



Clock upregulates intercellular adhesion molecule-1 expression and promotes mononuclear cells adhesion to endothelial cells



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ABSTRACT

Clock is a basic helix-loop-helix (bHLH) transcription factor that plays important role in circadian rhythms of various physiological functions. Previous study showed that the expression of intercellular adhesion molecule-1 (ICAM-1) was reduced in the liver tissues of Clock mutant mice. However, how Clock regulates ICAM-1 expression and whether Clock affects cell adhesion function remain unknown. In the present study, we found that exogenous expression of Clock upregulated the gene expressions of ICAM-1 and other adhesion-related genes including VCAM1 and CCL-2, and increased the transcriptional activity of ICAM-1 in mouse brain microvascular endothelial cell lines. In contrast, loss of Clock decreased these gene expressions and ICAM-1 transcriptional activity. Chromatin immunoprecipitation (ChIP) assay revealed that Clock binds to the E-box-like enhancer of ICAM-1 gene. ICAM-1 gene showed rhythmic expression in endothelial cells after serum shock *in vitro*, suggesting ICAM-1 may be a Clock-controlled gene. Clock regulates the adhesion of mononuclear cells to endothelial cells via ICAM-1. Together, our findings show that Clock is a positive regulator of ICAM-1, and promotes the adhesion of mononuclear cells to endothelial cells.

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1. Introduction

Almost all life forms display circadian rhythms of physiology and behavior that are entrained to a nearly 24-h cycle of light and darkness [1,2]. The circadian Clock arises from autoregulatory transcriptional, translational, and posttranslational feedback loops of few transcription factors known as Clock genes, including circadian locomotor output cycles kaput (Clock), brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (Bmal1), neuronal PAS-containing protein 2 (NPAS2), and period genes (Per1, Per2, and Per3), etc. [3–5]. Clock and Clock controlled gene reside not only in a central pacemaker, the suprachiasmatic nuclei (SCN) of hypothalamus in mammals, but also in all peripheral tissues except the testis, even in immortalized cells [6]. Clock is a Per-Arnt/AhR-Sim basic helix-loop-helix (bHLH-PAS) transcription factor and forms a Clock/Bmal1 heterodimer to drive the rhythmic transcription of Clock genes and Clock controlled genes via E-box or E-box-like enhancers located in their promoter or intronic regions [7–10]. The E-box or E-box-like sequences are necessary for circadian oscillation [11].

Previous study has identified more than 100 putative Clock-regulated circadian expressing genes in the mouse liver. In these genes, intercellular adhesion molecule-1/CD54 (ICAM-1) gene showed reduced expression level in Clock mutant mice [12]. However, how Clock regulates ICAM-1 expression remains unknown. ICAM-1 is a key molecule mediating adhesion of monocytes and T lymphocytes to endothelial surface followed by their migration into the vascular intima, which is the earliest event in both human and experimental atherosclerosis models [13–15]. Our previous studies have demonstrated that circadian Clock activities *in vivo* are closely associated with atherosclerosis development [16–19]. Thus, understanding the mechanisms underlying the regulation of ICAM-1 expression by circadian rhythms will be important for the pathobiology of atherosclerosis.

In the present study, our aim is to determine how Clock regulates ICAM-1 expression and whether Clock affects mononuclear cells adhesion to endothelial cell monolayer. By using *in vitro* endothelial cell culture model, we found that exogenous expression of Clock upregulated ICAM-1 gene expression and transcriptional activity, and Clock bound to the ICAM-1 gene E-box-like enhancer. There was a circadian variation of ICAM-1 gene expression in endothelial cells after serum shock *in vitro*, suggesting ICAM-1 may be a Clock-controlled gene. Clock regulates the adhesion of mononuclear cells to endothelial cells via ICAM-1.

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2. Materials and methods

2.1. Cell culture

The mouse brain microvascular endothelial cell lines (bEnd.3) were cultured in DMEM with 10% fetal bovine serum as described previously [20]. Peripheral blood mononuclear cells (PBMC) were separated from heparinized samples of peripheral blood obtained from the infraborbital veniplex of mice, using density gradient centrifugation on Ficoll-Paque (1.080). The cells were counted after double washing with RPMI-1640, and their viability was confirmed to be >90% by the trypan blue staining [21]. All culture reagents were from Invitrogen (Carlsbad, CA), unless otherwise stated.

