Original Research

Downregulation of *Clock* in circulatory system leads to an enhancement of fibrinolysis in mice

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Abstract

As a main component of circadian genes, *clock* plays not only an important role in circadian rhythm but also in the regulation of many physiological systems. The dysfunction of clock genes is associated with the development of various disorders. Many studies have investigated the association between clock genes and blood coagulation and the fibrinolytic system. The present study was designed to investigate the effect of downregulation of circulatory *Clock* on blood coagulation and fibrinolysis at the initial stage of active phase in male mice. Downregulation of the expression of the *Clock* gene by siRNA and, subsequently, its effect on the thrombotic potential and the expression of relative coagulative and/or fibrinolytic factors were investigated. It was found that the *Clock* interfered mice were less liable to thrombosis and showed prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT) at Zeitgeber time (ZT) 15. Meanwhile, these mice also showed an increase in factor VII (FVII) and a decrease in thrombomodulin (TM) and plasminogen activator inhibitor 1 (PAI-1) at ZT 15 at both transcriptional and translational levels. PT, APTT and mRNA expressions of *fvii*, *tm* and *pai-1* were analyzed with the least-squares fit of a 24-h cosine function by single cosinor method; no circadian rhythm was determined in PT and APTT, and a higher amplitude of *fvii* in the *Clock* RNAi group was found with a circadian phase shift, while lower amplitudes of *tm* and *pai-1* were found in the *Clock* RNAi group with nearly no phase shift. All these results suggest that downregulation of the *Clock* gene in circulatory system has an effect on factors involved in both blood coagulation and fibrinolysis resulting in an enhancement in mice. This may be considered as an indication that *Clock* regulates thrombotic homeostasis through the fibrinolytic system.

Keywords: *Clock*, factor VII, thrombomodulin, plasminogen activator inhibitor 1, fibrinolysis

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Introduction

Both blood coagulation and the fibrinolytic system are essential for maintaining a dynamic equilibrium of blood circulation. Once the balance is broken, it would lead to either a thromboembolic event or difficulty in hemostasis. It is well known that dysfunction of circadian genes is involved in many disorders. Circadian rhythms are demonstrated to have many correlations with the cardiovascular system[1,2] and the onset of myocardial infarction,[3–5] and the circadian genes have been increasingly investigated in thrombosis. Several studies have demonstrated circadian changes in human coagulation/fibrinolytic parameters, such as factor VII (FVII), thrombomodulin (TM) and plasminogen activator inhibitor 1 (PAI-1).[6–8] Circadian regulation of blood coagulation is also found in genetically engineered mice models.[9–11] Those studies described a robust circadian fluctuation of TM expression[9] and plasma PAI-1[10] in *Clock* mutant mice; the expression of PAI-1 was decreased in the PER2 overexpressed transgenic mice,[11] but the FVII level seemed not to be affected by the *Clock* gene mutation.[10]

Clock, the core component of the circadian system, was the first clock gene identified in vertebrates by forward mutagenesis using *N*-ethyl-*N*-nitrosourea in a behavioral screening,[12] and its significance is not only for the behavioral regulation but also for the molecular function. Maemura *et al.*[13] reported that CLOCK forms a heterodimer with CLIF and upregulates the PAI-1 gene through E-box sites in endothelial cells.

FVII plays a central role in the coagulation cascade. In conjunction with tissue factor,[14] FVII initiates the process of coagulation, and the positive feedback of extrinsic and common pathways of coagulation gets started. As a result, its activity is usually linked to increased risk for cardiovascular disease.[15] But it was also reported that certain FVII genotypes have a role in protection against myocardial infarction.[16] TM acts as an integral membrane protein that can inhibit the procoagulant effect of thrombin.[17,18] On the one hand, TM could function as a co-factor which speeds up the activation of protein C thousand fold in the thrombin-induced activation of protein C; on the other hand, it restrains thrombin activity by forming a complex with it.[14] Meanwhile, the TM–thrombin complex also inhibits fibrinolysis by cleaving thrombin-activatable fibrinolysis inhibitor into its active form.[19] PAI-1, a major regulator of the fibrinolytic system,[14] plays a vital role in regulating fibrinolysis within the circulation and is responsible for the controlled degradation of thrombi.[20] PAI-1 is widely produced by the endothelium and other tissue types;[21,22] it is the principal inhibitor of tissue plasminogen activator and urokinase, and hence an inhibitor of fibrinolysis.[23] While the association of elevated level of PAI-1 and an increased risk of thrombotic events has been widely investigated,[24,25] reports of PAI-1 deficiency are not so frequently seen, and it appears that lack of this protein is associated with a mild to moderate degree of bleeding disorder.[26,27] The first identified homozygous PAI-1 genetic defect that led to a bleeding disorder was documented in 1992.[26] Using homozygous PAI-1-deficient (PAI-$1^{-/-}$) mice, Carmeliet *et al.*[27] found that the disruption of the PAI-1 gene in mice appeared to induce a mild hyperfibrinolytic state and a greater resistance to venous thrombosis but not to impair hemostasis.

