1 (monocytic leukemia). Cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to the ATCC protocols. Cell transfection was performed by standard procedures described in detail in Data S1.

RNA isolation, qRT-PCR and Western blotting

Total RNA was isolated from cells using Trizol® Reagent (Invitrogen, Madrid, Spain) and a reverse transcription (RT; SuperScript First Strand, Invitrogen) reaction was performed using 200 ng of total RNA from each sample according to the manufacturer's instructions. MiRNAs and gene expression was quantified using Taqman® probes purchased from Applied Biosystems (Madrid, Spain) See Data S1 for qRT-PCR and Western blotting assays.

TF expression

Analysis of TF procoagulant activity was performed as previously described with some modifications [21]. Briefly, THP-1 cells (500 000 cells) were stimulated for 5 h (37 °C, 5%CO_2) with10 μg mL^{-1} lipolysaccharide (LPS) (#L-4391; Sigma-Aldrich) after transfection with pre-miR or antagomiR. Suspensions were centrifuged (350 × *g*, 5 min, 22 °C) and the pellet was resuspended in 100 μL phosphate buffered saline (PBS). Lysates were prepared by four times freeze/thawing in liquid N_2 and centrifuged (12 000 × *g*, 5 min, 22 °C). Supernatant were collected adjusted in protein content using BCA assay, and incubated with 10 μg mL^{-1} FX (Stago, Asnières, France), 5 U mL^{-1} rFVIIa (NovoSeven; NovoNordisk SA, Madrid, Spain) and 5 mmol L^{-1} $CaCl_2$. FXa generation was quantified in a Synergy 2 luminometer (Biotek, Bad Friedrichshall, Germany) at 405 nm after the addition of FXa substrate (Instrumentation Laboratory, Milan, Italy).

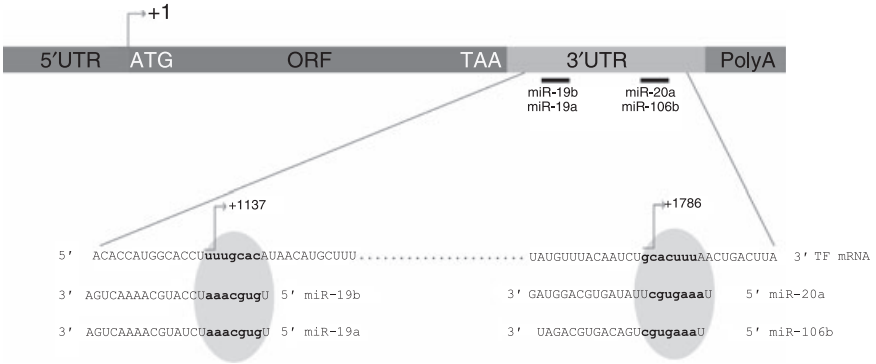

Fig. 1. Schematic representation of the binding sites of selected microRNAs (miRNAs) to tissue factor (TF) mRNA. (A) Putative binding sites localized in TF mRNA (Ensembl transcript IDENST00000334047). Nucleotide A from ATG is considered as position +1. Mir-19a and mir-19b seeds are located at +1137; miR-20a and miR-106b are located at position +1786. The ovals mark the position of the seed binding sites.

Plasmid/luciferase construct

Constructs for luciferase assays were designed as described in Data S1.

Luciferase assay

Twenty-four hours before transfection, HEK 293T (embryonic kidney cells) were plated at 10^5/well in 24-well plates with complete Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) without antibiotics. Cells were transfected with miR-20a precursor or scrambled control (Applied Biosystems) and 3′UTR firefly luciferase reporter plasmid (500 ng/well) along with 50 ng/well of renilla luciferase control plasmid (pRLTK; Promega, Madison, WI, USA) using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Luciferase assays were performed as described in Data S1.

Controls and patients

Ten patients [nine women, one man; 47 ± 13 years (range 35–71)] fulfiling the Sapporo criteria for APS [22] were recruited. Six patients suffered from arterial thrombosis (stroke; mean interval from last thrombotic event: 10.2 ± 3.3 years) and four patients had recurrent pregnancy loss (mean interval from last pregnancy loss: 8.5 ± 3.1 years). Eight patients were antibody positive. Twenty-seven patients [25 women, 2 men; 39 ± 14 years (range 18–68)] with SLE fulfiling the American College of Rheumatology classification criteria [23] were enrolled in this study. Three female patients had arterial thrombosis (two secondary SAF and one patient had factor V Leiden). Finally, 17 healthy controls were recruited from the general population [five men, 12 women; 33 ± 10 years (range 19–73)]. None of the controls had a history of autoimmune disease, bleeding disorders, thrombosis or pregnancy loss. All subjects gave their informed consent according to the Declaration of Helsinki to enter the study, which has been approved by the Local Ethics Committee.

