Coronary Heart Disease

Impaired Cholesterol Metabolism and Enhanced Atherosclerosis in Clock Mutant Mice

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Background—Clock is a key transcription factor that positively controls circadian regulation. However, its role in plasma cholesterol homeostasis and atherosclerosis has not been studied.

Methods and Results—We show for the first time that dominant-negative Clock mutant protein (ClockΔ19) enhances plasma cholesterol and atherosclerosis in 3 different mouse models. Detailed analyses revealed that ClkΔ19/Apoe−/− mice display hypercholesterolemia resulting from the accumulation of apolipoprotein B48-containing cholesteryl ester–rich lipoproteins. Physiological studies showed that enhanced cholesterol absorption by the intestine contributes to hypercholesterolemia. Molecular studies indicated that the expression of Niemann Pick C1 Like 1, Acyl-CoA:Cholesterol acyltransferase 1, and microsomal triglyceride transfer protein in the intestines of ClkΔ19/Apoe−/− mice was high and that enterocytes assembled and secreted more chylomicrons. Furthermore, we identified macrophage dysfunction as another potential cause of increased atherosclerosis in ClkΔ19/Apoe−/− mice. Macrophages from ClkΔ19/Apoe−/− mice expressed higher levels of scavenger receptors and took up more modified lipoproteins compared with Apoe−/− mice, but they expressed low levels of ATP binding cassette protein family A member 1 and were defective in cholesterol efflux. Molecular studies revealed that Clock regulates ATP binding cassette protein family A member 1 expression in macrophages by modulating upstream transcription factor 2 expression.

Conclusions—ClockΔ19 protein enhances atherosclerosis by increasing intestinal cholesterol absorption, augmenting uptake of modified lipoproteins by macrophages, and reducing cholesterol efflux from macrophages. These studies establish that circadian Clock activity is crucial in maintaining low plasma cholesterol levels and in reducing atherogenesis in mice. (Circulation. 2013;128:1758-1769.)

Key Words: atherosclerosis ■ ATP binding cassette transporter 1 ■ cholesterol ■ circadian rhythm ■ CLOCK proteins ■ lipid metabolism ■ upstream transcription factor 2

Circadian regulatory mechanisms synchronize biological functions to environmental stimuli such as light. Changes in light are transmitted from the eye by the retinal ganglion cells to the suprachiasmatic nuclei in the brain, where this information is translated into transcriptional regulation of certain transcription factors. Clock and Bmal1 are 2 transcription factors that increase the expression of other transcription factors to control the rhythmicity of different biological functions.1,2 Ablation of Clock has no significant effect on circadian rhythms because neuronal PAS domain containing protein 2 (NPAS2) can substitute for Clock deficiency by interacting with Bmal1.3,4 However, deletion of exon 19 in the Clock (Clk) gene results in the synthesis of ClockΔ19 mutant protein, which acts as a dominant-negative regulator and disrupts clock function.5,6 Mice expressing ClockΔ19 protein exhibit modest hypertriglyceridemia, hypercholesterolemia, hyperglycemia, and hyperleptinemia.7 We have previously shown that plasma triglycerides in ClkΔ19 mutant mice do not exhibit circadian rhythms; instead, they show modest hypertriglyceridemia.8,9 Molecular studies showed that ClockΔ19 protein disrupts plasma triglyceride homoeostasis by deregulating diurnal transcriptional regulation of short heterodimer partner (SHP) and microsomal triglyceride transfer protein (MTP).9 In this study, we examined the effects of ClockΔ19 protein on the regulation of plasma cholesterol and atherosclerosis. Here, we show that ClockΔ19 protein enhances atherosclerosis and identify different physiological pathways and molecular targets affected by the expression of ClockΔ19 protein that contribute to atherosclerosis.

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Methods

Animals
ClockΔ19, ClkΔ19/Ldlr−/−, and ClkΔ19/Apoε−/− mice were bred to obtain ClkΔ19/Ldlr−/−, ClkΔ19, ClkΔ19/Ldlr−/−, Ldlr−/−, ClkΔ19/Apoε−/−, and Apoε−/− mice. All mice on C57Bl6 background were housed with a 12-hour light schedule (7 AM–7 PM). Male 2- to 3-month-old mice were fed different diets (Table I in the online-only Data Supplement) for atherosclerosis studies. Animal experiments were approved by the Animal Care and Use Committee of the SUNY Downstate Medical Center and were performed in accordance with institutional guidelines.

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Macrophages
Bone marrow–derived macrophages obtained from Clk<sup>Δ19/Δ19</sup> Apoe<sup>/−</sup> mice and Apoe<sup>/−</sup> mice<sup>10</sup> were treated with or without oxidized low-density lipoprotein (ox-LDL) for 8 hours. For cholesterol efflux assays, macrophages were labeled with [3H]cholesterol for 24 hours, washed with PBS, and incubated in Dulbecco modified Eagle medium containing 0.2% BSA for 1 hour and then the same media in the absence or presence of apolipoprotein (Apo) AI (15 μg/mL) or high-density lipoprotein (HDL; 50 μg/mL) for 8 hours. The human monocyctic cell line THP-1 was maintained in RPMI 1640 media and differentiated by treatment with phorbol myristic acid.

Plasma Lipids
After a 4-hour fast, plasma was obtained to measure lipids with the use of kits. Plasma ApoA-I, ApoB, and ApoE were quantified by Western blotting. Mice were not fasted when daily changes in plasma and tissue lipids were studied.

In Vivo Absorption of Lipids
Mice were injected intraperitoneally with 0.5 mL Poloxamer 407 in PBS (1:6 vol/vol) and gavaged with [3H]cholesterol at noon.

Uptake and Secretion of Lipids by Enterocytes
To study uptake, enterocytes from Clk<sup>Δ19/Δ19</sup> Apoe<sup>/−</sup> mice and Apoe<sup>/−</sup> mice were incubated in triplicate with [3H]cholesterol (1 μC/mL) for different times. To measure secretion, enterocytes were incubated in triplicate with [3H]cholesterol for 1 hour, washed, and then incubated in fresh media containing oleic acid and taurocholate for different times.<sup>12</sup>

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation was used to study the binding of different transcription factors to the ABCA1 promoter using goat polyclonal antibodies against hypoxia-inducible factor (HIF)-1α, HIF1β, USF1, and USF2 with the use of kits as reported previously.<sup>9</sup> ABCA1 promoter sequences were amplified with primers (Table II in the online-only Data Supplement).

Bone Marrow Transplantation
Apoe<sup>/−</sup> mice (age 8 weeks) were lethally irradiated and transplanted with bone marrow cells derived from Clk<sup>Δ19/Δ19</sup> Apoe<sup>/−</sup> or Apoe<sup>/−</sup> mice.

Statistical Analyses
Data are presented as mean±SD (n=6–12 animals). Mice were euthanized at each time point, and plasma and tissue samples were collected. Statistical testing was performed by the paired Student t test. Temporal comparisons between 2 groups were performed with 2-way ANOVA followed by Bonferroni posttest (GraphPad Prism). Differences were considered statistically significant when P<0.05.

Results
Clk<sup>Δ19/Δ19</sup> Mice Develop Atherosclerosis on an Atherogenic Diet
To test the hypothesis that Clk<sup>Δ19/Δ19</sup> mice might be susceptible to atherosclerosis, WT (Clk<sup>+/+</sup>) and Clk<sup>Δ19/Δ19</sup> siblings were fed either chow or an atherogenic diet<sup>15</sup> ad libitum. Chow-fed Clk<sup>Δ19/Δ19</sup> mice had higher lipids compared with their WT siblings (Table) but did not show any atherosclerotic lesions. However, after being fed an atherogenic diet for 2 months, Clk<sup>Δ19/Δ19</sup> mice had 2- to 3-fold higher plasma cholesterol and triglyceride levels (Table), mainly very low-density lipoprotein/intermediate-density lipoprotein/LDL (Figure IA and IB in the online-only Data Supplement), increased amounts of ApoB100 and ApoB48 but reduced levels of ApoA1 and ApoE (Figure IC in the online-only Data Supplement), and higher ApoB/ApoA ratios (Figure ID in the online-only Data Supplement) compared with WT siblings. These mice had 2.3- and 1.6-fold more lesions at the aortic arches (Figure 1A) and aortic root (Figure 1B), respectively, and 3- to 4-fold elevated amounts of lipid lesions in the abdominal aorta (Figure 1C). Thus, Clk<sup>Δ19/Δ19</sup> mice show hyperlipidemia and develop more lesions on an atherogenic diet.