To understand the effect of disruption of the circulatory *Clock* gene on blood coagulation and the fibrinolytic system, RNA interference (RNAi) technology was employed in the present study to transiently disrupt the expression of *Clock* in mice. Three associated factors, FVII, TM and PAI-1, were chosen for analysis *in vitro* and *in vivo*, and the coagulation capacity and thrombotic potential of these *Clock* RNAi interfered mice were simultaneously evaluated. An enhancement of fibrinolysis was found in these *Clock* downregulated mice, and a possible pathway for this enhancement was discussed in this study.

Materials and methods

Cell culture

Mile sven 1 (MS1) cells (*Mus musculus* endothelial cell line) were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium (Hyclone Lab Inc., Nampa, ID, USA) supplemented with 18% fetal calf serum (Hyclone) and 100 IU/mL penicillin/streptomycin (BioWhittaker, Walkersville, MD, USA) at 37°C.

Husbandry

All studies were performed in male mice of the ICR strain (*Mus musculus*) (5 weeks old at the start of the experiments), which were purchased from Dossy Biotechnology Co, Ltd (Chengdu, China). Mice were kept in cages on a 12-h light–dark cycle with food and water available *ad libitum*. On the fourth day of the interference, 36 of these mice were sacrificed in batch with an interval of four hours, and others were sacrificed at ZT 15. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by the Ethical Committee of Investigation of Laboratory Animals of Sichuan University.

RNA interference

Cells were divided into two groups: (a) *Clock* RNAi group cells were transfected with the *pGenesil-Clock* siRNA plasmid (constructed by Wuhan Genesil Biotechnology Co., Ltd, Hubei, China) using Lipofectamine^{TM} 2000 transfection reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions; and (b) control group cells were treated with transfection reagent as the *Clock* RNAi group but no plasmid was added. Transfection was performed in triplicate.

Mice were divided into two groups with same amount: (a) the *Clock* RNAi group was transfected with the *pGenesil-Clock* siRNA plasmid (constructed by Wuhan Genesil Biotechnology Co., Ltd) by direct injection of the plasmid into the vena caudalis (40 μg plasmid DNA resolved in 200 μL saline per mouse); and (b) the control group was injected the same way with the identical amount of saline.

Blood collection

On the fourth day of the interference, the whole blood samples were collected from mice as follows: (a) the whole blood of six mice per group (1 mL/mouse) was collected without anticoagulation, and each sample was immediately placed in a Chandler loop to form artificial thrombus; (b) blood of 11 mice per group (1 mL/mouse) was collected and immediately mixed with 3.8% sodium citrate in tubes (blood:citrate, 5:1) under ether anesthesia before abdominal aorta isolation. Half of the blood samples of both groups were then centrifugated at 1500*g* for 15 min at 4°C to separate the plasma, and the plasma samples were stored at −20°C for later enzyme-linked immunosorbent assay analysis of FVII and PAI-1 levels. The remaining blood samples were analyzed within two hours of collection to evaluate coagulation capability; (c) blood of three mice per group (1 mL/mouse) was collected in batch with an interval of 4 h as described in (b), and was analyzed within two hours of collection to evaluate coagulation capability.

