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miR-133a Regulates Vitamin K 2,3-Epoxide Reductase Complex Subunit 1 (VKORC1), a Key Protein in the Vitamin K Cycle

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Regulation of key proteins by microRNAs (miRNAs) is an emergent field in biomedicine. Vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) is a relevant molecule for cardiovascular diseases, since it is the target of oral anticoagulant drugs and plays a role in soft tissue calcification. The objective of this study was to determine the influence of miRNAs on the expression of VKORC1. Potential miRNAs targeting *VKORC1* mRNA were searched by using online algorithms. Validation studies were carried out in HepG2 cells by using miRNA precursors; direct miRNA interaction was investigated with reporter assays. *In silico* studies identified two putative conserved binding sites for miR-133a and miR-137 on *VKORC1* mRNA. *Ex vivo* studies showed that only miR-133a was expressed in liver; transfection of miRNA precursors of miR-133a in HepG2 cells reduced *VKORC1* mRNA expression in a dose-dependent manner, as assessed by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) as well as protein expression. Reporter assays in HEK293T cells showed that miR-133a interacts with the 3′UTR of *VKORC1*. Additionally, miR-133a levels correlated inversely with *VKORC1* mRNA levels in 23 liver samples from healthy subjects. In conclusion, miR-133a appears to have a direct regulatory effect on expression of VKORC1 in humans; this regulation may have potential importance for anticoagulant therapy or aortic calcification.

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INTRODUCTION

Vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) is an essential element involved in the correct γ-carboxylation of vitamin K–dependent proteins such as Gas6, matrix-GLA protein and osteocalcin, as well as hemostatic proteins C, S and Z and coagulation factors II, VII, IX and X (1–3). As such, VKORC1 is the molecular target of vitamin K anticoagulants (VKAs) (4). Therapy with these drugs is the main strategy currently used for prevention and treatment of several diseases with a high thrombotic risk, including myocardial infarction, atrial fibrillation, stroke, prosthetic heart valve replacement and venous thrombosis (5). Common single nucleotide polymorphisms (SNPs) located at the 5′ and 3′ untranslated regions (UTRs) as well as within intronic sequences that modify VKORC1 protein levels are strong determinants of the oral anticoagulant requirements; however, along with other pharmacogenetic elements located in the *CYP2C9* or *CYP4F2* genes, they only partially explain the high intra-patient variability in VKA dose (6,7). Thus, the search for new elements that may regulate these proteins is not only relevant for VKA dose, but also for other pathologies where a dysregulation of these proteins may occur.

Identified in 1993, microRNAs (miRNAs) are a short class of noncoding RNA molecules (~22 nucleotides [nt]), which bind to the 3′UTR of target mRNAs by base pairing and regulate their degradation and/or translation (8). To date, the miRBase database has cataloged 21,643 mature miRNA products in 168 species; among these, 1,921 correspond to human mature miRNAs sequences (http://www.mirbase.org/

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search.shtml; release 18) involved in a wide range of processes, such as differentiation, development, apoptosis and tumorigenesis (9). miRNAs have also been related to disease progression and, interestingly, to potential drug efficacy (10). A growing number of studies have shown that miRNA expression unbalance may have dramatic effects in diverse cardiovascular pathologies such as arrhythmias, fibrosis, ischemic heart disease or cardiac hypertrophy (11,12).

Thus, our aim was to evaluate miRNAs as additional novel regulatory factors affecting VKORC1 expression, which might be of interest in areas where VKORC1 plays a role (that is, oral anticoagulant therapy and soft tissue calcification).

MATERIALS AND METHODS

In Silico Identification of *VKORC1* miRNA Binding Sites

We used various widely used algorithms for the target site prediction. Mature miRNAs against human *VKORC1* 3′UTR were queried by using the TargetScanS algorithm (release 5.1: http://www.targetscan.org). RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) (13) was used to calculate heteroduplex binding free energy values. Additionally, we used miRanda (http://www.microrna.org/microrna/home.do) to do a more restrictive miRNA selection, and only those miRNA target pairs identified by all three software programs as binding to the same location in the target *VKORC1* 3′UTR sequence were studied.

Tissue Samples and Cell Lines

A total of 23 frozen human liver samples were used to evaluate potential correlation between the expression of miR-133a and *VKORC1* mRNA. All the samples were provided by the Research Center of Experimental Pathology Department of La Fe Hospital and CIBERehd (Valencia, Spain). These samples, with no histological evidence of pathology, were obtained from discarded liver grafts or from elective liver biopsies after gaining informed consent that conformed to the rules of the hospital's ethics committee.