Clock Mutant Protein Increases Atherosclerosis in Ldlr<sup>/−</sup> Mice
Besides mice fed an atherogenic diet, atherosclerosis is commonly studied in mouse models deficient in LDL receptors and ApoE.<sup>16</sup> Clk<sup>Δ19/Δ19</sup> Ldlr<sup>/−</sup> mice had higher plasma lipids when fed chow and Western diets (Table) and developed more atherosclerotic lesions than Ldlr<sup>/−</sup> mice on both chow and Western diets, respectively (Figure 1D–1F).

Clock Mutant Protein Increases Atherosclerosis in Chow-Fed Apoe<sup>/−</sup> Mice
Next, we studied the effect of Clk<sup>Δ19/Δ19</sup> protein on atherosclerosis in Apoe<sup>/−</sup> mice. Clk<sup>Δ19/Δ19</sup> Apoe<sup>/−</sup> mice on a chow diet showed more extensive atherosclerotic lesions in the aortic arch than Apoe<sup>/−</sup> mice (Figure 2A). The whole aorta showed 34-fold increased lipid staining (Figure 2B), whereas the cardiac/aortic junctions of Apoe<sup>/−</sup> mice had 22-fold more lipid lesions. The lesions at the cardiac/aortic junction contained 4-fold more necrotic core (Figure 2C) and macrophages (Figure 2D). Furthermore, smooth muscle cells (Figure 2E) and collagen content (Figure 2F) were increased by 5-fold. These observations indicate the presence of advanced, stable plaques in Clk<sup>Δ19/Δ19</sup> Apoe<sup>/−</sup> mice. These lesions were seen more frequently in male than in female mice, and their size increased with age (Figure II in the online-only Data Supplement). The brachiocephalic arteries of these mice had higher amounts of cholesterol/cholesterol esters/triglycerides (Figure IIIA in the online-only Data Supplement), lipids (Figure IIIB in the online-only Data Supplement), necrotic area (Figure IIIC in the online-only Data Supplement), macrophages (Figure IIDD in the online-only Data Supplement), smooth muscle cells (Figure IIDE in the online-only Data Supplement).
Supplement), and collagen (Figure IIIF in the online-only Data Supplement). CLK\textsuperscript{wt/wt}\textsuperscript{Δ19Apoe−/−} mice developed more atherosclerotic lesions on a Western diet (Figure IV in the online-only Data Supplement). These studies indicate that CLK\textsuperscript{wt/wt}\textsuperscript{Δ19Apoe−/−} mice develop extensive lesions throughout the aorta that are rich in lipids, macrophages, smooth muscle cells, and collagen compared with Apoe−/− mice, most likely representing stable plaques.

**Plasma Lipids Are Higher in CLK\textsuperscript{Δ19Apoe−/−} Mice**

Plasma of chow-fed CLK\textsuperscript{Δ19Apoe−/−} mice was more turbid (Figure 3A) and had 2-fold more total and esterified cholesterol (Table). Plasma total cholesterol levels were significantly higher and triglyceride levels were lower in these mice at all time points (Figure V in the online-only Data Supplement). Cholesterol levels were higher in non-HDL lipoproteins but were lower in HDL (Table). Plasma ApoB100 and ApoAI levels were lower (Figure 3C, protein blot) but ApoB48 and ApoB/ApoAI ratios were higher in these mice (Figure 3C). These studies indicate that CLK\textsuperscript{Δ19Apoe−/−} mice accumulate more ApoB48-containing cholesteryl ester–rich lipoproteins.

**CLK\textsuperscript{Δ19Apoe−/−} Mice Absorb More Cholesterol**

Because lipoprotein catabolism is impaired in Apoe−/− mice, we hypothesized that higher plasma ApoB48-containing cholesteryl ester–rich lipoproteins are attributable to increased cholesterol absorption by the intestine. To study absorption, mice were gavaged with radiolabeled cholesterol. Radiolabeled cholesterol–derived lipids were higher at 2 to 4 hours in the plasma of CLK\textsuperscript{Δ19Apoe−/−} mice (Figure 3D). Increased absorption could be attributable to increased uptake or secretion by enterocytes. Isolated primary enterocytes from CLK\textsuperscript{Δ19Apoe−/−} mice took up more cholesterol in a time-dependent manner (Figure 3E). Furthermore, pulse-chase studies showed that enterocytes secrete more cholesterol (Figure 3F). These studies revealed that uptake and secretion of cholesterol are higher in CLK\textsuperscript{Δ19Apoe−/−} enterocytes.

Cholesterol uptake in enterocytes is a balance between import by NPC1L1 and export by ABCG5/ABCG8.\cite{17} Measurement of mRNA levels revealed no change in ABCG5/ABCG8 (Figure 3G); instead, we found significant increases in NPC1L1 mRNA (Figure 3G) and protein (Figure 3H).
levels. Therefore, increased expression of NPC1L1 might contribute to increased uptake of cholesterol in \( \text{Clk}^{\Delta 19/\Delta 19} \) enterocytes.

After uptake, cholesterol is secreted by enterocytes via HDL and chylomicrons. The HDL pathway transports free cholesterol involving ABCA1 and ApoAI. We found that ABCA1 was reduced but ApoAI mRNA was similar in the enterocytes of \( \text{Clk}^{\Delta 19/\Delta 19}\text{Apoe}^{-/-} \) and \( \text{Apoe}^{-/-} \) mice (Figure 3G). The transport of cholesterol via chylomicrons depends on ACAT enzymes and MTP because dietary cholesterol is esterified by ACAT1/ACAT2, packaged in chylomicrons by MTP, and secreted. We observed significant increases in ACAT2, but not ACAT1, mRNA levels in \( \text{Clk}^{\Delta 19/\Delta 19}\text{Apoe}^{-/-} \) mice (Figure 3G). Moreover, MTP mRNA, protein (Figure 3G and 3H), and activity (Figure 3I) were significantly higher in \( \text{Clk}^{\Delta 19/\Delta 19}\text{Apoe}^{-/-} \) mice. Increases in MTP and ACAT2 suggested that chylomicron assembly and secretion pathway might be augmented in \( \text{Clk}^{\Delta 19/\Delta 19}\text{Apoe}^{-/-} \) mice. To test this, we incubated enterocytes with radiolabeled cholesterol and subjected conditioned media to ultracentrifugation. Cholesterol counts were higher in chylomicrons but not in HDL fractions (Figure 3J), indicating that \( \text{Clk}^{\Delta 19/\Delta 19}\text{Apoe}^{-/-} \) mice absorb more cholesterol by enhancing assembly and secretion of chylomicrons.
Plasma Cytokines Are Higher in 

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**Figure 2.** Atherosclerosis in *Clk*Δ19/Δ19*Apoe−/−* mice. A and B, Chow-fed 8-month-old male *Clk*Δ19/Δ19*Apoe−/−* and *Apoe−/−* mice were dissected to visualize atherosclerotic lesions at the aortic arch (A). Whole aortas were stained with Oil Red O, and lesions were quantified (B). C, Sections from cardiac/aortic junctions were stained with hematoxylin and eosin followed by Oil Red O staining; lipid lesions and necrotic areas were quantified. Scale bar, 50 μm. D, Sections were stained with anti-macrophage antibodies and quantified. E, Sections were stained with β-actin to detect smooth muscle cells and were quantified. Scale bar, 400 μm. F, Sections were stained with Masson trichrome collagen staining kits and quantified. Mean±SD; n=12 to 15. Scale bar, 400 μm.*P<0.05, **P<0.01, ***P<0.001 vs *Apoe−/−* mice.