Evaluation of thrombotic potential in whole blood

Artificial thrombi were formed from whole blood using the Chandler loop method with some modifications.[28,29] Briefly, the thrombi (seven mice from each group) were prepared as described below. Each sample of the uncoagulated whole blood was placed in a single polyvinyl tube with a length

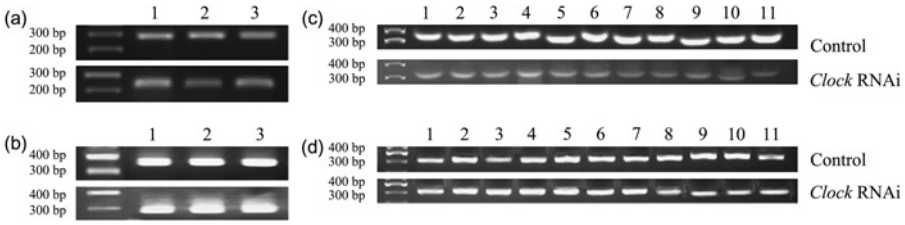

Figure 1 The transcriptional expression levels of CLOCK *in vitro* and *in vivo* were both downregulated. The electrophoresis results of reverse transcription polymerase chain reaction (RT-PCR) with cellular and vascular mRNA. This figure shows the electrophoresis results of RT-PCR with cellular and vascular mRNAs of *Clock* and *gapdh* at ZT 15. There are two groups: the *Clock* RNAi group, marked as *Clock* RNAi; and the Control group, marked as Control. (a) Cellular mRNA levels of *Clock*; (b) cellular mRNA levels of *gapdh*; (c) vascular mRNA levels of *clock*; and (d) vascular mRNA levels of *gapdh.* ZT, Zeitgeber time

of 250 mm (inner diameter = 4 mm, external diameter = 6 mm) and the two ends were joined by a silastic connector to form a Chandler loop with a diameter of 80 mm. The circular tube was placed and centered on a turntable, tilted to an angle of 74°, and rotated at 17 ± 1 rpm for 15 min at 37°C. Freshly prepared thrombi were removed, and blotted with filter paper. The humid weight of each embolus was assessed by an electronic balance capable of ± 0.01 mg accuracy, and the length was measured by a Vernier caliper capable of ± 0.02 mm accuracy. The emboli were then dried at 64°C for 20 min, and their dry weights were assessed once more.

Evaluation of plasmic coagulation capability

In coagulation screening tests, prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed by conventional clinical procedures, and a MTN-IV Coagulation Analyzer (Changchun Matenu Medical Equipment Co., Ltd, Jilin, China) was used according to the manufacturer's instructions. All reagents used in the coagulation screening tests were from Sichuan Maker Biotechnology (Sichuan, China). Note that the coagulation analyzer is designed for human use and, therefore, the data generated are best considered comparative and not quantitative.

Measurement of FVII and PAI-1 expression in plasma and TM expression in abdominal aortas

Abdominal aortas were removed immediately after the blood collection, and the protein was extracted with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. FVII and PAI-1 levels in plasma and TM levels in abdominal aortas were detected using commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Quantification of cellular and vascular *Clock*, *fvii*, *tm* and *pai-1* mRNA expression

All cells were harvested 48 h after transfection, and abdominal aortas were removed immediately after the blood collection. Total RNAs were extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. To quantify the expression of CLOCK, FVII, TM and PAI-1 at transcriptional level, reverse transcription polymerase chain reaction (RT-PCR) was performed on these RNAs. cDNA was synthesized by oligo(dT) priming with the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Inc., Burlington, ON, Canada). The primer sequences used for the PCR were as follows: *clock*: 5′ TGGCAAAATGTCACGAGC 3′ and 5′ TTAGAGGAGGCA GAAGGAGTT 3′; *fvii:* 5′ ATGGACTTTCCTGCTATTC 3′ and 5′ GCTGACTGACTGTGACTGC 3′; *tm*: 5′ CCTGCCT CAACATATCAGA 3′ and 5′ GTATTTCGGGACTACAAGG 3′; *pai-1*: 5′ TCCTGCTCAACCACCTTA 3′ and 5′ TTC AAGACCATGTGACCC 3′; and *gapdh* as a control: 5′ TCACTGCCACCCAGAAGA 3′ and 5′ AAGTCGCAGGA GACAACC 3′. RT-PCR products were detected by 1.5% agarose electrophoresis and analyzed according to the integral optical density method by using a Gel-Pro analyzer (Bio-Rad Laboratories, Irvine, CA, USA).