We used HepG2, a hepatocarcinoma cell line with endogenous expression of *VKORC1* mRNA and HEK-293T, a human embryonic kidney cell line obtained from ATCC (Manassas, VA, USA). Both cell lines were cultured according to ATCC protocols.

HepG2 Transfection

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Cells (10^5 for RNA extraction and 30×10^6 for protein extraction) were precultured for 24 h in complete medium without antibiotics and transfected at 40–60% confluence with three different concentrations (50–200 nmol/L) of precursor molecules (pre-miR) for miR-133a and scrambled control (SCR) (Life Technologies, Carlsbad, CA, USA) for mRNA analysis or with a unique concentration of 100 nmol/L for protein analysis by using the siPORT™ NeoFX™ transfection agent (Life Technologies). RNA or proteins were extracted from cells 48 h after transfection.

RNA Isolation, Quantification of miRNA and mRNA Expression Levels, and *VKORC1* Genotyping

Total RNA from human livers and from transfected cells was isolated using Trizol® Reagent (Life Technologies). RNA integrity was verified using an Experion™ 700-7030 bioanalyzer (Bio-Rad, Hercules, CA, USA). All samples showed a RNA quality indicator ≥8. RNA samples were stored at –80°C until used in the experiments.

miRNA and mRNA quantifications were carried out as previously described (14). Quantification of *VKORC1* was performed by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) by using TaqMan® Gene Expression Assay (Hs01653025_m1; Life Technologies). Expression of β-actin (Hs99999903_m1; Life Technologies) was used as an endogenous reference control. Antithrombin (*SERPINC1*) and coagulation factor 8 (*F8*) mRNAs were quantified by using designed oligonucleotides (SERPINC1_F, SERPINC1_R, F8_F and F8_R; Supplementary Table S1), and qRT-PCR was performed by using Sybrgreen (Takara Bio Europe, Saint-Germain-en-Laye, France).

A miRNA assay kit for miR-133a (Life Technologies) was used to quantify expression levels in human hepatocytes by qRT-PCR. Expression of U6 snRNA (Life Technologies) was used as an endogenous reference control.

Human livers were genotyped for rs9923231 (*VKORC1*) by using a metabolism drug assay (C_30403261_20; Life Technologies). PCRs were performed by using an LC480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany). Expression analysis was performed in triplicate for each sample. We used the $2^{-\Delta Ct}$ method to calculate the relative abundance of miRNA and mRNA compared with endogenous control expression. Ct is the threshold cycle, and ΔCt = Ct sample – Ct endogenous control.

Endogenous VKORC1 Analysis

Forty-eight hours after transfection, HepG2 cells were collected, washed with phosphate-buffered saline (PBS) and homogenized with ice-cold PBS supplemented with 0.5% Triton X-100, 0.5% NP-40, protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 12,000*g* for 20 min at 4ºC, and the supernatants were stored at –80°C until being used. Proteins were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes and immunostained with anti-VKORC1 polyclonal antibody (Abcam, Cambridge, UK) and anti-human β-actin monoclonal antibody (Sigma-Aldrich). Detection was performed by using adequate horseradish peroxidase conjugate

secondary antibodies (GE Healthcare, Waukesha, WI, USA) and SuperSignal West Dura Chemiluminescent Substrate (ThermoFisher Scientific, Rockford, IL, USA).

Reporter Plasmids

pMIR-REPORT. A PCR product (337 nt) containing the last 300 nt of VKORC1 3′UTR (AY587020, position 8887–9224), obtained by using primers 3′UTR_F_pMIR and 3′UTR_R_pMIR from genomic DNA, was cloned in the pCR 2.1 vector (Life Technologies) (Supplementary Table S1). Positive clones were digested with *Spe*I and *Xba*I and ligated into luciferase reporter plasmid pMIR-REPORT™ (Life Technologies) previously digested with *Spe*I (Figure 1A). Correct orientation was checked by sequencing (ABI3130 XL; Life Technologies). All sequence analyses and alignments were performed with the SeqmanPro program (Lasergene, version 7.1; DNASTAR, Madison, WI, USA).

pCMV6-VKORC1. Primer pairs containing the restriction sites for *Pme*I and *Fse*I were designed to amplify the *VKORC1* 3′UTR (3′UTR_F_pCMV6 and 3′UTR_R_pCMV6; Supplementary Table S1). PCR amplification was performed on genomic DNA. Yielded PCR products were cloned in the pCR 2.1 vector (Life Technologies). Positive clones were digested with *Pme*I and *Fse*I and ligated into an expression reporter plasmid pCMV6-VKORC1 with a c-myc tag (Origene Technologies, Rockville, MD, USA) (see Figure 1A).