**Plasma Cytokines Are Higher in *Clk*Δ19/Δ19*Apoe−/−* Mice**

Inflammation is a hallmark of atherosclerosis. Therefore, we measured cytokines in *Clk*Δ19/Δ19*Apoe−/−* and *Apoe−/−* mice. Plasma of *Clk*Δ19/Δ19*Apoe−/−* mice contained ≈2- to 4-fold higher levels of interleukin (IL)-12, IL17A, and granulocyte colony-stimulating factor (Figure VIA in the online-only Data Supplement). It is known that macrophages contribute to plasma cytokines. Therefore, we looked at the expression of several of these cytokines in bone marrow–derived macrophages. *Clk*Δ19/Δ19*Apoe−/−* macrophages had higher mRNA levels of IL12, IL6, tumor necrosis factor-α, and granulocyte colony-stimulating factor but not IL17A (Figure VIB in the online-only Data Supplement); IL17 is produced mainly by lymphocytes.19 To determine whether Clock plays a role in the regulation of cytokine expression, we reduced Clock levels using siRNA in WT macrophages. siClock reduced Clock mRNA by 80% (Figure V in the online-only Data Supplement) and increased granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. These studies suggest that Clock suppresses the expression of different cytokines in macrophages.
Figure 3. Plasma lipids and cholesterol absorption in Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} mice. A, Blood was centrifuged to separate plasma, and the tubes were photographed. B, Plasma was separated by fast protein liquid chromatography, and cholesterol was measured in fractions. C, Plasma (1 μL) was Western blotted (representative of n=10) to measure apolipoprotein (Apo) B100, ApoB48, and ApoAI (bottom). Bands were quantified and ApoB/ApoAI ratios were plotted (top). D, Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} mice absorb more cholesterol. Male mice were gavaged with radiolabeled cholesterol, and plasma was collected to measure radioactivity. E, To study uptake, isolated primary endothocytes were incubated with radiolabeled cholesterol. At different times, cells were collected to measure radioactivity and protein. F, To study lipid secretion, primary endothocytes were incubated with radiolabeled cholesterol for 1.5 hours, washed, and then incubated in fresh media. At the indicated times, media was collected to measure radioactivity. Two-way ANOVA was used in D through F. G, RNA was isolated from endothocytes to measure different transporters. H and I, Proximal jejunal pieces were homogenized, and 20 μg protein was used to detect MTP, NPC1L1, and GAPDH by Western blotting (H) and MTP activity (I). J, Enteroocytes isolated from Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} or Apoe\textsuperscript{−/−} small intestine were labeled for 1 hour with 0.5 μCi/mL \textsuperscript{3}H\textsuperscript{cholesterol}, washed, and incubated with fresh media containing 1.6 mmol/L oleic acid in taurocholate micelles for 2 hours. \textsuperscript{12,18} Media was used to separate lipoproteins by density gradient ultracentrifugation, and radioactivity was determined in all fractions. Fractions 1 through 3 represent chylomicrons (CM); fractions 8 to 10, high-density lipoprotein (HDL). Means±SD, n=10, ***P<0.05, **P<0.01, ***P<0.001 compared with Apoe\textsuperscript{−/−} mice.

**Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} Macrophages Take up More Modified Lipoproteins as a Result of Increased Expression of CD36 and SR-A1**

The studies described above showed that lesions in Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} mice were lipid and macrophage rich (Figure 2B and 2D and Figure IIIB and IIID in the online-only Data Supplement). To understand the mechanisms that might contribute to the accumulation of lipids in the aorta, we injected DiI-labeled acetylated LDL into Apoe\textsuperscript{−/−} and Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} mice. There was 2-fold higher DiI labeling in the aorta of Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} mice than Apoe\textsuperscript{−/−} mice (Figure 4A). It is known that macrophages are the principal cells that take up modified lipoproteins in the subintima; therefore, we studied the uptake of modified lipoproteins by bone marrow–derived macrophages from Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} and Apoe\textsuperscript{−/−} mice. Compared with Apoe\textsuperscript{−/−} mice, Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} macrophages took up 2-fold more DiI-labeled acetylated LDL and contained 2- to 3-fold more lipids, lipid peroxides, and total and esterified cholesterol (Figure 4B–4E). To explore reasons for lipid accumulation, we measured mRNA and protein levels of scavenger receptors involved in the uptake of modified lipoproteins. Clk\textsuperscript{A19/A19} Apoe\textsuperscript{−/−} macrophages expressed higher protein and mRNA levels of CD36 and SR-A1 (Figure 4F), suggesting that their increased expression could contribute to fat accumulation.

Clk\textsuperscript{A19/A19} reduces Clock activity by acting as a dominant-negative mutant.\textsuperscript{3} Therefore, to understand the mechanisms for increased expression of scavenger receptors, we reduced Clock expression using siRNA in Clk\textsuperscript{A19/A19} macrophages. siClock reduced Clock mRNA levels by ≈80% in WT macrophages, and these levels were unaffected by ox-LDL treatment (Figure 4G). Reductions in Clock had no effect on the mRNA (Figure 4G) and protein (Figure 4H) levels of CD36 and SR-A1 in normal macrophages. However, incubation of these macrophages with ox-LDL increased the expression of scavenger receptors in both siControl- and siClock-treated cells, but increases in the protein and mRNA levels of these scavenger receptors were higher in siClock-treated cells (Figure 4G and 4H). Furthermore, siClock-treated macrophages took up 2-fold more amounts of DiI-labeled acetylated LDL (Figure 4I). Similarly, siClock-treated human THP-1 macrophages took up more DiI-labeled acetylated LDL (Figure VIII in the online-only Data Supplement). These studies show that increases in scavenger receptors are higher when macrophages have...
reduced Clock expression and are exposed to ox-LDL. Thus, Clock reduces the expression of scavenger receptors when macrophages are exposed to modified lipoproteins.

**Clk\(^{ΔW/ΔD}\) Apoe\(^{−/−}\) Macrophages Are Defective in Cholesterol Efflux as a Result of Reduced ABCA1 Expression**

In addition to increased uptake, reduced efflux contributes to cholesterol accumulation in macrophages. Therefore, we studied in vivo reverse cholesterol transport from \(^3\)H-cholesterol–loaded \(J774\) macrophages in Apoe\(^{−/−}\) and \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) mice. The appearance of cholesterol into plasma, feces, and liver was significantly less in \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) mice compared with Apoe\(^{−/−}\) mice (Figure 5A), indicating that \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) plasma is less efficient in reverse cholesterol transport from \(J774\) macrophages, most likely secondary to low plasma HDL (Table) and ApoAI (Figure 3C) in these mice. Additionally, we studied the ability of \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages to give up cholesterol to plasma acceptors in WT mice. Injection of \(^3\)H-cholesterol–loaded \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) or Apoe\(^{−/−}\) macrophages into WT mice revealed that \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages are defective in giving off cholesterol, as evidenced by lower amounts of cholesterol in plasma, feces, and liver (Figure 5B). Furthermore, isolated \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages gave up less cholesterol to extracellular ApoAI and HDL in culture (Figure 5C). Thus, \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages are defective in cholesterol efflux.