Membrane tissue factor expression

Monocytes were purified using standard procedures [15]. Monocyte TF expression was measured by flow cytometry. See Data S1 for details.

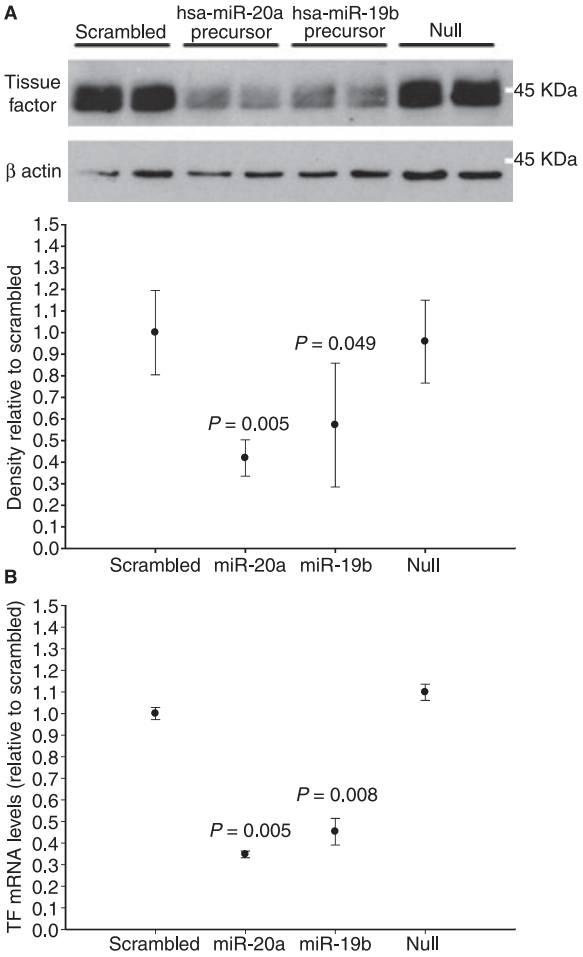

Fig. 2. Inhibition of tissue factor (TF) expression by miR-19b and miR-20a in MDA-MB-231. MDA-MB-231 were transfected with 100 nmol L^{-1} precursors of miR-19b and miR-20a or a scrambled precursor (A) TF expression was measured by Western blotting with β-actin used as a loading control. Densitometry was performed and normalized with respect to β-actin expression. (B) TF mRNA was measured by quantitative (qRT-PCR) and normalized with respect to β-actin mRNA. The normalized data were expressed as changes relative to the data of the cells transfected with scrambled pre-miR and set as 100%. Differences were analyzed by means of Student's *t*-test. Statistical significance was taken as $P < 0.05$. The data shown are mean ± SD representative of four independent experiments with three replicates each.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (version 15.0; SPSS, Chicago, IL, USA). See Data S1 for details.

Table 1 Binding *in silico* report

	TargetScan			PicTar (Krek et al. 2005)		
miRNA putative target site in TargetScan & Pictar	Seed match	TargetScan score percentile	PCT	PicTar score	Target site number	Free energies (Kcal mol^{-1})
hsa-miR-20a	8mer	97	0.95	3.20	1	−20.1
hsa-miR-106b	8mer	96	0.95	2.99	1	−19.6
hsa-miR-19a	8mer	92	0.65	2.84	1	−18.9
hsa-miR-19b	8mer	92	0.65	2.84	1	−21.2