Plasmids Directed Mutagenesis

To generate mutations in the predicted target site for miR-133a, seven nucleotides (GGACCAA) located in the seed sequence were deleted using the QuikChange site- directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) (Figure 1B). Sequencing was performed to check for the deletion of the seven nucleotides of the seed. The primers used (del_133_AS and Del_133_S) are detailed in Supplementary Table S1.

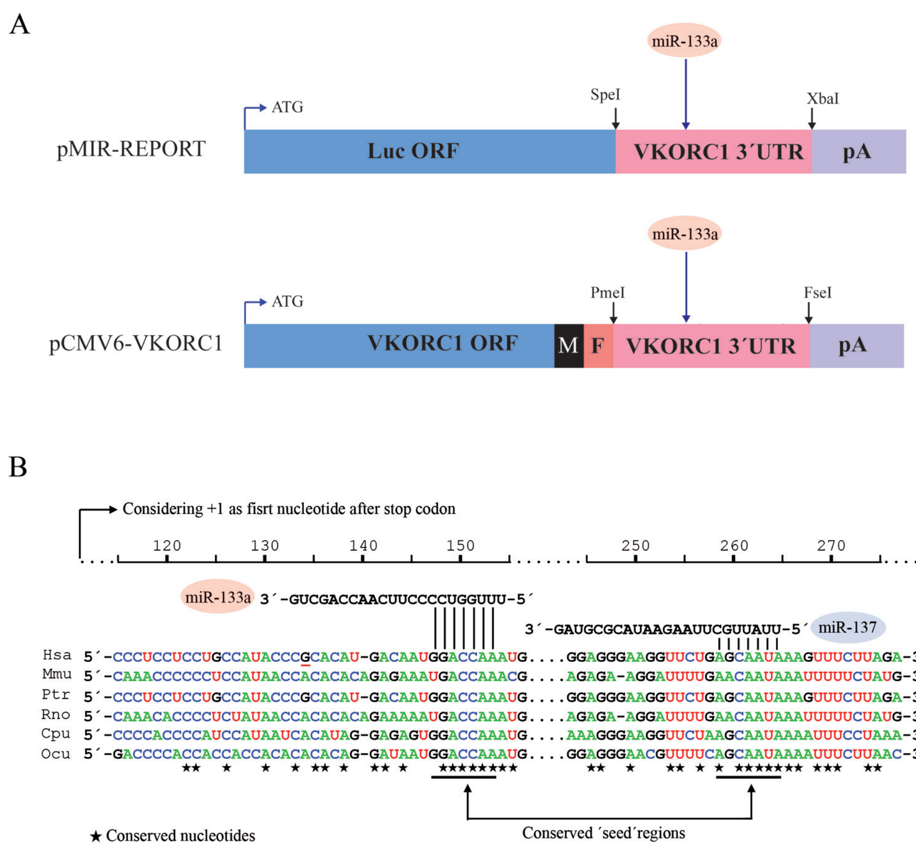

Figure 1. Reporter plasmids and miRNA binding sites in 3′UTR of *VKORC1*. (A) Schematic representation of the reporters used for the study. M, c-myc tag; F, flag tag; pA, polyA tail. (B) Multiple alignment of 3′UTR of *VKORC1* mRNA from different species.

Reporter Assays

Luciferase assay. Twenty-four hours before transfection, HEK-293T cells were plated at 100,000 cells/well in 24-well plates with complete DMEM supplemented with 10% fetal calf serum without antibiotics. Cells were cotransfected with miR-133a precursor or SCR, 3′UTR firefly luciferase reporter plasmid (500 ng/well) and 50 ng/well of renilla luciferase control plasmid (pRL-TK; Promega, Madison, WI, USA). Luciferase assays were performed as previously described (14). Briefly, luciferase assays were performed 48 h after transfection by using the Dual-Glo™ luciferase assay system (Promega). The enzymatic activities of renilla and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek, Winooski, VT, USA). Each combination of pMIR-REPORT (wild-type and mutated 3′UTR) and pRL-TK was tested in quadruplicate in four independent experiments. Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with SCR and pre-miR and set as 100%.