2.2. Transfection and viral infection

The mouse microvascular endothelial cell lines were transiently transfected with Clock or ICAM-1 siRNA (100 nM), or Non-silencing (control) siRNA (100 nM) with lipofectamine™ 2000 transfection reagent (Invitrogen) in 6-well plates according to the manufacturer's instructions respectively. Adenoviral vector expressing mouse Clock (Ad-Clock) was obtained from GeneChem (China), adenovirus expressing mouse GFP (Ad-GFP) was used as a control. The endothelial cell lines were infected by Ad-Clock or Ad-GFP at a multiplicity of infection (MOI) of 50. 4 h after infection, the cells were used for experiments. Cell transfection or infection was carried out in triplicates with three independent experiments.

2.3. Reverse transcription-PCR (RT-PCR) and real-time PCR

Total cellular RNA was isolated from bEnd.3 endothelial cells using Trizol reagent (Invitrogen, Carlsbad, CA). 2 µg of RNA was processed directly to cDNA with the first strand cDNA synthesis kit (Toyobo, Japan). Real-time PCR were performed with iQ™ SYBR Green Supermix (BioRad, Hercules, CA) according to the methods described previously [22]. The primers used are shown in Table 1. All samples were analyzed using a Bio-Rad real-time analyzer (Bio-Rad, Hercules, CA) and were normalized to GAPDH expression.

2.4. Plasmid construction, transient transfection and luciferase assay

Transient transfection and luciferase assay were performed as described previously [23]. A 401 bp (from –729 to –329 bp) mouse ICAM-1 gene promoter containing the E-box-like motif (CAAGTG, from –584 to –579) was cloned into the pGL3 luciferase reporter. The primers used were shown in Table 1. bEnd.3 endothelial cells were seeded in 12-well plates and cultured to 60–70% confluence. The cells were transiently transfected with ICAM-1 reporter and pCMV-β-galactosidase plasmid, with or without Clock siRNA using Lipofectamine 2000 from Invitrogen (Carlsbad, CA). In addition, the cells transfected with ICAM-1 reporter were infected by Ad-Clock or Ad-GFP. Cells were lysed with reporter lysis buffer 48 h later. Luciferase (Luc) activities of the cell extracts were determined using the Luciferase Assay System from Promega (Madison, WI). β-Galactosidase (β-gal) activity was measured, and relative Luc activity was calculated as the ratio of Luc/β-gal activity. All luciferase assays reported here represent at least three independent experiments, each consisting of three wells per transfection.

2.5. Chromatin immunoprecipitation (ChIP) assay

We carried out chromatin fixation and purification as described previously [24]. In brief, suspensions of mouse primary hepatocytes were fixed by adding formaldehyde to 1% (w/v) final concentration for 10 min at room temperature. Cells were then sonicated

Table 1

The primer sequences.