Statistical analysis

All data are presented as mean ± SEM. The temporal data of PT, APTT and the mRNA level of *fvii*, *tm* and *pai-1* in both group of mice were analyzed with the least-squares fit of a 24-h cosine function by single cosinor method.[30] The statistical significance of differences between the *Clock* RNAi group and the control group was calculated by Student's *t*-test. Probability values (*P*) of less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS software (version 11.0, SPSS Inc., Chicago, IL, USA).

Results

Clock was interfered by siRNA both *in vitro* and *in vivo*

Electrophoresis gel graphs of the RT-PCR of *Clock* in MS1 cells and abdominal aortas of mice are shown in Figure 1. Data of the integral optical densities obtained by a Gel-Pro analyzer are listed in Table 1. Lane-to-lane variation in the

Table 1 Comparisons of Clock mRNA levels *in vitro* and *in vivo*

	Clock RNAi group	Control group	*P*
In vitro ($n = 3$)	0.33 ± 0.03	0.73 ± 0.08	0.008
In vivo ($n = 11$)	0.36 ± 0.03	1.29 ± 0.1	<0.001

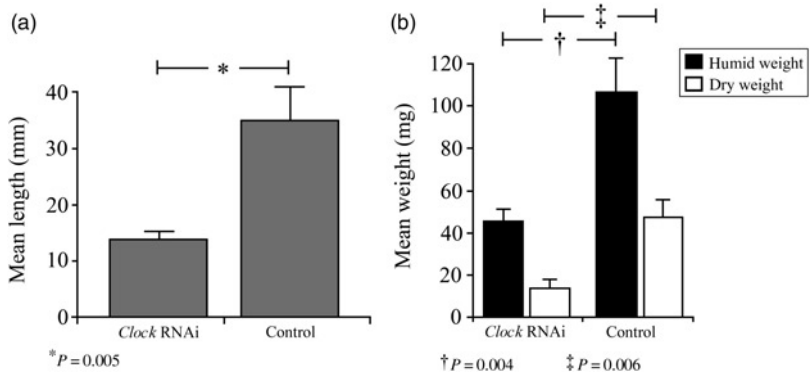

Figure 2 The comparison of length, humid weight and dry weight between two groups of artificial thrombi formed in the Chandler loop. This figure shows the characteristics of thrombi formed with mice whole blood collected at ZT 15 on the fourth day of interference. There are two groups: the *Clock* RNAi group, marked as *Clock* RNAi; and the Control group, marked as Control. (a) Mean lengths of thrombi and (b) mean humid weights and mean dry weights of thrombi. ZT, Zeitgeber time

amount of loaded mRNA was controlled internally by normalizing the level of each gene to that of *Gapdh*. In the *Clock* RNAi group, the *Clock* mRNA level was significantly decreased *in vitro* ($P = 0.008$) and *in vivo* ($P < 0.001$) compared with the control group.

The thrombotic potential was decreased in the *Clock* RNAi group

The comparison of artificial thrombi formed in the Chandler loop is shown in Figure 2. With measurement, the average length ($P = 0.005$) was found shortened, and both humid