VKORC1 vector expression assay. Twenty-four hours before transfection, HEK-293T cells were plated at 100,000 cells/well in 24-well plates with complete DMEM supplemented with 10% fetal calf serum without antibiotics. Cells were cotransfected with miR-133a precursor or SCR and pCMV6-VKORC1 by using Lipofectamine LTX (Life Technologies); 48 h after transfection, HEK-293T cells were collected, washed with PBS and homogenized as described above with ice-cold PBS supplemented with

Table 1. Specific scores, free energy values and characteristics of the *VKORC1* 3′UTR/miRNAs binding.

MicroRNA (hsa-miR)	Context score percentile	Conserved branch length score	p^{Ct}	Start site	End site	Binding free energy (kcal/mol)
Conserved sites for miRNA families						
133	94	1.659	0.77	145	151	–18.7
137	86	1.937	0.87	254	260	–19.3
Poorly conserved sites and sites for poorly conserved miRNA families						
147b	75	0.01	NA	131	137	–19.5
330-3p	50	1.396	NA	30	36	–17.6
330-3p	56	1.396	NA	35	41	–17.6
1296	80	0.517	NA	85	91	–14
765	44	0.01	NA	116	122	–9.3
644	76	0.396	NA	156	162	–11.4
1276	75	0.01	NA	167	176	–8.6
1276	90	0.01	NA	221	226	–8.6
612	85	0.319	NA	232	238	–11.5
1285	85	0.319	NA	232	238	–22
1207-5p	42	0.323	<0.1	123	127	–9.2
183	7	0.323	NA	123	129	–10.8
609	37	0.01	NA	177	183	–10.2
620	28	0.048	NA	213	219	–8.1
1270	17	0.448	NA	213	219	–19.7
642	48	1.282	NA	240	246	–20.4
1178	84	1.207	NA	252	258	–9.3

Details of these scores can be found at http://www.targetscan.org. Binding free energy values were calculated by using the RNAHybrid algorithm (see Materials and Methods). NA, not applicable.

0.5% Triton X-100, 0.5 % NP-40, protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The homogenates were centrifuged at 12,000*g* for 20 min at 4ºC, and the supernatants were stored at –80°C until being used. BCA assays (ThermoFisher, Waltham, MA, USA) were performed to determine protein concentration of the lysates. Proteins (30 μg) were electrophoresed by SDS-PAGE, transferred onto PVDF membranes and immunostained with anti-myc monoclonal antibody (Clontech Laboratories, Mountain View, CA, USA) and anti-human β-actin monoclonal antibody (Sigma-Aldrich). Detection was performed by using adequate horseradish peroxidase conjugate secondary antibodies and the use of an ECL kit (GE Healthcare). Densitometric analysis was performed with Quantity One 1-D analysis software (Bio-Rad). Data were expressed as changes (ratio VKORC1–myc/β-actin) relative to the values of the cells transfected with SCR and pre-miRs and set as 100%. All experiments were performed five times in triplicate.

Statistical Analysis

Comparisons between groups were performed by an unpaired *t* test. Correlations were analyzed by the Pearson correlation test. We evaluated statistical significance with $P < 0.05$ regarded as significant. Analyses were carried out by using R version 2.12.0 software (http://www.r-project.org) and Statistical Package for Social Science (version 15.0; SPSS, Chicago, IL, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

In Silico Prediction Suggests the Binding of miRNAs to *VKORC1* mRNA

Two conserved binding sites in the 3′UTR of *VKORC1* were found with potential to bind miR-133a and miR-137, located at 145 and 254 bp downstream from the stop codon, respectively (see Figure 1B). Furthermore, *VKORC1* 3′UTR contains about 20 additional nonconserved binding sites also susceptible to regulation by miRNAs. Scores, free energy values and characteristics of mRNA/miRNA interactions (Table 1) encouraged us to validate these *in silico* data by performing additional *in vitro* experiments.

miRNA Expression in Human Hepatic Tissues

We checked the expression of miR-133a and miR-137 in 23 healthy hepatic tissues. By using qRT-PCR, we showed that only miR-133a was constitutively co-expressed with *VKORC1* in human hepatocytes (n = 3; miR-133a Ct = 31.5 ± 0.8 and snU6 Ct = 17.4 ± 0.9; VKORC1 Ct = 30 ± 0.36 and β-actin Ct = 27.1 ± 0.37).

miR-133a Inhibits VKORC1 Expression

To specifically investigate whether *VKORC1* expression could be regulated

at its 3′UTR by the previously mentioned miRNA, we overexpressed miR-133a in HepG2 cells, which constitutively express *VKORC1* mRNA.