**Clock Regulates ABCA1 Expression**

To understand reasons for reduced cholesterol efflux, we measured mRNA and protein levels of transporters involved in cholesterol efflux and found lower amounts of ABCA1 and ABCG1 mRNA and protein levels in \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages but no change in SR-B1 and ABCG4 expression (Figure 5D). To determine whether low expression of ABCA1 was contributing to reduced cholesterol efflux, we expressed ABCA1 under the control of cytomegalovirus promoter. Overexpression of ABCA1 increased cholesterol efflux from \(Clk^{ΔW/ΔD}\) Apoe\(^{−/−}\) macrophages (Figure 5E).
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Next, we asked whether Clock regulates ABCA1. First, we asked whether APOE deficiency is required for Clock−/− mice to reduce ABCA1. This was not the case because ABCA1 levels were low in Clock−/− macrophages compared with their WT littermates (Figure 5F). Second, knockdown of Clock in Clk ΔΔ macrophages reduced ABCA1 mRNA (Figure 5G) and protein (Figure 5H, inset) levels, as well as efflux to ApoAI (Figure 5H). Similarly, Clock knockdown in human THP-1 macrophages reduced cholesterol efflux to HDL and ApoAI (Figure VIIIB and VIIC in the online-only Data Supplement). In contrast, knockdown of PER1, CRY1, or BMAL1 in Clock−/− macrophages had no effect on ABCA1 mRNA (Figure VIIIA in the online-only Data Supplement) and cholesterol efflux (Figure VIIIB in the online-only Data Supplement). These data suggest that Clock regulates ABCA1 expression and cholesterol efflux.
Clock Modulates ABCA1 Expression Involving USF2

To determine whether Clock regulates ABCA1 at the transcriptional level, we expressed luciferase under the control of 1.3 kB ABCA1 promoter, along with a Clock expression plasmid or lentiviruses expressing shClock in WT macrophages. Overexpression of Clock increased whereas its knockdown significantly reduced promoter activity (Figure 6A), suggesting that Clock increases ABCA1 transcription. To identify transcription factors regulated by Clock and those involved in ABCA1 expression, we measured mRNA levels of various activators and repressors known to regulate ABCA1 gene expression. Activators of ABCA1 either were reduced or did not change in ClkΔ19Apoe−/− macrophages (Figure IXA in the online-only Data Supplement). Quantification of various repressors showed that USF1, USF2, and TRβ were significantly increased (Figure IXB in the online-only Data Supplement). Furthermore, knockdown of Clock in WT macrophages either reduced or had no effect on activators (Figure IXC in the online-only Data Supplement). Although siClock had no significant effect on various repressors, it significantly increased mRNA expression.21

Involving USF1, USF2, and HIF1β had no effect on Clock mRNA, suggesting that Clock is not regulated by them (Figure X in the online-only Data Supplement). siUSF1 and siUSF2 increased ABCA1 expression, but siHIF1α and siHIF1β had no effect (Figure 6C), indicating that USF1 and USF2 suppress ABCA1 expression. Therefore, we asked whether Clock needs these transcription factors to regulate ABCA1. siClock reduced ABCA1 expression in siHIF1α-, siHIF1β-, and siUSF1-treated cells but not in siUSF2-treated cells (Figure 6C), indicating that siClock needs USF2 to reduce ABCA1 expression. To confirm the role of USF2 in ABCA1 regulation, we performed chromatin immunoprecipitation in Apoe−/− and ClkΔ19Apoe−/− macrophages. In Apoe−/− macrophages, ABCA1 promoter was occupied by HIF1β, USF1,
and USF2 (Figure 6D). However, in ClkΔ19/Apo−/− macrophages, only USF1 and USF2 were found to be associated with the promoter. The amounts of USF2 associated with the promoter were higher in ClkΔ19/Apo−/− macrophages. Therefore, increased binding of USF2 to the ABCA1 promoter in ClkΔ19/Apo−/− macrophages might reduce expression.

To garner the in vivo significance of USF2 in cholesterol efflux, we hypothesized that a reduction of USF2 in ClkΔ19/Apo−/− macrophages might enhance reverse cholesterol transport. To test this, bone marrow–derived ClkΔ19/Apo−/− macrophages were treated with siControl or siUSF2, loaded with 3H-cholesterol, and injected in WT mice. After 48 hours, mice receiving siUSF2-treated macrophages contained higher 3H-cholesterol levels in the plasma, liver, and feces (Figure 6E). These studies indicate that siUSF2 increases reverse cholesterol transport from macrophages.

Cyclic Expression of ABCA1 and USF2 in Macrophages

The above studies indicated that Clock regulates macrophage ABCA1 expression and cholesterol efflux by regulating USF2. Nothing is known about the circadian regulation of ABCA1 and USF2 in macrophages or in other cells. To determine whether ABCA1 and USF2 expression shows diurnal changes, WT bone marrow macrophage cultures treated with siClock or not treated were synchronized by incubating them in 50% serum for 2 hours. Subsequently, changes in macrophage ABCA1 and USF2 were measured at different times. ABCA1 and USF2 expression showed cyclic expression in synchronized WT macrophages. ABCA1 and USF2 levels increased and decreased, respectively, in siClock-treated macrophages (Figure 6F). Furthermore, ABCA1 mRNA levels were low when USF2 levels were high. These studies indicate that ABCA1 and USF2 expression in macrophages shows cyclic change and that Clock plays an important role in these changes.

Effect of ClkΔ19/Apo−/− Bone Marrow Cell Transplantation on Atherosclerosis in Apo−/− Mice

To determine whether macrophage dysfunction contributes to increased atherosclerosis independently of hyperlipidemia, we transplanted bone marrow cells obtained from ClkΔ19/Apo−/− or Apo−/− mice into lethally irradiated Apo−/− mice. Bone marrow transplantation slightly reduced total plasma cholesterol in Apo−/− mice (Figure 7A). However, Apo−/− mice that received bone marrow cells from ClkΔ19/Apo−/− mice had 2- to 2.7-fold more atherosclerotic plaques in the ascending aortas and 3 main branching arteries (Figure 7B) and at the cardiac/aortic junctions (Figure 7C). Furthermore, there was 3-fold more lipid staining in the aorta (Figure 7D). Moreover, macrophages obtained from mice transplanted with bone marrow cells from ClkΔ19/Apo−/− were defective in cholesterol efflux to ApoAI and HDL (Figure 7E). Gene expression analysis showed that macrophages isolated from Apo−/− mice transplanted with ClkΔ19/Apo−/− bone marrow cells had low mRNA levels of ABCA1/ABCG1 and higher levels of CD36/SR-A1 (Figure 7F). Further analysis of transcription factors that regulate ABCA1 revealed that these macrophages had higher levels of USF2 (Figure 7G). Thus, macrophage dysfunction caused by the expression of ClockΔ19/Apo−/− protein contributes to atherosclerosis in Apo−/− mice.

Discussion

Using 3 different mouse models and 3 different diets, we show for the first time that Clock dysfunction resulting from the expression of a dominant-negative ClockΔ19/Apo−/− protein increases atherosclerosis in mice. Different mouse models carrying ClockΔ19/Apo−/− protein had higher cholesterol in ApoB-containing non-HDL lipoproteins. Mechanistic studies revealed that ClockΔ19/Apo−/− protein enhances cholesterol absorption by enterocytes and uptake of modified lipoproteins by macrophages in Apo−/− mice. In contrast, it reduces cholesterol efflux from macrophages. Thus, Clock plays an important and novel role in the regulation of cholesterol metabolism in enterocytes and macrophages to prevent hypercholesterolemia and atherosclerosis.

Biochemical analysis showed that hypercholesterolemia in ClkΔ19/Apo−/− mice was attributable to accumulation of cholestereryl ester–rich ApoB48-containing lipoproteins. Physiological studies showed that enterocytes expressing ClockΔ19/Apo−/− protein take up more cholesterol from the intestinal lumen and secrete more cholesterol with chylomicrons. Molecular studies demonstrated that increased cholesterol uptake was associated with enhanced expression of NPC1L1 with no significant changes in the cholesterol exporters ABCG1/ABCG8. After uptake, cholesterol is transported to plasma involving HDL and chylomicrons. The HDL pathway was not affected, but the chylomicron pathway was upregulated in ClkΔ19/Apo−/− mice. Two proteins, ACAT2 and MTP, that are involved in the assembly of chylomicrons were increased in ClkΔ19/Apo−/− mice. Thus, Clock regulates cholesterol absorption by modulating cholesterol uptake, cholesterol esterification, and chylomicron assembly.