Gene names	Primers	Primer sequences
<i>Clones</i>		
mlCAM-1 promoter	Sense	5'-CGGGGTACCAGTCAAAGCAGAAA-3'
	Antisense	5'-CCGCTCGAGGAGTACGCCTCTCGTT-3'
<i>ChIP</i>		
mlCAM-1	Sense	5'-AGGCAGGACTTTCTCACAGC-3'
	Antisense	5'-CAAGCCTCTTTAACCACCA-3'
mlCAM1-NC	Sense	5'-TGGTGGGTAAAGAGGCTTG-3'
	Antisense	5'-CAGGTGAGTCCGGAGAGAAG-3'
mPAI-1	Sense	5'-GGCTCAGTGGGTAGAACACG-3'
	Antisense	5'-AGGTGAGGACAGCTTTCAG-3'
mDBP-2	Sense	5'-GGACTTTTCAGTGGCTCGTG-3'
	Antisense	5'-GAGTTCACGACAGCCAGAA-3'
<i>Real time-PCR</i>		
mCLOCK	Sense	5'-ATGAGCACCAAGACCATTCC-3'
	Antisense	5'-GCTTCAGTGCTCCCACTTC-3'
mlCAM-1	Sense	5'-AGCACCTCCCCACCTACTTT-3'
	Antisense	5'-AGCTTGACGACCCCTTCTAA-3'
mVCAM-1	Sense	5'-ACCCAACAGAGGCAGAG-3'
	Antisense	5'-GAGCAGGTGAGGTTCACA-3'
mCCL-2	Sense	5'-GGCTCCAGACATACATTAA-3'
	Antisense	5'-ACGGGTCAACTTCACATT-3'
mCCL-5	Sense	5'-ACCACTCCCTGCTGCTTT-3'
	Antisense	5'-ACACTTGGCGGTTCCTTC-3'
<i>RNA interfering</i>		
Target sequence		5'-GGACAAGTCTACTGTCTA-3'
mClock-siRNA	Sense	5'-GGACAAGUCUACUGUUCUAdTdT-3'
	Antisense	5'-UAGAACAGUAGACUUGUCCdTdT-3'
Target sequence		5'-GGACCTTAACAGTCTACAA-3'
mlcam1-siRNA	Sense	5'-GGACCUAAACAGUCUACAAdTdT-3'
	Antisense	5'-UUGUAGACUGUUAAGGUCCdTdT-3'

to prepare a chromatin suspension of 200–500 bp DNA. Immunoprecipitations were carried out using anti-Clock antibody (Abcam, Cambridge, UK) or normal mouse IgG (Abcam, Cambridge, UK). Realtime-PCR was performed using the primers described in the Table 1. Relative enrichment was calculated as the difference between specific antibody and normal IgG signals that were normalized to the respective input signals. The ChIP assay was performed with five independent experiments.

2.6. Serum shock procedures

The serum shock procedures were performed as described previously [25]. bEnd.3 cells were grown in DMEM supplemented with 10% fetal calf serum and a mixture of penicillin–streptomycin–glutamine (PSG). Approximately 5×10^5 cells/10 cm dish were plated 2 days before experiment. At circadian time (CT) = 0, the medium was exchanged with serum-rich medium (DMEM + PSG, supplemented with 50% horse serum), and after 2 h this medium was replaced by serum-free DMEM. At the indicated time, the dishes were washed twice with ice-cold PBS, frozen on a layer of liquid nitrogen and kept at –70 °C until the extraction of whole-cell RNA. The experiments were performed with three independent times.

2.7. Mononuclear cells to endothelial cell monolayer adhesion assay

Mononuclear cells to endothelial cell monolayer adhesion were assessed according to the procedures described previously with some modifications [26]. The bEnd.3 endothelial cells were seeded into matrigel-coated 96-well plates until confluence was reached. Peripheral blood mononuclear cells were added to the endothelial cell monolayer. After the plate was incubated at 37 °C for 1 h, non-adherent cells were removed by gently washing the plate three times with PBS. Adherent cells were then stained with 0.1% crystal

violet. Then, the dye was extracted with Triton X-100. The absorbance at 570 nm, which correlates with the amount of attached cells, was measured with a microtiter plate reader. The assays were carried out in triplicate. Each experiment was repeated 3 times.

2.8. Statistical analysis

Statistical comparisons were made using the unpaired two-tailed Student's *t*-test when two groups were compared. One-way analysis of variance was used when more than two groups were compared. The 0.05 level of probability was used as the criterion of significance.

3. Results

3.1. Clock increases the expressions of ICAM-1 and adhesion-related molecules

We overexpressed or knocked down Clock using adenovirus or siRNA in the bEnd.3 endothelial cells, and evaluated the gene expressions of ICAM-1 and adhesion-related molecules using RT-PCR. As shown in Fig. 1A, increasing Clock expression increased the expression of ICAM-1 ($P < 0.01$), and other adhesion-related genes, including VCAM1 and CCL-2 ($P < 0.05$). Conversely, with knockdown of Clock, we observed the decrease of these gene expressions (Fig. 1B, $P < 0.01$).