Our results showed that miR-133a decreased the *VKORC1* mRNA expression in a dose-dependent fashion. A maximal decrease of ~50 % was obtained by using 200 nmol/L ($P < 0.001$; Figure 2A). The use of 50 nmol/L pre-miR also provoked a statistically significant *VKORC1* mRNA decrease ($P < 0.05$; see Figure 2A). Additionally, miR-133a precursor did not exert any significant effect at the highest dose on either *SERPINC1* or *F8* mRNA levels (Figure 2B). Thus, these experiments further support the specificity of miR-133a on *VKORC1* expression. Next, we tested endogenous VKORC1 expression in HepG2 cells. Our results showed that cells transfected with a scrambled mimic had a faint expression of VKORC1, whereas protein was mostly undetectable in cells transfected with miR-133a (Figure 2C). We further tested any potential correlation between the expression of *VKORC1* mRNA and miR-133a in the liver of 20 additional healthy subjects. As shown in Figure 3B, levels of *VKORC1* mRNA statistically and inversely correlated with those of miR-133a (Pearson correlation = –0.44; P = 0.009). To avoid the potential bias caused by genetic factors already affecting *VKORC1* mRNA levels, we also genotyped the *VKORC1* rs992323 in these samples, a SNP that is located in the promoter region of the *VKORC1* gene and has a strong impact on gene transcription. In accordance with previous reports, we confirmed that the G allele of this SNP is associated with a higher transcription rate of *VKORC1* mRNA (1) (Figure 3A). Importantly, the correlation between miR-133a and *VKORC1* mRNA levels observed for the whole sample was also confirmed for subjects carrying the GG (n = 7, P = 0.039) and GA (n = 13, P = 0.036) genotypes (Figures 3C, D). We were not able to find this correlation in polymorphic homozygote subjects (AA), probably because of the small sample size (n = 3) (data not shown).

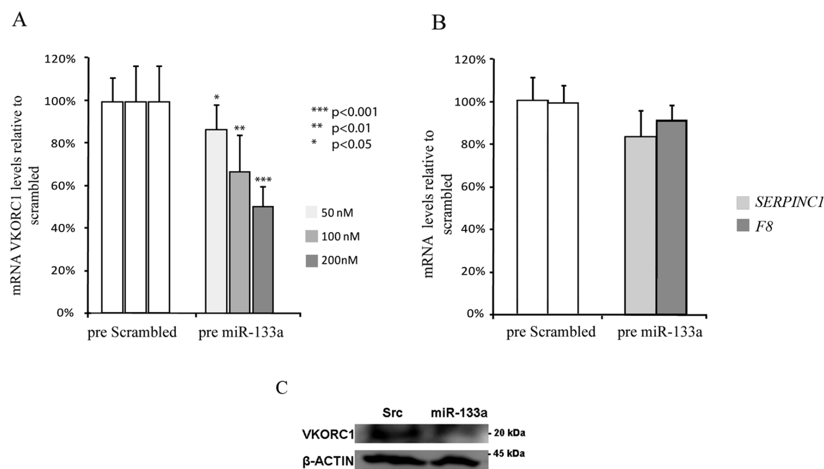

Figure 2. Validation and specificity of *VKORC1* mRNA-miRNA interaction in HepG2 cells. (A) HepG2 cells were transfected with precursor molecules for miR-133a and with an SCR at three different concentrations. (B) HepG2 cells were transfected with 200 nmol/L of precursors for miR-133a and with an SCR control and tested for *F8* and *SERPINC1* mRNA expression by qRT-PCR. Results are represented as means ± standard deviation (SD) from five experiments performed in triplicate. Student *t* test was calculated in SCR versus pre-miR (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (C) Expression of endogenous VKORC1 in HepG2 after transfection with miR-133a (100 nmol/L). β-actin serves as the loading control.