This study shows that ClockΔ19/Apo−/− protein disrupts several macrophage functions: secretion of cytokines, uptake of oxidized lipoproteins, and cholesterol efflux. ClkΔ19/Apo−/− macrophages secrete more IL12, IL17, and granulocyte colony-stimulating factor. They take up more modified lipoproteins and retain more oxidized lipids. Additionally, we showed that ClkΔ19/Apo−/− mice were defective in reverse cholesterol transport as a result of a combination of lower plasma cholesterol acceptors, ApoAI/HDL, and reduced macrophage expression of ABCA1 and ABCG1 transporters. Molecular studies revealed that Clock regulates ABCA1 and that USF2 might be an intermediary repressor that is regulated by Clock to modulate ABCA1 expression in macrophages. During Clock deficiency, USF2 levels are increased. Further binding of this repressor to the ABCA1 promoter is enhanced.

Consideration was given to the possibility that the effect observed in ClkΔ19/Apo−/− mice might not be specific to Clock dysfunction. Instead, it could be a general effect resulting from deficiencies in other Clock genes or from off-target effects of the mutation. To address this, we performed several Clock knockdown experiments in WT macrophages. ABCA1 levels were reduced after siClock treatment. Furthermore, there were no significant differences, except for
Apoe−/− mice transplanted with bone marrow cells obtained from ClkΔ19/Δ19 Apoe−/− mice. Lethally irradiated Apoe−/− mice were transplanted with bone marrow cells obtained from ClkΔ19/Δ19 Apoe−/− or Apoe−/− mice. After 3 months, animals were started on a Western diet. Lesions and plasma lipids were quantified after 1 month. A, Plasma was used to measure cholesterol.

B, Aortic arches were dissected and photographed, and lesions were quantified with Image-Pro. C, Sections from cardiac/aortic junctions were stained with hematoxylin and eosin followed by Oil Red O staining, and lipid lesions and necrotic areas were quantified. Scale bar, 500 μm. D, Aortas were dissected, stained with Oil Red O, and quantified. E, Bone marrow macrophages from Apoe−/− mice transplanted with ClkΔ19/Δ19 Apoe−/− or Apoe−/− bone marrow cells were incubated with [3H]-cholesterol and acetylated low-density lipoprotein for 18 hours, washed, and incubated with purified apolipoprotein (Apo) AI or high-density lipoprotein (HDL) for 8 hours. Cholesterol efflux to media acceptors was expressed as percent of cellular cholesterol. F and G, Bone marrow macrophages from irradiated Apoe−/− mice transplanted with ClkΔ19/Δ19 Apoe−/− and Apoe−/− bone marrow cells were used to measure mRNA levels of different lipid transporters (F) and transcription factors (G) that regulate ABCA1. Means ± SD; n = 9 to 10. Hif indicates hypoxia-inducible factor; LDLr, low-density lipoprotein receptor; LXRα, liver X receptor-α; PPAR, peroxisome proliferator-activated receptor. *P<0.05, **P<0.01, ***P<0.001 vs control.

Per3 and Cry2 mRNA levels, in macrophages obtained from ClkΔ19/Δ19 Apoe−/− and Apoe−/− mice (Figure XI in the online-only Data Supplement). Thus, Clock has a specific effect on lipid metabolism. It is known that Clock affects cholesterol synthesis and degradation by the liver. In our studies, we observed no significant increase in lipoprotein production by the liver. Therefore, we did not address whether hepatic cholesterol metabolism was affected in these mice.

Myocardial infarctions occur predominantly in the morning. It is known that circadian clocks regulate arhythmo-genes, myocardiad contractility, and oxidative metabolism. Using an isograft model, Cheng et al. have shown that transplantation of arteries from Bmal1 and Per-deficient mice into WT mice elicits a pathological response resulting in intestinal hyperplasia and wall thickening. This response was attributable to infiltration of WT cells and hyperplastic response by the Clock-deficient arteries. Here, we provide evidence that Clock deficiency alters lipid metabolism and macrophage function to enhance atherosclerosis. Thus, circadian Clock might play an important protective role against hyperlipidemia and atherosclerosis.

Conclusions
These studies show that Clock regulates cholesterol metabolism in the intestine and macrophages and acts as an antiatherogenic gene (Figure XII in the online-only Data Supplement). In the intestine, Clock deficiency increases lipid absorption. In macrophages, it augments uptake of modified lipoproteins and diminishes cholesterol efflux. These changes could contribute to enhanced atherosclerosis in ClkΔ19/Δ19 Apoe−/− mice.

Acknowledgments
We are grateful to Drs Lita Freeman and Alan Remaley of the National Institutes of Health for plasmids expressing luciferase under the control of various ABCA1 promoter regions; Drs Roman Kondrakov and Antoch Mariko for plasmids expressing Clock; Yan Li, and Liana Kondrakov for technical assistance in monitoring luciferase activity.
Li and Joyce Queiroz for technical assistance in the early analysis of atherosclerotic plaques; and Wei Quan for technical assistance in fluorescence microscope.

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**Disclosures**
None.

**References**

**CLINICAL PERSPECTIVE**

Heart attacks happen mainly in the early hours of the day, suggesting that their occurrence might be related to circadian rhythms seen in various behavioral, physiological, and biochemical activities. Here, we show that disruption of circadian Clock activity as a result of a dominant-negative mutation (ClockΔ19/Δ19) increases susceptibility to atherosclerosis in various mouse models. ClockΔ19/Δ19 mice fed an atherogenic diet had increased plasma cholesterol, triglycerides, and atherosclerotic lesions compared with their wild-type siblings. Similarly, ClockΔ19/Δ19 protein increased cholesterolemia and atherosclerosis in Ldlr−/− and Apoe−/− mice fed a chow or Western diets. Physiological studies revealed that high plasma cholesterol in ClockΔ19/Δ19Apoe−/− mice was due in part to increased cholesterol uptake by enterocytes. In addition, macrophages in ClockΔ19/Δ19Apoe−/− mice displayed higher lipid uptake and reduced cholesterol efflux compared with Apoe−/− siblings. Molecular studies demonstrated that knockdown of Clock gene expression in wild-type macrophages reduces ABCA1 expression and cholesterol efflux. Furthermore, Clock overexpression increases ABCA1 transcription. Evidence is presented suggesting that USF2 could participate in the modulatory effect of Clock on ABCA1 expression. These studies provide significant evidence for the importance of Clock in the proper physiological functioning of enterocytes and macrophages. Hence, disruptions in Clock function as a result of either mutations or other environmental factors such as a high-fat diet, transcontinental flights, and night shift work might deregulate enterocyte and macrophage function, increasing the risk for atherosclerosis.
Impaired Cholesterol Metabolism and Enhanced Atherosclerosis in Clock Mutant Mice
Xiaoyue Pan, Xian-Cheng Jiang and M. Mahmood Hussain

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Supplemental Methods

**Materials:** siHif1α (sc-35562), siHif1β (sc-29734), siUSF1 (sc-36784), siUSF2 (sc-36785) as well as lentiviruses expressing shRNA against Clock (sc-35075-v) were from Santa Cruz Biotechnology. Antibodies against NPC1L1 (Novus, NB400-128), ABCA1 (Abcom, NB400-105), ABCG1 (Abcom, ab52617), HIF1α (sc-35562PR), HIF1β (sc-8077), USF1 (SC-36784PR), USF2 (SC-36785PR), and other antibodies (1) were purchased. [14C]Cholesterol, [3H]cholesterol, [3H]oleic acid and [3H]triolein were from NEN LifeScience Products. Alexa Flour 488 labeled AcLDL (L-23380) was from Invitrogen.