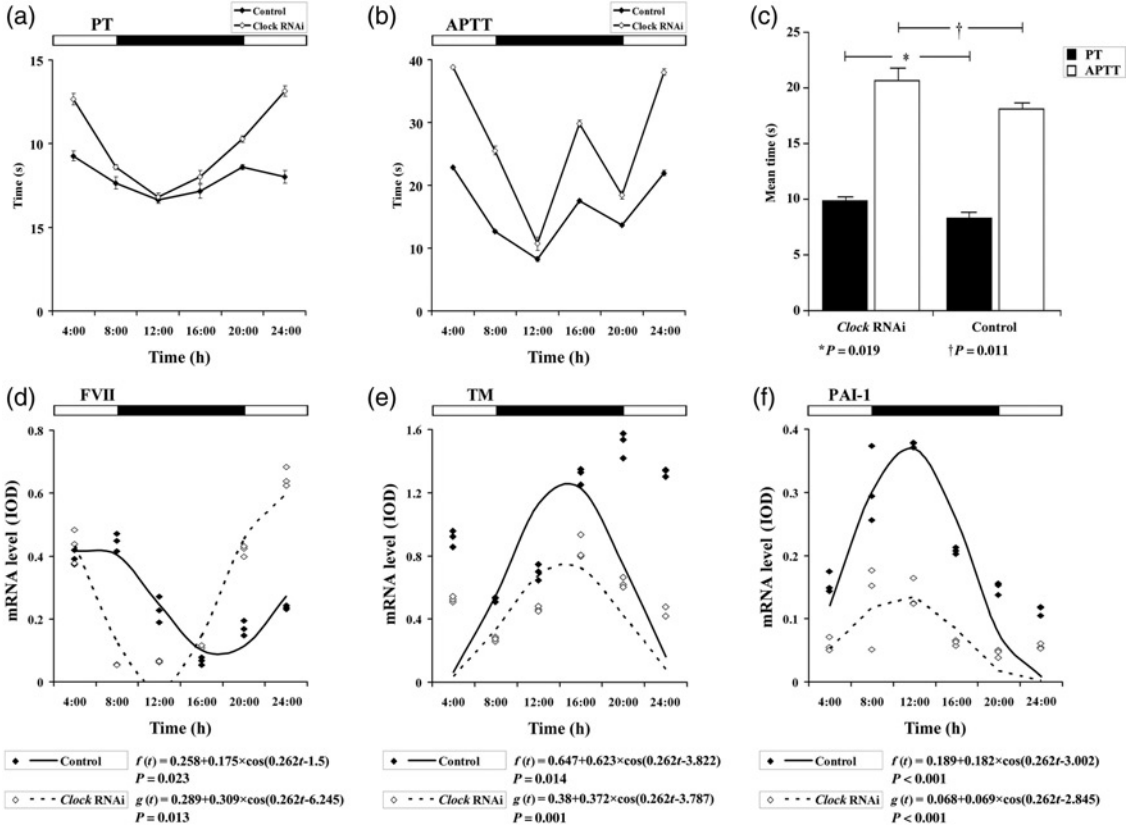

Figure 3 The temporal analysis of PT, APTT and the expressions of *fvii*, *tm* and *pai-1*. This figure shows the temporal values of PT and APTT, and the best-fitting cosine curve of vascular *fvii*, *tm*, *pai-1* mRNA expressions by single cosinor analysis. White and black bars indicate the duration of light and dark phases of the 24-h LD cycle. There are two groups: the *Clock* RNAi group, marked as *Clock* RNAi; and the Control group, marked as Control. (a) PT values of six time points in a circadian period of 24 h; (b) APTT values of six time points in a circadian period of 24 h; (c) the comparison of PT and APTT tests with mice whole blood at ZT 15, significant difference is found both in the results of PT and APTT; (d) the best-fitting cosine curve of vascular *fvii* expression with a function fitted by single cosinor analysis; (e) the best-fitting cosine curve of vascular *tm* expression with a function fitted by single cosinor analysis; and (f) the best-fitting cosine curve of vascular *pai-1* expression with a function fitted by single cosinor analysis. ZT, Zeitgeber time; PT, prothrombin time; APTT, activated partial thromboplastin time; LD, light–dark

weight ($P = 0.004$) and dry weight ($P = 0.006$) were found lessened in the *Clock* RNAi group compared with the control group.

PT and APTT values were both increased in the *Clock* RNAi group

The temporal results of the coagulation tests were analyzed with single cosinor method; the *P* values of both were greater than 0.05, so the circadian rhythm was not determined in PT and APTT. The Student's *t*-test for each time point indicated a prolongation in both PT and APTT with *P* values less than 0.05 except for 12:00 and 16:00 of PT. The comparisons of each time point are shown in Figures 3a and b. The results of PT and APTT at ZT 15 were obtained for 16 of the 22 mice. Fibrin strands were present in six out of 22 samples, which made them unsuitable for analysis. The comparisons of PT and APTT values are shown in Figure 3c. In the *Clock* RNAi group, both PT ($P = 0.019$) and APTT ($P = 0.011$) were prolonged compared with the control group.

Cellular and vascular FVII, TM and PAI-1 levels of MS1 cells were affected by *Clock* RNAi transfection at the level of transcription. The temporal mRNA levels of vascular *fvii*, *tm* and *pai-1* were analyzed with single cosinor method, and circadian rhythms were determined in both groups as shown in Figures 3d–f. There were higher amplitudes of *fvii* in the *Clock* RNAi group with a phase shift, and lower amplitudes of *tm* and *pai-1* in the *Clock* RNAi group with no phase shift. The comparison of transcriptional expression levels of FVII, TM and PAI-1 in MS1 cells and abdominal aortas of mice at ZT 15 are shown in Figure 4.