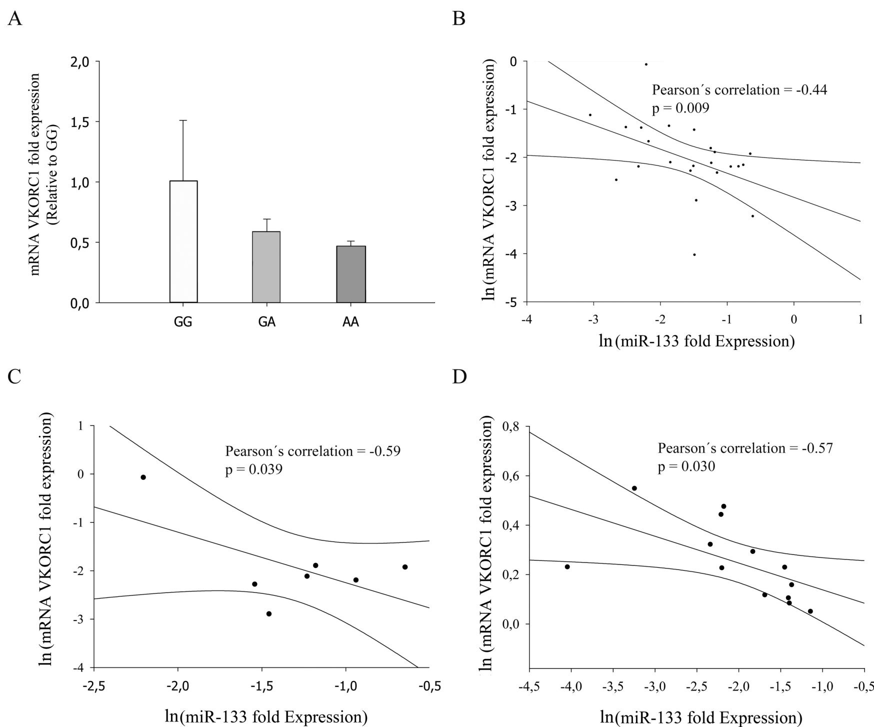

Figure 3. *VKORC1* mRNA and miR-133a levels in healthy livers. (A) Genotyping of rs9923231 was performed in 23 human healthy livers by using a validated TaqMan SNP Genotyping Assay (GG, wild-type homozygotes; GA, heterozygotes; AA, polymorphic homozygotes). Pearson correlation between miR-133a and *VKORC1* mRNA was performed in all the samples (B), GG samples (C) and GA samples (D). Statistical significance was taken as $P < 0.05$. The results are presented as ln fold change.

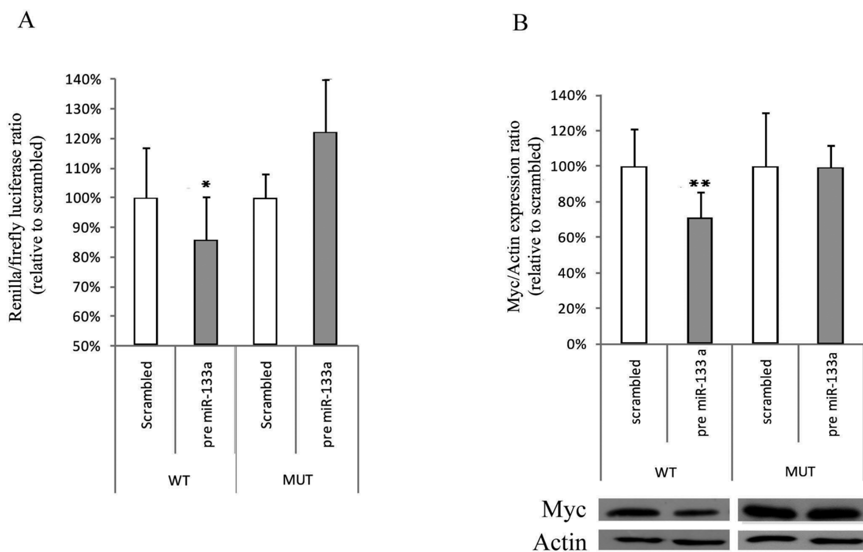

Figure 4. Reporter studies of the direct *VKORC1* mRNA-miRNA interaction. (A) pMIR-REPORT with the *VKORC1* 3′UTR binding site for miR-133a (wild-type (WT) or mutated for the miR-133a binding site (MUT)) were cotransfected with pRL-TK vector and scrambled control or pre–miR-133a. Each combination of pMIR-REPORT (WT and MUT) and pRL-TK was tested in quadruplicate in four independent experiments. Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with SCR and pre-miR and set as 100%. (B) Western blot analysis of myc-VKORC1 expression levels in HEK293T cells after cotransfection with pCMV6-*VKORC1* 3′UTR WT or MUT and scrambled control or pre–miR-133a. Fusion protein was detected with an anti-c-myc antibody. Densitometric analysis was performed with Quantity One 1-D analysis software. A representative Western blot is shown. Statistical significance was taken as $P < 0.05$. Results are represented as mean ± SD from four (A) and six (B) experiments performed in triplicate. t-Student was calculated in scrambled control versus pre-miR (*$P < 0.05$; **$P < 0.01$).