**Animals and diets:** All single knockout mice on C57Bl6J background were purchased from the Jackson Laboratory. Double knockout mice were generated at SUNY Downstate. ClkΔ19/Δ19 mice are infertile. Therefore, ClkΔ19/wt, ClkΔ19/wt Ldlr−/−, and ClkΔ19/wt Apoe−/− mice were bred to obtained ClkΔ19/Δ19, ClkΔ19/wt, ClkΔ19/Δ19 Ldlr−/−, Ldlr−/−, ClkΔ19/Δ19 Apoe−/− and Apoe−/− mice. Male, 2-3 months old ClkΔ19/Δ19 and ClkΔ19/wt siblings were placed on Paigen’s atherogenic diet (TD88051; Harlan Teklad; Madison, WI) containing 1.25% cholesterol and 0.5% cholic acid for 8 weeks. Male (2-3 months) ClkΔ19/Δ19 Ldlr−/− and Ldlr−/− mice were fed a western diet (0.15% cholesterol, 20% saturated fat; Research Diets) for 2 months for atherosclerosis studies. Apoe−/− and ClkΔ19/Δ19 Apoe−/− mice were maintained on a regular chow diet from weaning until sacrifice or fed a western diet as indicated. Mice were housed in an air-conditioned room at 22°C±0.5°C with a 12-hour lighting schedule (700–1900 hours). Animal experiments were approved by the Animal Care and Use Committee of the SUNY Downstate Medical Center.

**Macrophages:** Bone marrow-derived macrophages were obtained from ClkΔ19/Δ19 Apoe−/− mice and Apoe−/− mice (2;3). For gene expression studies, cells were placed in 10% DMEM plus 25% L-cell-conditioned medium for 1 week (2). Cells were then treated with or without oxLDL (200 μg/ml) as indicated for 8 hours. Total RNA was extracted and analyzed by real-time PCR (4). For cholesterol efflux assays, bone marrow derived macrophages were labeled with [3H]cholesterol (5.0 μCi/ml) for 24 h, washed with PBS, incubated in DMEM containing 0.2% BSA for 1 h and then in the same media in the absence or presence of apoAI (15 μg/ml) or HDL (50 μg/ml) for 8 hours. Radioactivity in the medium and total cell-associated radioactivity was determined by scintillation counting. The assays were performed in quadruplicate and are presented as percent efflux (2).
The human monocytic cell line THP-1 (TIB-202) was maintained in RPMI 1640 media supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal bovine serum. To induce differentiation, cells (5 × 10^5 to 10^6 per ml) were seeded in serum-free medium with 200 nM phorbol myristic acid (cat#16561-29-8, Sigma) for 24 h. After incubation, nonattached cells were removed by aspiration, and the adherent cells were washed three times with RPMI 1640 and were transfected with indicated siRNAs as suggested by the manufacturer (Santa Cruz). Cells were then cultured for 48 h in RPMI containing 5% heat-inactivated fetal bovine serum. Human THP-1 macrophages were treated with siControl or siClock for 48 h and used for different experiments.

**Quantification of plasma lipids, lipoproteins and apolipoproteins as well as tissue lipids:** After a 4-h fast, blood was obtained from mice anesthetized with methoxyflurane (Pitman–Moore, Mundelein, IL), placed into pre-cooled tubes containing EDTA (final concentration 4 mM), and centrifuged (2500 × g for 20 min at 4°C) to obtain plasma. Total cholesterol (TC), triglycerides (TG), phospholipids (PL), and free cholesterol (FC) were assayed using commercial kits (5). Plasma high density lipoproteins (HDL) were isolated after the precipitation of apoB-lipoproteins with phosphotungstate/MgCl2 reagent (HDL-cholesterol; Sigma). Total and HDL triglycerides and cholesterol were measured using commercial kits. Non-HDL apoB-lipoprotein triglyceride and cholesterol were determined by subtracting HDL lipid values from totals. Plasma levels of murine apoA-I, apoB and apoE were quantified by western blotting using polyclonal antibodies as previously reported (4).

**In vivo absorption of lipids:** Mice were injected intraperitoneally with 0.5 ml of Poloxamer P407 in PBS (1:6, v/v) and then gavaged with 50 µl of olive oil containing [3H]cholesterol with cold cholesterol at 12:00. Blood was collected from the tail, and plasma was used for liquid scintillation counting (5).

**Uptake and secretion of lipids by enterocytes:** Enterocytes were isolated using EDTA (5-8) as described earlier. To study uptake, isolated enterocytes from Clk^{Δ19/Δ19}Apoe^{-/-} mice and Apoe^{-/-} mice were incubated in triplicate with 1 µCi/ml of [3H]cholesterol. At different times, enterocytes were centrifuged, washed and counted. To measure secretion, enterocytes were incubated in triplicate with 1 µCi/ml of [3H]cholesterol for 1 h, centrifuged, washed and then incubated in fresh media for different time. Media were collected and counted as previously reported (1).
To study the distribution of cholesterol in chylomicrons and HDL, conditioned media was adjusted to a density of 1.10 g/ml by the addition of KBr and overlaid with different density solution as described earlier (9). After ultracentrifugation large chylomicrons (fraction 1), small chylomicrons (fraction 2), VLDL (fraction 3) and other lipoproteins were collected from the top of the gradient (9) and radioactivity was measured.

**Evaluation of atherosclerosis:** The proximal aorta was collected after saline perfusion through the left ventricle. Aortas were placed in 4% phosphate buffered formaldehyde at 4°C for 24 h. The aortic root and ascending aorta were sectioned at a thickness of 10 µm, and alternate sections were saved on slides and stained with Oil-Red-O for neutral lipids and hematoxylin. Ten cross sections/animal from the aortic root were evaluated for lesion areas as previously reported (10;11). Moreover, ten sequential sections (10 µm thick) of the brachiocephalic artery per animal were stained and mean areas of lipid staining were determined. Sections were also stained with trichrome (collagen), hematoxylin/eosin (cytoplasm and nucleus), and Chromaview kit (elastin). Necrotic areas with no collagen and eosin staining were measured. The percent collagen and necrotic area per total plaque area (n=20 plaques) were determined. To evaluate cellularity of the lesion, sections were immunostained for macrophages (AIA31240, Accurate Chemical and Scientific Corp) and smooth muscle cells (anti-SMC actin 1A4, Zymed). Primary antibodies were incubated at room temperature in 3% serum matched to the species of the secondary antibodies. Biotinylated secondary antibodies were incubated for 30 minutes followed by 45 minute horseradish peroxidase conjugated streptavidin and visualized with diaminobenzidine. Nuclei were counterstained with hemalaune. The mean area of staining per section per animal for 8 sections was determined for each animal. Positive macroscopic lesions, and the mean areas of lipid staining were quantified using Image Pro Plus software (10-12). For *en face* analysis, whole aortas were isolated, and the fatty streaks in the lumen were stained with Oil Red O, and the percentage staining was quantified for each animal (10-12).

**In vivo macrophage cholesterol efflux measurement** (2;13;14): J774A.1 cells were grown in suspension in DMEM medium supplemented with 10% fetal bovine serum. Cells were radiolabeled with 5 µCi/mL \(^3\text{H}\)-cholesterol and 50 µg/mL acetylated LDL for 48 hours. These labeled foam cells were washed twice, equilibrated in medium with 0.2% bovine serum albumin (BSA) for 8 hours, centrifuged, and resuspended in RPMI medium immediately before use. The...
labeled foam cells were injected intraperitoneally into ClkΔ19/Δ19 Apoε−/− mice and Apoε−/− mice and were caged individually with unlimited access to food and water. Blood was collected at 6, 12, 24, and 48 hours, and plasma samples were used for liquid scintillation counting. Feces were collected over 48 hours and stored at 4°C before extraction and counting. At 48 hours after injection, mice were exsanguinated and perfused with cold phosphate-buffered saline (PBS), and portions of the liver were removed and flash-frozen for lipid extraction and quantification of tracer counts.