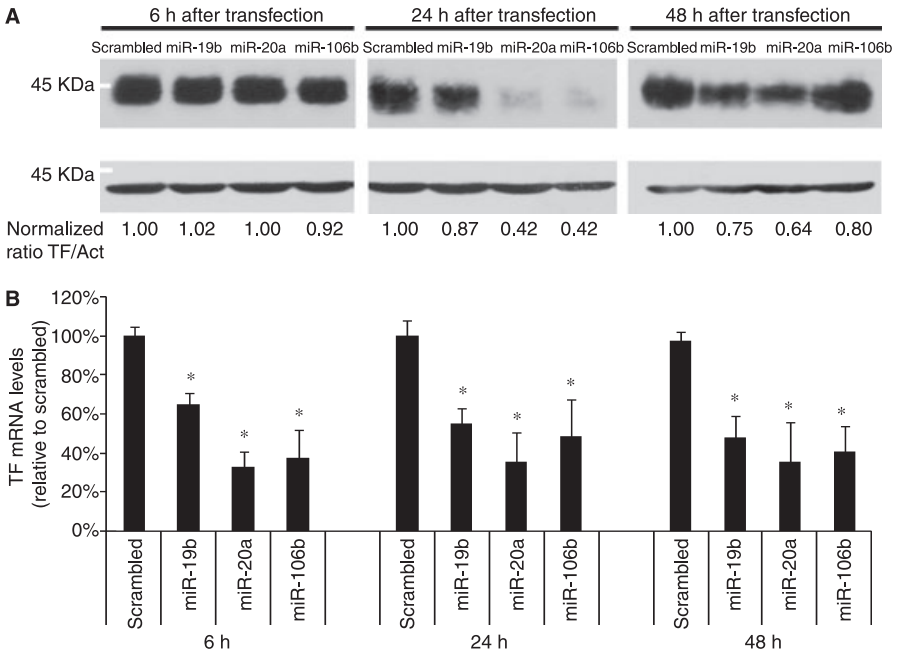

Fig. 3. Time course inhibition of tissue factor (TF) expression in MDA-MB-231. MDA-MB-231 were transfected with 100 nmol L^{-1} precursors of miR-19b, miR-20a, miR-106b or a scrambled precursor and collected after 6, 24 and 48 h. (A) TF expression was measured by Western blotting with s-actin used as a loading control. Densitometry was performed and normalized with respect to β-actin expression. (B) TF mRNA was measured by qRT-PCR and normalized with respect to β-actin mRNA. The normalized data were expressed as changes relative to the data of the cells transfected with scrambled pre-miR and set as 100%. Differences were analyzed by means of Student's *t*-test. Statistical significance was taken as $P < 0.05$. The data shown are mean ± SD representative of two independent experiments with duplicates (western blot) and three independent experiments with three replicates each (qRT-PCR).

Results

In silico *study of miRNA–TF mRNA interaction*

The seed regions binding of miR-19a/miR-19b and miR-20a/miR-106b were located at 1137 and 1786 bp from the beginning of the ATG codon, respectively (Fig. 1). Scores, free energies and characteristics of the seed region calculated by Pictar and Targetscan algorithms are specified in Table 1. We principally focused for the rest of the study on miR-20a. Additionally, we also performed studies with miR-19b as miR-19a has been recently shown to interact with TF mRNA [17].

Mir-19b and miR-20a inhibit TF expression

To validate the inhibition of endogenous TF transcripts by miR-19b and miR-20a, we looked for a model of a tumor cell line constitutively expressing TF. We chose the breast carcinoma cell line MDA-MB-231 which has been shown to express TF at high levels [24]. Overexpression of both miRNAs by transfecting MDA-MB-231 cells with oligonucleotide precursors significantly reduced the expression of endogenous TF 48 h after transfection (Fig. 2A). We observed an almost 60% decrease of TF protein expression as assayed with densitometry after Western blot. The inhibition of TF synthesis by miR-19b and miR-20a correlated with a statistically significant reduction of TF mRNA (Fig. 2B). To further validate the effect of miR-20a, we transfected cells with miR-106b that shares the same binding site. Time course experiments showed that only 6 h after transfection, the three miRNAs decreased TF mRNA levels by 20–60% (Fig. 3B). Concerning protein levels, the inhibition effect of the three miRNAs only appeared after 24-h transfection and was maximal at that time (Fig. 3A).

Aiming to verify the effect of these miRNAs in a cell type that inducibly expresses TF, we investigated their potential role in inhibiting TF mRNA transcripted in THP-1, a human acute monocytic leukemia cell line. In our experiment, we first transfected THP-1 with pre-miR precursors and after 24-h cell settlement, we challenged THP-1 with LPS for 2 h and checked for the expression of TF mRNA. The present results showed that overexpression of miR-19b and miR-20a reduced the levels of TF mRNA by 40–60% in comparison with cells transfected with the scrambled precursor and later activated with LPS (Fig. 4A). This reduction in mRNA was also accompanied by a statistically significant reduction of TF protein expression as measured by TF procoagulant activity (Fig. 4B). Concordantly, the inhibition of miR-19b and miR-20a increased the levels of TF mRNA by 15% and 12%, respectively (Fig. 4A). On the other hand, the procoagulant activity in cells transfected with anti-miR-20a significantly augmented by 50% in comparison with cells transfected with the scrambled control (Fig. 4B).