Validation of miRNA-*VKORC1* mRNA Interaction

To test the hypothesis that miR-133a can directly modulate VKORC1 expression, *VKORC1* 3′UTR was cloned downstream from the firefly luciferase open reading frame. The wild-type reporter construct or miRNA binding site deleted construct were cotransfected in the HEK-293T cell line, with an SCR or miR-133a precursor.

Our results showed that the relative luciferase activity significantly decreased in cells cotransfected with the wild-type construct and miR-133a precursors (86 ± 15%) ($P < 0.05$) (Figure 4A). However, the binding of miR-133a was completely inhibited in HEK-293T cells cotransfected with the miR-133a binding site deleted construct (122 ± 15%), indicating that *VKORC1* mRNA could be a direct target of miR-133a.

To validate these results, we performed a different experimental approach by cloning the 3′UTR of *VKORC1* in a vector expressing the VKORC1 protein (pCMV6-VKORC1). This vector expressed VKORC1 with a myc tag that allowed the visualization of VKORC1 by Western blotting techniques by using an antibody against c-myc. In addition, this experiment was designed by hypothesizing that *VKORC1* mRNA 3′UTR cloned downstream to the *VKORC1* mRNA transcribed by pCMV6-VKORC1 may have a secondary structure closer to the physiological one than that transcribed in pMIR-REPORT. We further hypothesized that this more physiological structure would potentially favor the interaction of *VKORC1* mRNA 3′UTR with miR-133a. The analysis of myc-VKORC1 protein levels 48 h after cotransfection of HEK-293T cells with a wild-type expression vector pCMV6-VKORC1 and the different miRNA precursors confirmed the previous results, with a statistically significant reduction of the protein expression for miR-133a (29%, $P < 0.01$) with respect to the nonspecific control (Figure 4B). In contrast, when we cotransfected each of the pCMV6-VKORC1 mutant constructs with the pre-miR oligonucleotides, we did not find any difference in myc-VKORC1 protein expression levels in comparison to those cells cotransfected with the SCR, indicating again that *VKORC1* seemed to be a direct target of miR-133a.

DISCUSSION

Our results indicate, for the first time, that miR-133a exerts a novel regulatory mechanism on the expression of the main genetic factor on coumarins dose requirements, VKORC1. After the *in silico* identification of potential miRNAs involved in VKORC1 expression (miR-133a and miR-137), also suggested in a previous report (15), we showed that only miR-133a was coexpressed in human healthy livers with *VKORC1*, which enables this molecule to exert a potential biological effect on *VKORC1* levels in the liver. These results are in accordance with the data from the miRNA array published by Tzur *et al.* (16). We next checked and quantified this possibility in HepG2 cells that constitutively express *VKORC1*. The results showed that miR-133a significantly decreased *VKORC1* mRNA levels in a dose-dependent manner. Additionally, we were also able to observe a decrease of endogenous VKORC1 in HepG2 cells transfected with miR-133a. In an attempt to study the nature of the interaction between miR-133a and *VKORC1* in greater depth, the entire 3′UTR of *VKORC1* was subcloned in a reporter system downstream of luciferase, and results showed an effect for precursors of miR-133a (see Figure 4A). However, it was published that the 3′UTR from a target mRNA attached to luciferase mRNA could create secondary structures that may be different from the physiological target mRNA framework (17). To further confirm our previous results, a vector

expressing VKORC1 with an attached c-myc tag allowed us to quantify by immunoblotting using an anti-c-myc antibody and the VKORC1 expression and to obtain additional and robust evidence that miR-133a binds to *VKORC1* 3′UTR.

Finally, the significant and inverse correlation found between miR-133a and VKORC1 levels in 23 liver samples from healthy subjects strongly suggests that VKORC1 expression in human liver may be controlled by a fine-tuning regulation of at least miR-133a. Thus, we reported multiple evidences by using five different approaches based in *in silico*, *in vitro* and *ex vivo* experiments supporting that miR-133a regulates VKORC1 expression.

Additionally, it may be logical to think that inter-individual variations in miR-133a levels, which may be explained by genetic factors such as SNPs in the miRNA gene, as well as SNPs affecting the 3′UTR of target genes, may have pathophysiological and pharmacogenetic consequences because of the key relevance in these two fields. In fact, evidence suggests that a gain or a loss of miRNA function is associated with disease progression and prognosis (18–20), and several studies show that miRNAs are differentially expressed in several diseases (21).