In cellular results, *fvii* mRNA levels of the *Clock* RNAi group was higher than that of the control group ($P = 0.036$), while *tm* mRNA levels ($P = 0.002$) and *pai-1* mRNA levels ($P = 0.001$) were lower. In vascular results, *fvii* mRNA levels of the *Clock* RNAi group was much higher than that of the control group ($P = 0.001$), while *tm* mRNA levels ($P < 0.001$) and *pai-1* mRNA levels ($P < 0.001$) were lower.

FVII and PAI-1 levels in plasma and TM levels in abdominal aortas were affected by *Clock* RNAi transfection

The results of protein expression levels of FVII, TM and PAI-1 at ZT 15 are shown in Table 2. In the *Clock* RNAi group, FVII level was higher than that of the control group ($P = 0.039$), while TM levels ($P = 0.01$) and PAI-1 levels ($P = 0.045$) were lower.

Discussion

For nocturnal organisms, the active phase begins at ZT 12. In this study, the lights for the animals began at 07:00, the light phase would be from 07:00 to 19:00, and the dark phase from 19:00 to the next 07:00. ZT 15 was selected to study the fibrinolysis state at the initial stage of active phase.

The results of the Chandler loop and prolonged PT and APTT at ZT 15 in *Clock* interfered mice showed a tendency of enhancement of fibrinolysis. Three important factors involved in the processes of blood coagulation and fibrinolysis, TM, PAI-1 and FVII, which can be regulated by