3.2. Clock induces ICAM-1 transcriptional activation

We investigated the potential involvement of Clock in the transcriptional activation of ICAM1. The bEnd.3 endothelial cells were transfected with the ICAM-1 promoter reporter containing the E-box-like motif (Fig. 2A). As expected, overexpression of Clock enhanced the ICAM-1 transcriptional activity ($P < 0.01$, Fig. 2B). In

contrast, inhibition of Clock gene expression by Clock siRNA inhibited the ICAM-1 reporter activation ($P < 0.01$, Fig. 2C).

3.3. Clock binds to the ICAM-1 gene E-box-like enhancer

We analyzed the E-box or E-box-like motifs within the -2 kb promoter regions of the mouse ICAM-1 genes. A 401 bp (from -729 to -329 bp) mouse ICAM-1 gene promoter containing the E-box-like motif (CAAGTG, from -584 to -579 bp) was cloned. We examined whether Clock binds to this ICAM-1 gene promoter using chromatin immunoprecipitation (ChIP) assays in hepatocytes. We also examined Clock binding to the PAI-1 (Plasminogen Activator Inhibitor-1) or DBP-2 (D-site Binding Protein-2) gene promoter, which contain the E-box-like motif, as the positive control. We found that endogenous Clock was clearly recruited to the ICAM-1 promoter by the standard and the quantitative ChIP assays (Fig. 3), indicating that Clock binds to the ICAM-1 gene E-box-like enhancer.

3.4. Cyclic expression of ICAM-1 after serum shock

It is known that brief exposure of cells to 50% horse serum induces circadian expression of various genes [5]. We examined whether ICAM-1 gene shows rhythmic expression after 50% serum exposure in endothelial cells. Per2, a well-defined Clock-controlled gene, was used as the positive control (Fig. 4A). As expected, ICAM-1 mRNA (Fig. 4B) showed cyclic expression after 50% serum exposure, with significant variations within 48 h. Thus, our results suggest that ICAM-1 may be a Clock-controlled gene.

3.5. Overexpression of Clock in endothelial cells induced the adhesion of mononuclear cells to endothelial cells is mediated by ICAM-1

We then determined the effect of Clock on mononuclear cells adhesion to endothelial cells. We found that downregulation of Clock expression in endothelial cells decreased the adhesion of mononuclear cells to endothelial cells, and overexpression of Clock promoted mononuclear cells adhesion to endothelial cells (Fig. 4C and D). Knockdown of ICAM-1 decreased Clock induced the adhesion of mononuclear cells to endothelial cells (Fig. 4D, $P < 0.01$).

4. Discussion

The circadian clock plays important roles in the physiological function regulation and the pathological situations including atherosclerosis [27–29]. Our previous studies have shown that the circadian disruption is closely associated with the process of atherosclerosis [16–19]. However, the molecular mechanisms how Clock affects the development of atherosclerosis are not well understood. In the present study, we found that clock regulates the transcription of ICAM-1 gene through binding the E-box-like enhancer, and promotes the adhesion of mononuclear cells to endothelial cells through ICAM-1.

We showed that exogenous expression of Clock upregulated the gene expressions of ICAM-1 and other adhesion-related genes including VCAM1 and CCL-2, and increased the transcriptional activity of ICAM-1 in the endothelial cell lines. In contrast, loss of Clock decreased these gene expressions and ICAM-1 transcriptional activity, indicating that Clock induces ICAM-1 transcription activation. The 5'-region of the human ICAM-1 gene has been cloned, and consensus sequences for promoter elements, e.g. TATA and CAAT boxes and Sp1-binding sites were identified within the 5'-flanking region of human ICAM-1 gene [30]. We analyzed the E-box or E-box-like motifs within the -2 kb promoter regions of the mouse ICAM-1 gene promoter. The E-box-like motif (CAAGTG,