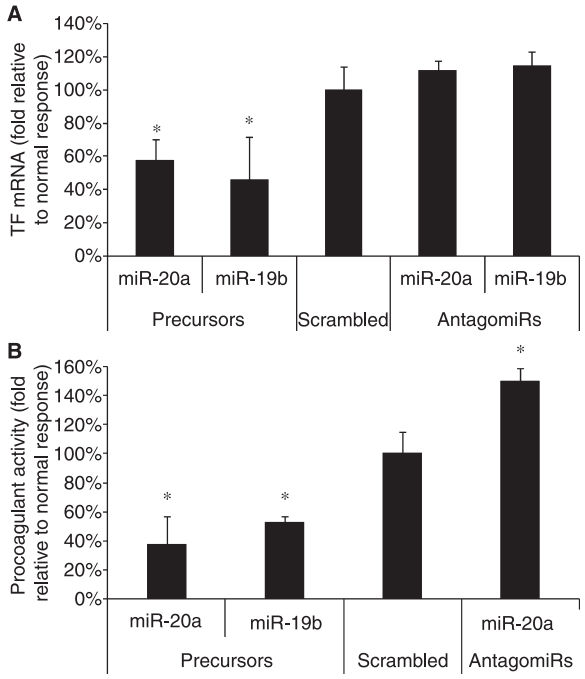

Fig. 4. Inhibition of tissue factor (TF) expression by miR-19b and miR-20a in THP-1. THP-1 cells were transfected with 100 nmol L^{-1} precursors of miR-19b and miR-20a or a scrambled precursor and with 100 *n* miRNA antagonists (antagomiRs) and (A) activated after 24 h with 10 μg mL^{-1} lipolysaccharide (LPS) for 2 h. TF mRNA was measured by qRT-PCR and normalized with respect to β-actin mRNA, (B) activated after 24 h with 10 μg mL^{-1} LPS for 5 h. Cells were lysed and the cytosolic fraction was incubated with 10 μg mL^{-1} FX, 5 U mL^{-1} rFVIIa and 5 mmol L^{-1} CaCl2. FXa generation was quantified at 405 nm after addition of FXa substrate. The normal response was TF mRNA or procoagulant activity difference between THP-1 cells incubated with or without LPS, transfected with 100 nmol L^{-1} scrambled control and set as 100%. Differences with cells transfected with scrambled controls were analyzed by means of Student's *t*-test, $*P < 0.05$. The data shown are mean ± SD of five independent experiments with three replicates each (mRNA) and three independent experiments with three replicates each (procoagulant activity).

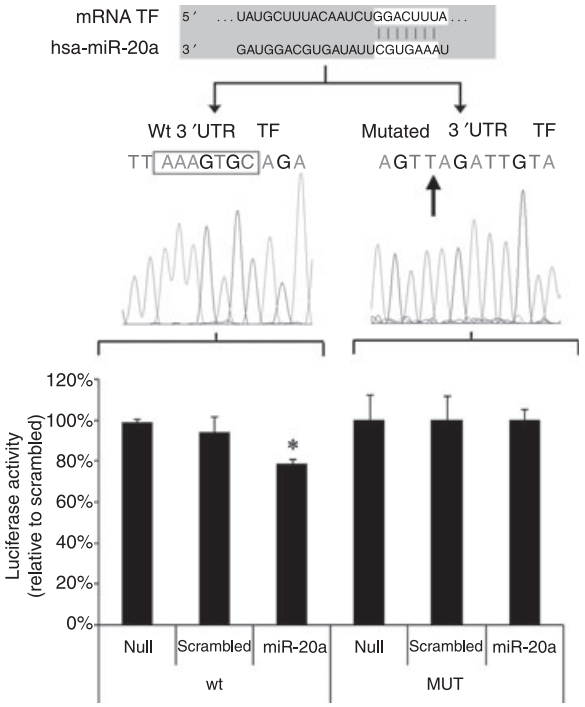

Fig. 5. Luciferase expression assay. HEK293T cells were transfected with either wild-type (WT) 3′UTR TF or Mutant 3′UTR TF in which the seven nucleotides forming the seed region were deleted, along with miR-20a precursor (100 nmol L^{-1}). A scrambled precursor was used as a control. Luciferase activities were normalized to renilla activities. Differences between WT and mutants were analyzed by means of Student's *t*-test, $*P < 0.01$. Results (mean ± SD) were obtained from four independent experiments performed in quadruplicate.