CONCLUSION

One of the most important potential effects of VKORC1 regulation by miRNAs may be the consequences on oral anticoagulant therapies that target this protein. Our results show that miR-133a inversely correlates with *VKORC1* mRNA in liver samples, regardless of the *VKORC1* genotype considered. Although *VKORC1* mRNA levels are crucial to vitamin K antagonist dose (1), the potential role of the VKORC1 regulation by miRNAs on anticoagulant therapy should be further confirmed. Moreover, the relevance that inter-individual variations of miRNA levels may have for anticoagulant drug requirement is another aspect that deserves further research. Finally, VKORC1 is not only expressed in liver but also in other tissues such as endothelium and heart. The involvement of the VKORC1 γ-carboxylation system in the inhibition of soft tissue calcification was demonstrated in both matrix-GLA–deficient mice (22) and patients with aortic calcification (23). On the other hand, it was recently reported that miR-133a is necessary for proper skeletal and cardiac muscle development and function and has a profound influence on multiple myopathies, such as hypertrophy, dystrophy and conduction defects (24). The potential consequences of the direct interaction between *VKORC1* and miR-133a in arterial vascular diseases may deserve further attention in the area of cardiovascular research.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Rieder MJ, *et al.* (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N. Engl. J. Med.* 352:2285–93.
2. Stafford DW. (2005) The vitamin K cycle. *J. Thromb. Haemost.* 3:1873–8.
3. Teichert M, *et al.* (2008) Vitamin K epoxide reductase complex subunit 1 (VKORC1) polymorphism and aortic calcification: the Rotterdam Study. *Arterioscler. Thromb. Vasc. Biol.* 28:771–6.
4. Oldenburg J, Marinova M, Muller-Reible C, Watzka M. (2008) The vitamin K cycle. *Vitam. Horm.* 78:35–62.
5. Salem DN, O'Gara PT, Madias C, Pauker SG. (2008) Valvular and structural heart disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest.* 133:593S–629S.
6. Klein TE, *et al.* (2009) Estimation of the warfarin dose with clinical and pharmacogenetic data. *N. Engl. J. Med.* 360:753–64.
7. Perez-Andreu V, *et al.* (2009) Pharmacogenetic relevance of CYP4F2 V433M polymorphism on acenocoumarol therapy. *Blood.* 113:4977–9.
8. Bartel DP. (2009) MicroRNAs: target recognition and regulatory functions. *Cell.* 136:215–33.
9. Esteller M. (2011) Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12:861–74.
10. Mishra PJ, *et al.* (2007) A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc. Natl. Acad. Sci. U. S. A.* 104:13513–8.
11. Han M, Toli J, Abdellatif M. (2011) MicroRNAs in the cardiovascular system. *Curr. Opin. Cardiol.* 26:181–9.
12. Small EM, Olson EN. (2011) Pervasive roles of microRNAs in cardiovascular biology. *Nature.* 469:336–42.
13. Kruger J, Rehmsmeier M. (2006) RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res.* 34:W451–4.
14. Teruel R, *et al.* (2011) Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. *J. Thromb. Haemost.* 9:1985–92.
15. Shomron N. (2010) MicroRNAs and pharmacogenomics. *Pharmacogenomics.* 11:629–32.
16. Tzur G, *et al.* (2009) Comprehensive gene and microRNA expression profiling reveals a role for microRNAs in human liver development. *PLoS One.* 4:e7511.
17. Brodersen P, Voinnet O. (2009) Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol.* 10:141–8.
18. Chen K, *et al.* (2008) Polymorphisms in microRNA targets: a gold mine for molecular epidemiology. *Carcinogenesis.* 29:1306–11.
19. Ryan BM, Robles AI, Harris CC. (2010) Genetic variation in microRNA networks: the implications for cancer research. *Nat. Rev. Cancer.* 10:389–402.
20. Sun G, *et al.* (2009) SNPs in human miRNA genes affect biogenesis and function. *RNA.* 15:1640–51.
21. Croce CM. (2009) Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet.* 10:704–14.
22. Luo G, *et al.* (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature.* 386:78–81.
23. Koos R, *et al.* (2009) Relation of circulating Matrix Gla-Protein and anticoagulation status in patients with aortic valve calcification. *Thromb. Haemost.* 101:706–13.
24. Townley-Tilson WH, Callis TE, Wang D. (2010) MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. *Int. J. Biochem. Cell Biol.* 42:1252–5.