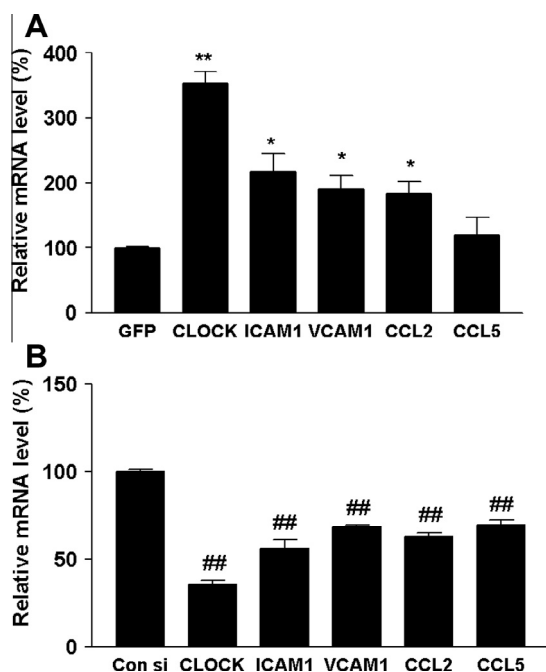


Fig. 1. Clock increases the expressions of ICAM-1 and adhesion-related molecules. (A) bEnd.3 endothelial cell lines were infected by Ad-Clock or Ad-GFP at a multiplicity of infection (MOI) of 50 for 48 h; (B) Cells were transfected with Clock siRNA or the control siRNA for 48 h. RNA was isolated and subjected to quantitative reverse transcription-polymerase chain reaction for the gene expressions of ICAM-1 and other adhesion-related molecules. The mRNA levels are normalized to the Ad-GFP group or control siRNA group ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. Ad-GFP group; ### $P < 0.01$ vs. the control siRNA group.

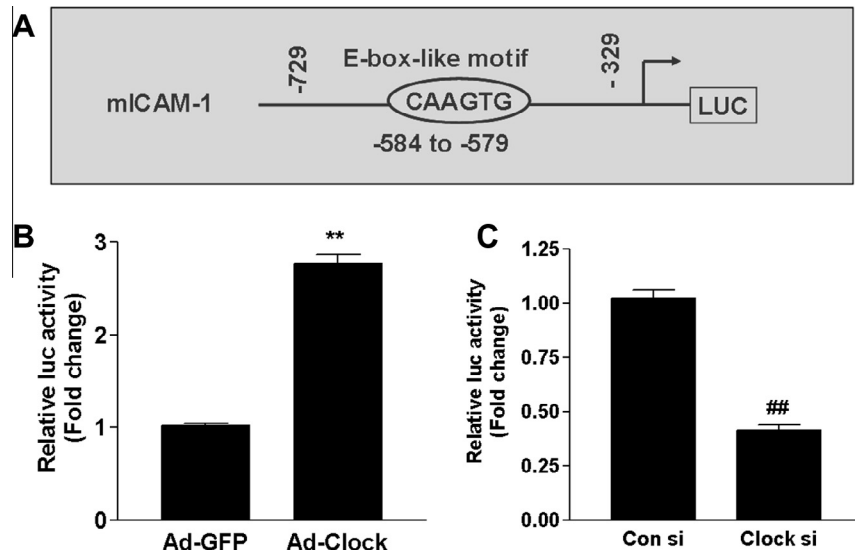


Fig. 2. Clock induces ICAM-1 transcriptional activation. (A) A 401 bp (from –729 to –329 bp) mouse ICAM-1 gene promoter containing the E-box-like motif (CAAGTG, from –584 to –579) was cloned into the pGL3 luciferase reporter. (B) bEnd.3 endothelial cells were co-transfected with the ICAM-1 reporter and β -gal plasmid, and then were infected by Ad-Clock or Ad-GFP. (C) The ICAM-1 reporter and β -gal plasmids were cotransfected with Clock siRNA or the control siRNA. 48 h later, relative luciferase activity was determined by the ratio of luciferase/ β -galactosidase activity, and normalized to that of the control. ** $P < 0.01$ vs. Ad-GFP group; ## $P < 0.01$ vs. the control siRNA group.