TF is a direct target of miR-20a

Recently, miR-19b has been shown to directly bind to TF mRNA [17]. Aiming to test for a miR-20a interaction, we generated two different luciferase constructs carrying the 3′UTR sequence of TF downstream of the luciferase coding region, and containing the wild-type (WT 3′UTR TF) or mutated (Mutate 3′UTR TF) 3′UTR sequence of TF without the 7-mer seed that interacts with miR-20a. HEK-293T cells, expressing low levels of miR-20a, were transfected with the luciferase vectors and the miR-20a precursor together with a pRL-TK control vector for normalization. Overexpression of miR-20a substantially reduced luciferase activity in WT 3′UTR FT but it exhibited no effect on the mutated construct lacking seven nucleotides forming the 8-mer seed core (Fig. 5).

Mir-19b and mir-20a are decreased in APS and SLE patients

APS and SLE are two conditions known to provoke a higher level of TF in monocytes and endothelial cells. We checked in patients with APS ($n = 10$) or SLE ($n = 23$), TF levels in monocytes purified by negative selection using specific antibodies and compared with that obtained in a group of healthy individuals ($n = 17$). As shown in Fig. 6A, differences in TF levels in monocyte membrane and mRNA were significantly higher in patients with APS and in a lesser degree from SLE than in controls. Aiming to test the hypothesis that miRNA may influence TF levels from patients with APS and SLE, we measured using qRT-PCR the levels of miR-19b and mir-20a in monocytes from APS and SLE patients. In APS patients, the levels of these two miRNAs have an approximate seven-fold decrease in comparison with monocytes from healthy controls (Fig. 6B). In monocytes from SLE patients, miR-20a levels were also lower than those from healthy subjects (five-fold decrease) (Fig. 6B). As expected, Pearson's correlation using data from all individuals showed a statistically significant positive score for the expression of both miRNAs and, importantly, the correlation study also indicated statistically significant negative scores of −0.373 for miR-19b and of −0.608 for miR-20a between TF expression on the cell surface and endogenous mature miRNAs, indicating that while one parameter decreases (miRNA) the other increases (TF) (Fig. 6C). As expected, expression of miR-19b and miR-20a was highly correlated (Fig. 6D).