Bone marrow-derived macrophages derived from ClockΔ19/Δ19 Apoε−/− mice and Apoε−/− mice were loaded with cholesterol by incubation with acetylated LDL (50 µg protein/ml) and 5 µCi of [3H]cholesterol for 24 hr. The labeled macrophages were injected intraperitoneally into WT mice. Plasma was collected at 6, 12, 24 and 48 hours and feces were collected at 48 hours to measure tracer counts. ClkΔ19/Δ19 Apoε−/− mice and Apoε−/− mice were injected intraperitoneally with 3H-cholesterol labeled J774 foam cells.

**Ex vivo cholesterol efflux from macrophages:** Bone marrow-derived macrophages were cultured for 7 days, then labeled with [3H]cholesterol carried by acetylated LDL as described above for J774A.1 cells. After labeling, cells were washed 3 times with PBS, then equilibrated with DMEM containing 0.2% BSA for 1 hour, and incubated with purified human HDL or apoA-I in 0.5 ml of DMEM containing 0.2% BSA. The medium was collected at 8 hours and centrifuged at 6000 x g for 10 min to remove cell debris. Radioactivity in the medium and total cell-associated radioactivity was determined by scintillation counting. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells and medium as previously reported (3).

**Alexa Fluor 488–AcLDL uptake:** For acetylated low-density lipoprotein (AcLDL) uptake, bone marrow-derived macrophages from wildtype C57Bl6J mice were transfected with siClock or siControl for 48 hours and then incubated with Alexa Fluor 488–AcLDL (L-23380) (5 µg/mL) in serum-free DMEM media at 37°C for 3 hours. Bone marrow-derived macrophages derived from ClockΔ19/Δ19 Apoε−/− mice and Apoε−/− mice were placed in 10% DMEM plus 25% L-cell-conditioned medium for 1 week, then cells were incubated with Alexa Fluor 488–AcLDL (5 µg/mL) in FBS free DMEM at 37°C for 3 hours. Cells were photographed using a BX-50-FLA fluorescence microscope (Olympus) at a magnification of ×40.
Measurement of lipid peroxides: Tissue samples were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 1.15% KCl and centrifuged. The supernatants were used to measure lipid peroxides using TBARS assay kit (Cat#10009055, Cayman Chemical Company).

Determination of MTP Activity: After extensive washes with ice-cold PBS, 1-cm segments of proximal small intestine were homogenized with 1 ml of ice-cold 1 mM Tris-HCl (pH 7.6), 1 mM EGTA, and 1 mM MgCl2 in a glass homogenizer. The homogenates were centrifuged (SW55 Ti rotor, 50,000 rpm, 10 °C, 1 h), and supernatants were used for an MTP assay (15) using a kit (Chylos, Inc.).

Immunoblot analysis: Proteins were separated under non-reducing conditions, transferred to nitrocellulose membranes, blocked for 2 h in 20 mM Tris, 137 mM NaCl, pH 7.5, containing 0.1% Tween 20 and 5% nonfat dry milk at room temperature. The blots were washed three times and incubated overnight at 4 °C in the same buffer containing 0.5% dry milk and a primary antibody (1:100-1:1000 dilution), washed, and then incubated with mouse horseradish peroxidase-conjugated secondary antibody (1:1000-1:4000) in 1.0% skim milk for 1 h at room temperature. Immune reactivity was detected by chemiluminescence as previously described (4).

Real-time PCR analysis: Total RNA was isolated using TRIzol™ (Invitrogen). Isolated total RNA from different tissues were reverse transcribed as previously described (1), and the resultant reaction mixtures were used for real-time PCR. 18S rRNA was used as the reference gene. The mRNA expression level of GAPDH was also measured as an internal control with GAPDH control Reagent (Applied Biosystems).

Luciferase reporter assays: Plasmids expressing luciferase under different ABCA1 promoter sequences (16) were used. For co-transfection studies FLAG-Clock plasmids (17;18), or siRNA, shRNA plasmids were utilized. Macrophages from wildtype C57Bl6J mice were seeded onto 48-well plates, transfected with 1.5 µg of a plasmid expressing firefly luciferase under the control of the ABCA1 promoter (16). Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Assay System (Promega) was previously reported (5).

Chromatin immunoprecipitation (ChIP) assay: ChIP was used to study the binding of different transcription factors to the ABCA1 promoter using goat polyclonal antibodies against HIF1α, HIF1β, USF1, and USF2 using kits as reported (5). DNA samples (n=3-4) recovered after
immunoprecipitation were subjected to PCR to detect coimmunoprecipitated DNA using the $ABCA1$ promoter–specific primers for E-boxes (16) that flank the consensus HIF1α or Hif1β or USF1, USF2 binding sites in the human $ABCA1$ promoter. As negative controls, ChIP was performed in the absence of antibody or in the presence of rabbit IgG.

**Bone marrow transplantation:** Bone marrow cells were harvested from the tibias of donor mice ($Clk^{Δ19/Δ19} Apoe^{-/-}$ mice and $Apoe^{-/-}$ mice) as previously described (2). A total of 20 $Apoe^{-/-}$ mice (age 8 weeks) were lethally irradiated with 1000 rads (10Gy). Ten animals each were transplanted with bone marrow cells ($5×10^6$) derived from $Clk^{Δ19/Δ19} Apoe^{-/-}$ or $Apoe^{-/-}$ mice via tail vein within 2 hours of irradiation. We monitored the process of cell replacement by polymerase chain reaction (PCR), using genomic DNA from mouse white blood cells as a template.
Supplemental Tables

Table S1: Chemical composition (% weight) of different diets used in the study:

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Table S2: Oligonucleotide sequences of primers used

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mIL-17A | GGAAGCTCAGTGCCGCGCACC | CCGACAATGGGAGGCCACGCA
mG-CSF | CAGCGCTCTGCCACCATCCC | TGCAGGCTTGGCTAGAGCA
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hCLOCK | AAAAACCCCCATAGTGCTTTGA | CTGACACCACATCGCTGTAAAA
hABCA1 | TGGTCTCCAAAGCAGAGTGTG | GAGCAGCAGCTCCAAATAC
hABCG1 | ACAACTTCACAGAGGCCCAG | TTTCCAGAGATCCCTTCTCA
hSBR1 | CTGTTGGGATGATCATGTG | GCCGAAGTGCAACCTGGTC
hHIF1α | CCACAGCTGACCAGTTATGATTG | GAGTAATTCTTCACCTGGGAGTAGGT
hHIF1β | CTGTCAGATATGGTACCCCTG | GCCATGCCTAGATGGTCTAGCT
hUSF1 | AACCACCGCTTTGCTGAAGCAAC | GTGTGAAGCAGCTCATGGTC
hUSF2 | AGGCCAGTTTCAGTCATGATGA | GATCGTCTTCTTGTTCTCTCT
hβ-actin | CACACTTGCCATCATCTACGA | TTCTCTACGGAAGAGCTTGA

**Chip assay primers**
Forward (5′-3′) | Reverse (5′-3′)
mABCA1 | GGACCCTAAGACACCTGGCT | TCCCGGCTCCGTGTTATGTA

**Genotyping primers**
mClockΔ19/Δ19 | AGCACCTTCCTTTGCAGTTTCG | TGTGCTCAGACAGAAATAAGTA
mClockwt/wt | GGTCAAAGGCTAAGTAGTA | TGGGTAAAAGACCTCTTGCTC
mApoewt/wt | ACTACTCACAGGATGCCTAGCC | AGGTGAAGAGCTGGAGCACCTC
mApoewt/wt | TGTGACCTGGAAGCTCTGGAC | GCCTAGAGGGAGGAGGAGC
mLDlr-/- | AATCCAATGGCTTAGCATGGC | CCAATGCTACAATGGACTC
mLDlrvt/wt | GCGATGTAATCTACGGC | CCAATGCTACAATGGACTC
Fig S1: Changes in Plasma and hepatic lipids in ClkΔ19/Δ19 mice fed an atherogenic diet.