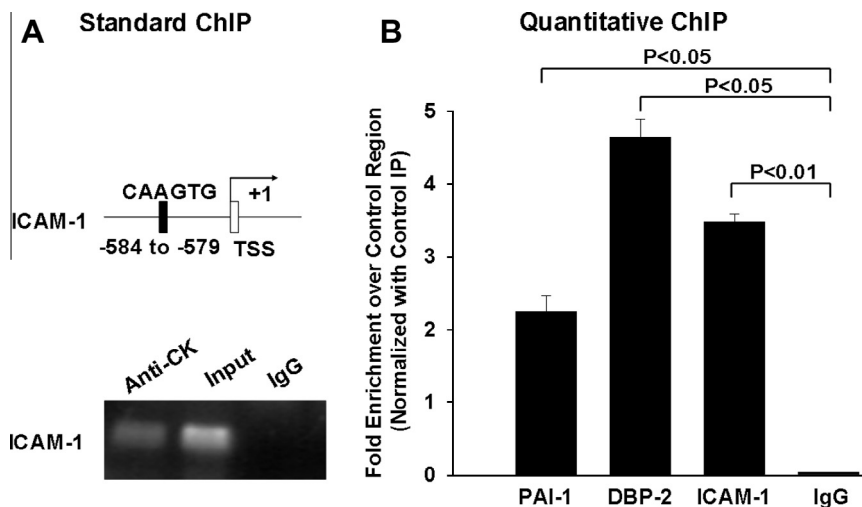


Fig. 3. Clock binds to the ICAM-1 gene E-box-like enhancer. ChIP assays were carried out with Clock antibody or control IgG. (A) Gel images of PCR products of ICAM-1 promoter using input and precipitated chromatin as template were shown. (B) Relative levels of enrichment of genomic DNA region were quantified by real time PCR. PAI-1 and DBP-2 were used as the positive controls ($n = 3$).

from –584 to –579 bp) was identified. By chromatin immunoprecipitation (ChIP) assay, our data revealed that Clock binds to the ICAM-1 gene E-box-like enhancer. Thus, our results suggest that Clock may regulate ICAM-1 transcription activation through binding to the E-box-like enhancer.

Moreover, ICAM-1 mRNA showed cyclic expression after 50% serum exposure, with significant variations within 24 h, suggesting that ICAM-1 is a clock-controlled gene. Our previous study has demonstrated that the expression of ICAM-1 gene show circadian rhythm in the aortic intima in both C57BL/6J and ApoE knockout mice [18]. In fact, the previous study has hypothesized that ICAM-1 might be a clock-regulated circadian expressing gene [12]. Thus, the present study provides the evidence to this hypothesis.

Vascular inflammation is a primary event in the pathogenesis of atherosclerosis [31,32]. The interaction of leukocytes with endothelial cells mediated by adhesion molecules and subsequent transmigration seems to play a pathogenetic role in the early phase of atherosclerosis [33]. Various adhesion molecules have been identi-

fied on endothelial cells, including ICAM-1 [34]. ICAM-1, a transmembrane glycoprotein that belongs to the immunoglobulin superfamily, is up-regulated in patients with atherosclerosis [35]. Our results show that Clock induced the adhesion of mononuclear cells to endothelial cells was significantly decreased by knockdown of ICAM-1, suggesting that Clock regulates the adhesion of mononuclear cells to endothelial cells via ICAM-1. Our previous study have demonstrated that Clock genes including Clock, Bmal1, Per1, Per2, and Cry1 in ApoE knockout mice showed increased expression levels, enhanced amplitudes, and different circadian rhythms compared to the normal mice. Interestingly, abnormalities of circadian genes were more severe in ApoE knockout mice at the advanced stage of atherosclerosis than in the mice at the early stage [18]. Thus, these results suggest that circadian genes expressions are closely association with the progression of atherosclerosis.