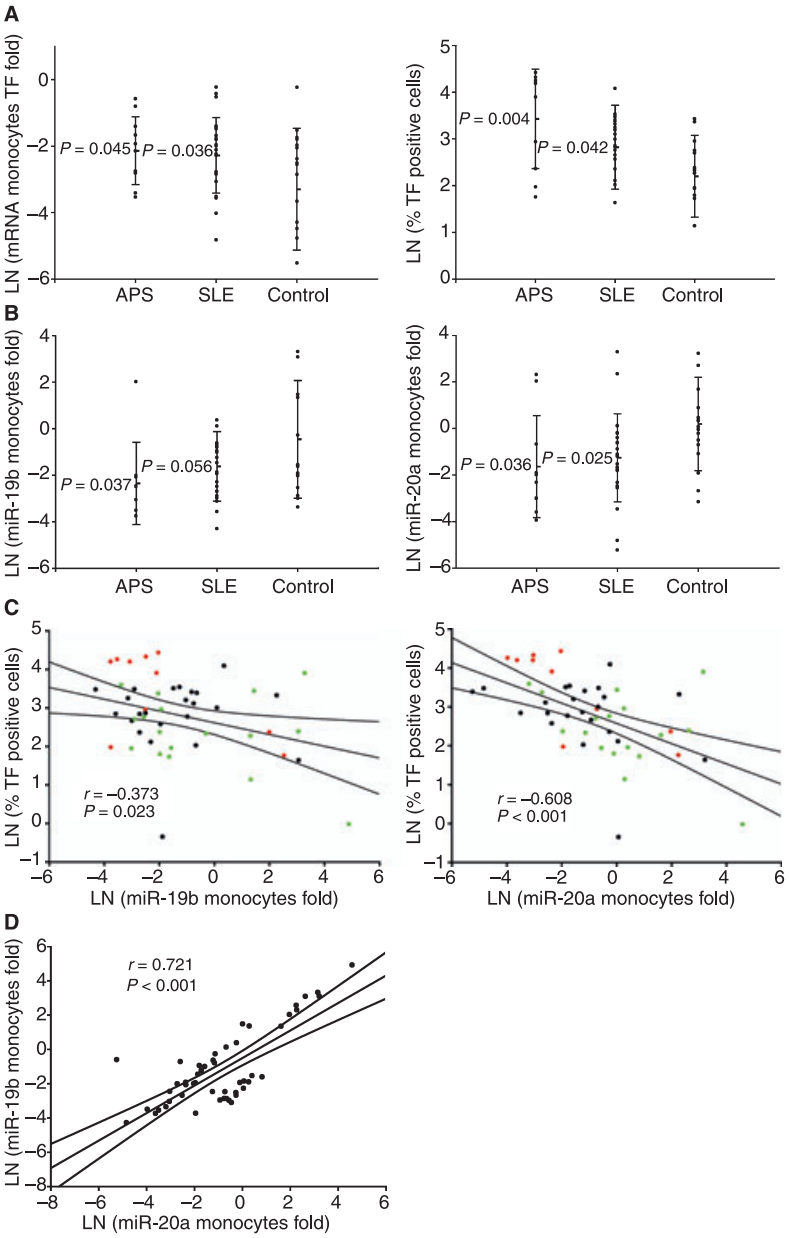

Fig. 6. Tissue factor (TF) membrane, miR-19b, and miR-20a expression levels in antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) patients. (A) TF membrane and mRNA levels in monocytes purified by negative selection were measured by flow cytometry and qRT-PCR normalized with β-actin in APS ($n = 10$) and SLE ($n = 27$) patients and healthy controls ($n = 17$). (B) miRNAs levels in patients and healthy controls were measured by qRT-PCR and normalized with U6 snRNA. Differences were analyzed by means of Student's *t*-test. Statistical significance was taken as $P < 0.05$. (C) Pearson's correlations between endogenous mature miRNAs (miR-19b and miR-20a) and TF expression on cell surface. Black dots are SLE patients, red dots are APS patients, and green dots are controls. (D) Pearson's correlation between both endogenous mature miRNAs in all subjects. Statistical significance was taken as $P < 0.05$. The results were normalized and presented as Ln (fold or % TF positive cells).

Discussion

The last few years have been marked by the emergence of miRNAs as fundamental regulators of different biological and pathophysiological processes. The present results demonstrate that miR-19b and miR-20a, miRNAs from the miR 17∼92 cluster, could be modulators of TF expression.

Our *in silico* results showed that the 3′UTR of TF mRNA contains conserved target sites of miR-20a; these *in silico* data were validated by *in vitro* reporter assays indicating that miR-20a may directly inhibit TF expression. Indeed, the rapid and profound TF mRNA decrease observed only after 6-h transfection with miRNA precursors (Fig. 3B) further suggest that the miRNA inhibition is performed through a direct mechanism. To note, these results are in accord with a recent paper showing that in mammalians, the protein decrease owing to miRNA regulation is mainly done through the destabilization and degradation of the target mRNA [25]. A recent report showed that miR-19a (from miR 17∼92 cluster) directly

inhibits TF in MDA-MB-231 cells in accordance with the results obtained in the present study [17].

SLE and mainly APS are pathologies with a high incidence of thrombotic episodes: deep vein thrombosis, stroke and recurrent fetal loss among others. In particular, APS is characterized by the presence of aPLs that may play a significant role in the development of thrombosis. Several studies have shown that aPLs induce the transcription of TF in endothelial cells and monocytes [26–29]. However, the mechanism that promotes this hypercoagulable state in patients with APS is not yet fully elucidated. The levels of TF observed in patients with APS and SLE are heterogeneous [16]. This variation can be explained by differences in transcription, stability, or regulatory elements such as miRNAs. In the present study, we confirm that monocytes from patients with APS and SLE have a significantly higher expression of membrane TF, directly related to the procoagulant activity of the molecule [30,31], in comparison with controls. In addition, we showed that miRNA levels were inversely correlated with the membrane TF levels. Obviously, this type of correlation does not necessarily imply that the decrease levels of miRNAs cause the increase levels of TF, but our *in vitro* data are sufficiently suggestive to propose that a low expression of miR-19b and miR-20a (~30% of the mean level of healthy controls) may increase TF expression. Indeed, the present study suggests that endogenous levels of miR-19b and miR-20a may be important for the control of TF expression. Indeed, inhibition of miR-20a in THP-1 cells provoked a 50% increase of their procoagulant activity after LPS induction in comparison with cells transfected with a scrambled control. The mechanism by which miRNAs are down-regulated in monocytes from APS and SLE patients is a crucial point that remains to be clarified. A recent work showed that LPS selectively increases the transcription of certain miRNAs in a NF-κB-dependent manner in human monocytes [32]. Thus, other processes not yet characterized may be involved in the regulation of these miRNAs.