10 weeks old male ClkΔ19/Δ19 mice and wild type littermates were fed an atherogenic diet for 2 months. Plasma and liver were collected at 12:00. Plasma was subjected to FPLC and total triglyceride (A) and cholesterol (B) were measured in different fractions. Plasma was fractionated on SDS-PAGE and subjected to western blotting using anti-ApoB, anti-ApoA1 or anti-ApoE antibodies (C). Bands corresponding to ApoB100 and ApoB48, and ApoA1 were quantified and ApoB/ApoA1 ratios were plotted (D). Each group represents mean±SD, n=6-9. ** P < 0.01; *** P < 0.001, compared to Clkwt/wt mice.
Fig S2: Effect of sex and age on the development of atherosclerosis in $Clk^{Δ19/Δ19}Apoe^{-/-}$ mice.

(A) Proximal aortic arches were exposed to visualize atherosclerotic lesions in male and female mice of different ages (n=10-15).

(B) Oil Red O staining of whole aortae obtained from male and female mice of different ages (n=10-15). Representative pictures are shown.
Fig S3: Atherosclerotic lesions in the brachiocephalic artery (BCA) of \textit{Clk}^{\Delta 19/\Delta 19} Apoe^{-/-} mice. (A) BCA was collected as marked in the inset of Panel A, lipids were extracted, and cholesterol, cholesterol esters and triglycerides were measured. (B) Serial sections of BCA were stained with Oil Red O and lesion areas were quantified. (C) Hematoxylin and eosin stained lesions and their quantifications. (D) Sections were stained to determine macrophages and quantified. (E) Sections were stained to quantify smooth muscle cells. (F) Collagen content was quantified after Trichrome staining. Each group represents mean±SD, n=10-15. ** P < 0.01; *** P < 0.001, Scale bars: 40 μM.
Fig S4: Effect of a western diet on atherosclerosis in $\text{Clk}^{\Delta 19/\Delta 19} \text{Apoe}^{-/-}$ mice.
$\text{Clk}^{\Delta 19/\Delta 19} \text{Apoe}^{-/-}$ mice and $\text{Apoe}^{-/-}$ mice (male, 8-12 weeks old, n=12) were fed a western diet for one month and used for analysis. (A) Proximal aortic arches were exposed to visualize atherosclerotic lesions in male (n=10-15). (B) Aortic sections were stained with hematoxylin and eosin and lesion areas were quantified. (C) Sections were stained with Trichrome to measure collagen content. Each group represents mean±SD, n=12. *** P < 0.001, scale bars: 100 μM.
Fig S5: Diurnal rhythm of total plasma triglyceride and cholesterol in

Clk^{Δ19/Δ19} Apoe^{-/-} mice.

Plasma were collected from Clk^{Δ19/Δ19} Apoe^{-/-} mice and Apoe^{-/-} mice (male, 10 weeks old) at different time of a day, mice were kept in a 12 h light/dark cycle with free access to chow and water. Total Plasma triglycerides (A) and cholesterol (B) were measured. Each group represents mean±SD, n=9, ***P <0.001.
**Fig S6: Increased inflammatory response in Clk<sup>Δ19/Δ19</sup> Apoe<sup>-/-</sup> mice.**

(A) Plasma cytokines were measured using inflammatory kit (mouse inflammatory Cytokines #MEM-004A, SABioscience). (B) mRNA expression of inflammatory marker genes in bone marrow macrophages of male mice (8 months old) was measured by RT-PCR. (C) Isolated bone marrow macrophages from wild type C57BI/6J mice were treated with siControl or siClock. After 48 h, RNA was isolated to quantify mRNA levels of Clock, and inflammatory marker genes. Each group represents mean±SD, n=6-9.* P < 0.05, ** P < 0.01; *** P < 0.001.
Fig S7: Clock knockdown increases uptake of modified lipoproteins and reduces cholesterol efflux in human THP-1 macrophages:

(A) After 48 h, they were incubated with Dil-labeled AcLDL (5 µg/ml) in the presence or absence of cold AcLDL (200 µg/ml) for 6 h and then visualized under a fluorescent microscope, Scale bars: 50 µM.

(B-C) For efflux studies, cells were labeled with cholesterol by incubating cells with [3H]cholesterol and acetylated LDL. After labeling, cells were washed 3 times with PBS, then equilibrated with DMEM, 0.2% BSA for 1 hour, and incubated with 10 µg/ml purified human HDL (B) or apoA-I (C) in 0.5 ml of DMEM containing 0.2% BSA. The medium was collected at 8 hours and centrifuged (6000 x g, 10 minutes) to remove cell debris. Radioactivity in the medium and total cell-associated radioactivity was determined by scintillation counting. Mean± SD, n=6. * P < 0.05, *** P < 0.001.
**Fig S8: Effect of knockdown of different circadian clock genes on ABCA1 expression and cholesterol efflux.**

(A) Wild type bone marrow macrophages were transfected with different indicated siRNAs. After 48 h, ABCA1 mRNA levels were quantified in these cells.

(B) Wildtype bone marrow macrophages were transfected with siRNAs against few clock genes and then used to measure cholesterol efflux to ApoAl. Each group represents mean±SD, n=6, * P < 0.05.
Fig S9: Gene expression of different transcription factors in bone marrow macrophages of $Clk^{Δ19/Δ19}Apoe^{-/-}$ mice.

(A-B) Bone marrow macrophages isolated from $Clk^{Δ19/Δ19}Apoe^{-/-}$ and $Apoe^{-/-}$ mice were used to measure mRNA levels of different transcription factors that are known to regulate ABCA1.

(C-D) Macrophages from wildtype C57Bl6J mice were treated with siControl or siClock for 48 h. mRNA levels of different transcription factors that are known to regulate ABCA1 were quantified. Mean±SD, n=6, * P < 0.05, ** P < 0.01, *** P < 0.001.
Fig S10: Clock is not regulated by USF1, USF2, HIF1α and HIF1β. Wildtype macrophages were transfected with indicated siRNAs and changes in Clock mRNA were measured. Mean±SD, n=6.
**Fig S11**: Expression of Clock genes in bone marrow macrophages of *Clk*<sup>Δ19/Δ19</sup>*Apoe<sup>−/−</sup>* mice.

Bone marrow macrophages isolated from *Clk*<sup>Δ19/Δ19</sup>*Apoe<sup>−/−</sup>* and *Apoe<sup>−/−</sup>* mice were used to measure different Clock genes mRNA levels. Mean±SD, n=9, *** P < 0.001.
Fig S12: A schematic diagram explaining the regulation of different pathways and molecules modulated by Clock in enterocytes and macrophages. Data presented in this manuscript show that Clock regulates cholesterol metabolism in enterocytes and macrophages. Expression of a Clock mutant protein increases expression of NPC1L1, ACAT2 and MTP in enterocytes leading to enhanced uptake and secretion of cholesterol with chylomicrons. In the presence of the Clock mutant protein, macrophages take up more modified lipoproteins due to increased expression of scavenger receptors such as CD36. Further, these macrophages are defective in cholesterol efflux due to reduced expression of ABCA1. Molecular studies suggest that reduced expression of ABCA1 might be secondary to increased expression and binding of the USF2 repressor to the ABCA1 promoter. BBM, brush border membrane; BLM, basolateral membrane; Chol, cholesterol.
Reference List