In conclusion, we demonstrated that Clock is a positive regulator of ICAM-1 through binding the E-box-like enhancer of ICAM-1 gene. Clock regulates the adhesion of mononuclear cells to

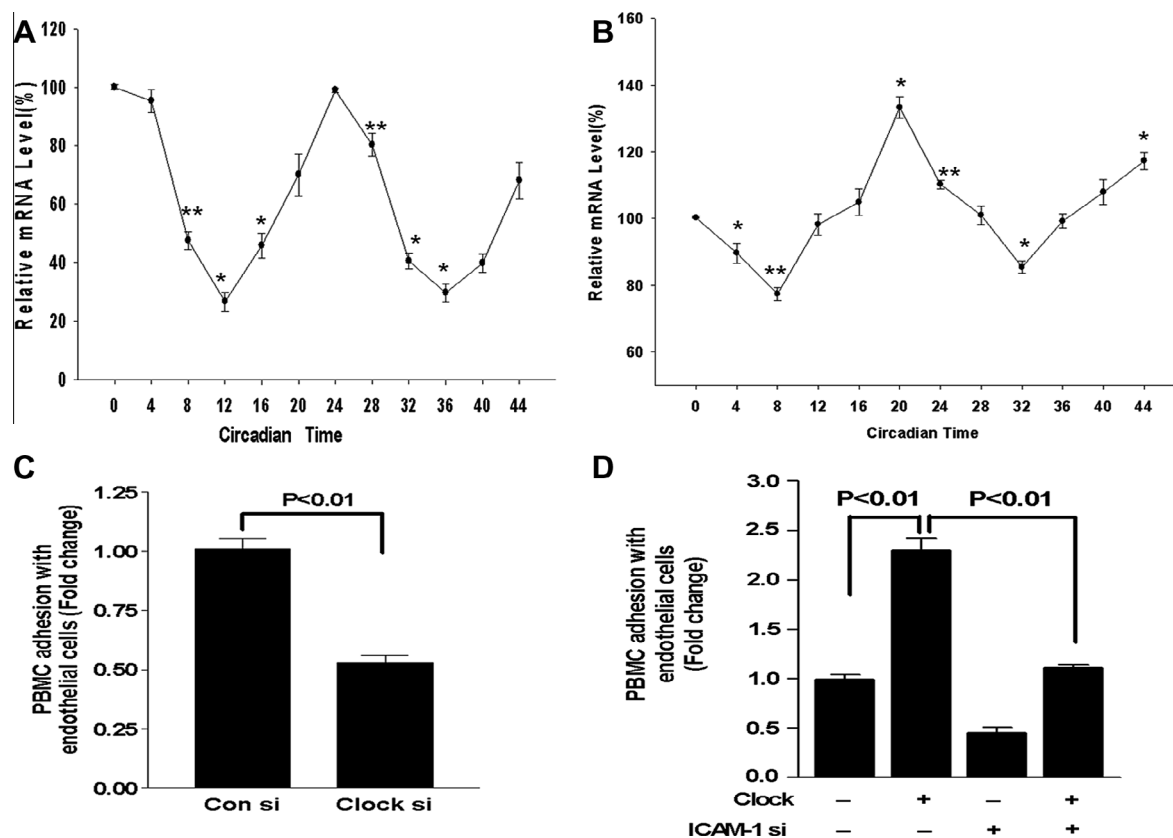


Fig. 4. Cyclic Expression of ICAM-1 after serum shock and Clock regulates endothelial cell adhesion via ICAM-1. Endothelial cell lines were incubated in media containing 50% fetal calf serum for 2 h. Cells were washed and incubated in serum-free media. At indicated times, cells were harvested to measure Per-2 (A) or ICAM-1 (B) mRNA expression by real time PCR. The values at 0 h were normalized to 100%. Other values represent the percentage change compared to 0 h ($n = 3$). Endothelial cell lines were transfected with Clock siRNA or the control siRNA (C), or infected with Ad-Clock or Ad-GFP together with siRNA against ICAM-1 or the control siRNA as indicated (D). 48 h later, the endothelial cells were seeded into matrigel-coated 96-well plates until confluence was reached. Peripheral blood mononuclear cells were added to the endothelial cell monolayer, and incubated at 37 °C for 1 h. Non-adherent cells were removed by gently washing the plate three times with PBS. The attached cells were measured as described in Section 2 ($n = 3$).

endothelial cells via ICAM-1. This study will provide new insights to better understand the role of the circadian Clock in atherosclerosis.

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