On the other hand, multiple causes may be responsible of interindividual variations in miRNAs levels such as the presence of single nucleotide polymorphisms (SNPs). In fact, several studies suggest that a gain or a loss of miRNA function is associated with disease progression and prognosis (see review [33–35]) and that miRNAs are differentially expressed in several diseases (see review [36]). A recent study [37] identified 16 miRNAs differentially expressed in peripheral blood mononuclear cells from a SLE patient, showing that miR-17, a member of the miR-17~92 cluster which may potentially inhibit TF expression as our *in silico* results show (Table 1), was downregulated. Thus, SNPs present in pri-, pre- and mature-miRNA can potentially impair or enhance the processing and expression of miRNAs and/or modulate the target selection of these miRNAs [34]. These interindividual variations may subtly and precisely affect TF expression and thus be a relevant element affecting the risk of thrombosis.

Finally, our studies could have potential therapeutic utility in diseases such as SLE and APS where TF plays an important role in the development of thrombotic episodes. Although improvements in tissue specificity is required, therapies based on miRNAs have already been tested [36,38] and certainly in the coming years targeted therapeutics will emerge as new and important instruments to control certain diseases.

In conclusion, the present study suggests for the first time a potential role of miRNAs in patients with SLE and APS, that have a reduced expression of miR-19b and miR-20a that is inversely correlated with TF membrane expression. These results shed new light on new mechanisms that may regulate the expression of TF and thus the occurrence of thrombotic events. Inhibitors of TF expression are attractive therapeutic targets for thrombosis occurrence. The results shown here provide a target that may accomplish this objective and potentially decrease the thrombotic risk.

Addendum

R. Teruel, C. Pérez-Sánchez designed research, performed research and analyzed data. J. Corral and C. López-Pedrera designed research, analyzed data and critically revised the manuscript. M.T. Herranz, E. Saiz and V. Roldán collected data and samples. V. Pérez-Andreu and I. Martínez-Martínez performed research and analyzed data. N. García-Barberá performed research. V. Vicente critically revised the manuscript. C. Martínez designed research, performed research, analyzed data and wrote the manuscript.

Acknowledgements

The authors are grateful to J. Lozano, T. Iturbe and P. Conesa for patient recruitment. R.T. holds FPI fellowships from the Spanish Ministry of Science and Innovation (BES-2007-16973). V.P.A. holds a Río Hortega Fellowship from ISCIII. C.M. is an investigator from Fundación para la Formación e Investigación Sanitarias de la Región de Murcia (FFIS). This work was supported by research grants from ISCIII and FEDER (PI08/1506, EMER07/035), ISCIII (Red RECAVA RD06/0014/0039), Fundación Séneca (07703/GERM/07), Research Grants from Junta de Andalucía (PI0246 y P08-CVI-04234), and Fondo de Investigación Sanitaria (PS09/01809).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied