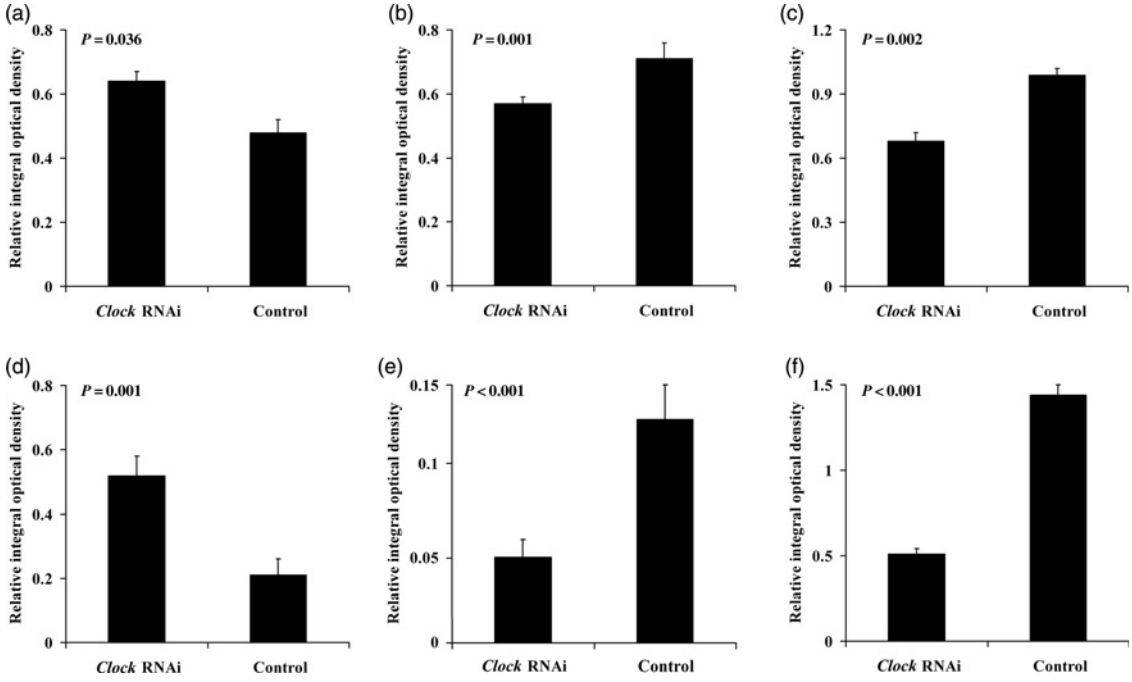

Figure 4 The comparison of transcriptional expression levels of FVII, TM and PAI-1 *in vitro* and *in vivo*. This figure shows the relative cellular and vascular mRNA levels of *fvii*, *tm* and *pai-1* at ZT 15. There are two groups: the *Clock* RNAi group, marked as *Clock* RNAi; and the Control group, marked as Control. (a) The mRNA levels of *fvii* in MS1 cells; (b) the mRNA levels of *pai-1* in MS1 cells; (c) the mRNA levels of *tm* in MS1 cells; (d) the mRNA levels of *fvii* in abdominal aortas of mice; (e) the mRNA levels of *pai-1* in abdominal aortas of mice and (f) the mRNA levels of *tm* in abdominal aortas of mice. ZT, Zeitgeber time; FVII, factor VII; PAI-1, plasminogen activator inhibitor 1; TM, thrombomodulin; MS1, Mile sven 1

Table 2 Protein expression levels of FVII, PAI-1 and TM by ELISA assay

	Clock RNAi group ($n = 11$)	Control group ($n = 11$)	*P*
FVII	48.52 ± 1.40	44.02 ± 1.46	0.039
PAI-1	41.11 ± 1.86	48.09 ± 2.69	0.045
TM	27.17 ± 1.71	41.31 ± 1.21	0.011

FVII, factor VII; PAI-1, plasminogen activator inhibitor 1; TM, thrombomodulin; ELISA, enzyme-linked immunosorbent assay

CLOCK/BMAL2 heterodimer or CLOCK by combining with E-Box in their promotor sequences,[9,13,31] were therefore chosen in the present study. The mRNA levels of *fvii*, *tm* and *pai-1* at ZT 15 were measured. Their temporal values were also analyzed with the least-squares fit of a 24-h cosine function by single cosinor method. As shown in Figures 3d–f, circadian rhythms were determined in the mRNA expressions of *fvii*, *tm* and *pai-1*. We found that expression levels of TM and PAI-1 were decreased with the interfered *Clock* gene, and the findings were consistent with those in other researches.[9,13] The expression of FVII, at both mRNA and protein levels, was however significantly increased in the *Clock* RNAi group at ZT 15, and had a phase shift between two groups; this was different from what Ohkura *et al.*[10] have reported in 2006. They pointed out that FVII remained constant throughout the day and was not affected by *Clock* gene mutations.

In Figures 3a and b, the temporal values of PT and APTT fluctuated with time, but their *P* values of single cosinor method analysis are greater than 0.05 both in the *Clock* RNAi group and in the control group. As a result, PT and APTT are not considered to be of 24-h rhythm. However, the overall durations of PT and APTT were prolonged in the *Clock* RNAi group to different degrees at different time points, and all of these prolongations, compared with the control group, are significant except for the time points of 12:00 and 16:00 in PT. It seems that the constant PT and APTT can also be regulated by *Clock* gene expression.

CLOCK seems to be a positive regulator of TM and PAI-1 mRNA expressions as previously reported;[9–11] while circadian expression of FVII mRNA seems to be negatively regulated by CLOCK protein, because both the peak and averaged expression levels were increased in *Clock* RNAi mice. However, the phase of *fvii* significantly shifted and its amplitude increased; whereas no significant differences are found in the phases of the best-fitting cosine curves in *tm* and *pai-1* between two groups. As for *tm* and *pai-1*, both their temporal expressions were decreased more in the rest phase than those in the active phase in *Clock* RNAi mice, and the maximum decreases were found constant with their respective acrophases. As for *fvii*, CLOCK appears to be involved in not only the regulation of expression level but also its circadian phase, which leads to a great phase shift in *fvii*; its acrophase was shifted from the end of the active phase in control mice to the beginning of the active phase in *Clock* RNAi mice.

From all data obtained in this study, we concluded that the interfered circadian *Clock* could lead to an enhancement on fibrinolysis. We also suspect that there might be less tendency for infarction in mice when *Clock* in the circulatory system was interfered; however, it has not been proved in experiments. It may also be inferred that the regulation of *Clock* on *pai-1* may be positive in this enhancement of fibrinolysis. Whether increased *fvii* and decreased *tm* play a positive or negative role in this enhancement is still unknown, and further research is needed to clarify it.

Author contributions: ZJ, YW and SC carried out conception and design; ZJ, YW and SC carried out analysis and interpretation of data; SC, YZ and CC carried out acquisition of data; SC drafted the article; JX and HG critically revised the article; ZW made the final approval of the article; and YL carried out statistical analysis. SC and ZJ contributed equally to this work.

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