by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1 Boyd SD. Everything you wanted to know about small RNA but were afraid to ask. *Lab Invest* 2008; **88**: 569–78.
2 Fabbri M, Croce CM, Calin GA. MicroRNAs. *Cancer J* 2008; **14**: 1–6.
3 Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. *Trends Mol Med* 2006; **12**: 580–7.
4 Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer. *Annu Rev Med* 2009; **60**: 167–79.
5 Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. *Circulation* 2010; **121**: 1022–32.
6 Zhang H, Li Y, Lai M. The microRNA network and tumor metastasis. *Oncogene* 2010; **29**: 937–48.
7 Fort A, Borel C, Migliavacca E, Antonarakis SE, Fish RJ, Neerman-Arbez M. Regulation of fibrinogen production by microRNAs. *Blood* 2010; **116**: 2608–15.
8 Muth M, Theophile K, Hussein K, Jacobi C, Kreipe H, Bock O. Hypoxia-induced down-regulation of microRNA-449a/b impairs control over targeted SERPINE1 (PAI-1) m. *J Transl Med* 2010; **8**: 33.
9 Teruel R, Corral J, Perez-Andreu V, Martinez-Martinez I, Vicente V, Martinez C. Potential role of miRNAs in developmental haemostasis. *PLoS ONE* 2011; **6**: e17648.
10 Arnout J, Hoylaerts MF, Lijnen HR. Haemostasis. *Handb Exp Pharmacol* 2006; **176 Pt2**: 1–41.
11 Furie B. Pathogenesis of thrombosis. *Hematology Am Soc Hematol Educ Program* 2009; 255–8.
12 Shantsila E, Lip GY. The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. *Thromb Haemost* 2009; **102**: 916–24.
13 Zwicker JI, Liebman HA, Neuberg D, Lacroix R, Bauer KA, Furie BC, Furie B. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res* 2009; **15**: 6830–40.
14 Dobado-Berrios PM, Lopez-Pedrera C, Velasco F, Aguirre MA, Torres A, Cuadrado MJ. Increased levels of tissue factor mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. *Thromb Haemost* 1999; **82**: 1578–82.
15 Lopez-Pedrera C, Buendia P, Cuadrado MJ, Siendones E, Aguirre MA, Barbarroja N, Montiel-Duarte C, Torres A, Khamashta M, Velasco F. Antiphospholipid antibodies from patients with the antiphospholipid syndrome induce monocyte tissue factor expression through the simultaneous activation of NF-kappaB/Rel proteins via the p38 mitogen-activated protein kinase pathway, and of the MEK-1/ERK pathway. *Arthritis Rheum* 2006; **54**: 301–11.
16 Nojima J, Masuda Y, Iwatani Y, Suehisa E, Futsukaichi Y, Kuratsune H, Watanabe Y, Takano T, Hidaka Y, Kanakura Y. Tissue factor expression on monocytes induced by anti-phospholipid antibodies as a strong risk factor for thromboembolic complications in SLE patients. *Biochem Biophys Res Commun* 2008; **365**: 195–200.
17 Zhang X, Yu H, Lou JR, Zheng J, Zhu H, Popescu NI, Lupu F, Lind SE, Ding WQ. MicroRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells. *J Biol Chem* 2011; **286**: 1429–35.
18 Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; **120**: 15–20.
19 Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, Macmenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet* 2005; **37**: 495–500.
20 John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol* 2004; **2**: e363.
21 Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. *J Thromb Haemost* 2009; **7**: 198–205.
22 Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, Brey R, Derksen R, Harris EN, Hughes GR, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum* 1999; **42**: 1309–11.
23 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; **40**: 1725.
24 Zhou JN, Ljungdahl S, Shoshan MC, Swedenborg J, Linder S. Activation of tissue-factor gene expression in breast carcinoma cells by stimulation of the RAF-ERK signaling pathway. *Mol Carcinog* 1998; **21**: 234–43.
25 Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010; **466**: 835–40.
26 Amengual O, Atsumi T, Khamashta MA, Hughes GR. The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb Haemost* 1998; **79**: 276–81.
27 Kornberg A, Blank M, Kaufman S, Shoenfeld Y. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. *J Immunol* 1994; **153**: 1328–32.
28 Reverter JC, Tassies D, Font J, Monteagudo J, Escolar G, Ingelmo M, Ordinas A. Hypercoagulable state in patients with antiphospholipid syndrome is related to high induced tissue factor expression on monocytes and to low free protein s. *Arterioscler Thromb Vasc Biol* 1996; **16**: 1319–26.
29 Zhou H, Wolberg AS, Roubey RA. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. *Blood* 2004; **104**: 2353–8.
30 Cuadrado MJ, Lopez-Pedrera C, Khamashta MA, Camps MT, Tinahones F, Torres A, Hughes GR, Velasco F. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. *Arthritis Rheum* 1997; **40**: 834–41.
31 Noguchi M, Sakai T, Kisiel W. Correlation between antigenic and functional expression of tissue factor on the surface of cultured human endothelial cells following stimulation by lipopolysaccharide endotoxin. *Thromb Res* 1989; **55**: 87–97.
32 Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, Tamassia N, Mantovani A, Cassatella MA, Locati M. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A* 2009; **106**: 5282–7.
33 Chen K, Song F, Calin GA, Wei Q, Hao X, Zhang W. Polymorphisms in microRNA targets: a gold mine for molecular epidemiology. *Carcinogenesis* 2008; **29**: 1306–11.
34 Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* 2010; **10**: 389–402.
35 Sun G, Yan J, Noltner K, Feng J, Li H, Sarkis DA, Sommer SS, Rossi JJ. SNPs in human miRNA genes affect biogenesis and function. *RNA* 2009; **15**: 1640–51.
36 Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 2009; **10**: 704–14.
37 Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, Xu ZM, Yin YB. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus* 2007; **16**: 939–46.
38 Petri A, Lindow M, Kauppinen S. MicroRNA silencing in primates: towards development of novel therapeutics. *Cancer Res* 2009; **69**: 393–5.