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Bioinformatic Analysis of Circadian Gene Oscillation in Mouse Aorta

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- *Background*—Circadian rhythmicity of many aspects of cardiovascular function—blood pressure, coagulation and contractile function—is well established, as is diurnal variation in important clinical events, such as myocardial infarction and stroke. Here, we undertake studies to globally assess circadian gene expression in murine aorta.
- *Methods and Results*—Aortae from mice were harvested at 4-hour intervals for 2 circadian cycles (48 hours). Gene expression was assessed by expression profiling and subjected to a gene ontology bioinformatics analysis. Three hundred thirty transcripts exhibited a circadian pattern of oscillation in mouse aorta, including those intrinsic to the function of the molecular clock. In addition, many genes relevant to protein folding, protein degradation, glucose and lipid metabolism, adipocyte maturation, vascular integrity, and the response to injury are also included in this subset of roughly 7000 genes screened for circadian oscillation.
- *Conclusions*—Detection of functional cassettes of vascular genes that exhibit circadian regulation in the mouse will facilitate elucidation of the mechanisms by which the molecular clock may interact with environmental variables to modulate cardiovascular function and the response to therapeutic interventions. (*Circulation.* 2005;112:2716-2724.)

Key Words: aorta ■ circadian rhythm ■ metabolism ■ vasculature ■ genes

T emporal integration of physiology and behavior with the environment is orchestrated by the circadian clock. This master clock, which, in mammals, resides in the hypothalamic suprachiasmatic nucleus (SCN), synchronizes multiple peripheral oscillators to ensure temporally coordinated physiology. Recently, we characterized the circadian clock in blood vessels, identifying both known and novel clock components.¹ In addition, we identified a mechanism by which humoral signals may reset or entrain this peripheral oscillator. We also wished to identify genes under the transcriptional control of the vascular clock and have undertaken a genome-wide screen using high-density oligonucleotide arrays to interrogate \approx 7000 distinct genes.

Methods

Animals

All animal studies were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Male C57BL/6J mice 4 to 6 weeks old were initially acclimatized for 2 weeks in 12-hour/12-hour light/dark cycles before being subjected to constant darkness for a minimum of 18 hours, followed by experimentation in darkness. Experiments were conducted in constant darkness (free running conditions) to permit identification of genes under the control of the molecular clock, as

Clinical Perspective p 2724

opposed to light-driven signals.² Experimental chronology is measured in circadian time, subjective day beginning at 7 AM (CT0) and subjective night beginning at 7 PM (CT12). Wild-type mice were euthanized at 4-hour intervals for 44 hours and exsanguinated. Twelve thoracic aortae were harvested per time point from CT18 to CT64, encompassing 2 complete circadian cycles. Two groups of 6 aortae were pooled and immediately snap-frozen in liquid nitrogen and stored at -70° C.

DNA Microarray Hybridization

Each pool of 6 aortae was pulverized on dry ice with a Plattner's diamond mortar and pestle set (Fisher Scientific). Total RNA was extracted by use of Trizol reagent (Invitrogen). The total RNA was then subjected to purification by use of the RNeasy spin column kit (Qiagen), which improves cRNA yields.³ After aortic RNA had been quantified by spectrophotometer, target cRNA was prepared and hybridized using 20 μ g of cRNA.

Data Analysis

All protocols were conducted as described in the Affymetrix Gene-Chip Expression Analysis Technical Manual. Affymetrix Microarray Suite 4.0 was used to quantify expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. Briefly, border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each

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probe. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in that sector. Probe pairs were scored positive or negative for detection of the targeted sequence by comparing signals from the perfect match and mismatch probe features. The number of probe pairs meeting ratio and difference thresholds was used to assign a call of absent, present, or marginal for each assayed gene. The average signal difference (Avg Diff) between perfect match and mismatch probes was calculated after high- and low-intensity outlier pairs had been excluded. This Avg Diff value, a relative measure of the expression level, was computed for each assayed gene. Global scaling was applied to allow comparison of Avg Diff values across multiple microarrays; after exclusion of the highest and lowest 2%, the average Avg Diff was calculated and used to determine what scaling factor was required to adjust the chip average to an arbitrary target of 150. All Avg Diff values from one microarray were then multiplied by the appropriate scaling factor. The methodology implemented permitted comparisons with other tissues that were processed identically.3 The mean hybridization signal strength and the standard error of the mean for each probe, set at each time point, were calculated from the duplicate hybridizations for circadian expression analysis. For analysis of circadian gene expression, statistically significant correlations between the temporal expression profiles of each probe set and cosine waves of defined period and phase were sought empirically. Genes with >95% probable correlation with a cosine test wave with a period between 20 and 28 hours were scored as circadian-regulated. This analysis is independent of signal strength and imposes no minimal change in amplitude.

Data are available at http://expression.gnf.org/circadian or http:// www.ncbi.nlm.nih.gov/geo/, accession No. GDS404.

Informatic Analysis of Functional Classifications of Oscillating Genes

Three separate analyses were performed. (1) Cycling genes were tested for gene ontology (GO) classes relative to expressed genes using DAVID (http://david.niaid.nih.gov/david)4 and EASE.5 Of 307 cycling genes, 255 genes (83.1%) were GO annotated. Of these, 108 genes were assigned to 31 overrepresented GO classifications (EASE score <0.05), including 20 terms that described biological processes. (2) Cycling genes were clustered on the basis of shared GO annotations.6 Fifty clusters were obtained and mapped to 18 broader terms of classes, which represented 158 genes. Seventy-six of these genes were also assigned to enriched functional classes in the EASE analysis. (3) Three hundred thirty oscillating Affimetrix identifiers were mapped to 324 genes on the basis of the Ingenuity database and with 202 functional annotations (62.3%). One hundred forty of these genes were statistically assigned to 45 high-level functions, which is composed of 160 distinct functional terms in the Ingenuity database. The 45 high-level functions were reduced to 20 broader functional terms on the basis of overlap and similarity.

Quantitative Polymerase Chain Reaction

In a de novo set of experiments, single aortae were isolated from CT24 to CT60 at 6-hour intervals. Total RNA was isolated from individual aortae (unpooled) and used to confirm microarray oscillations by quantitative polymerase chain reaction (Q-PCR). RNA was quantified by use of a fluorescence-based real-time PCR technology (TaqMan Real-Time PCR, Applied Biosystems) using an ABI 7000 or 7900 light cycler. Each PCR program started by initial denaturation at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing, and polymerization at 60°C for 1 minute. The SYBR Green detection method was used to quantify each gene analyzed, and melting (dissociation) analysis was performed to ensure that consistent target sequences were amplified. To eliminate the possibility of contaminating genomic DNA amplification, DNase I treatment was incorporated into RNA extraction procedures, and an additional control analysis was performed for each primer set using a sample reaction devoid of reverse transcriptase. For control reactions, mouse GAPDH mRNA was amplified from the same RNA samples.

Relative mRNA abundance was calculated by use of ABI SDS software. All final measurements were normalized by the target mRNA/GAPDH average value for all samples.

Vascular Leakage Assay

Unanesthetized male C57BL/6J mice were injected intravenously with Evans blue dye via the tail vein (50 μ L). Ears were then systematically injured by uniformly clamping the ear. Thirty minutes after injury, the mice were killed and ears were harvested. Ears were placed in formamide (5 μ L/mg tissue) overnight. Extracted Evans blue dye was diluted 1:1 with formamide to obtain an optical density reading between 0 and 1 for spectrophotometric analysis. Optical density was measured at 610 nm.

Informatic Analysis of Common Transcriptional Regulatory Motifs

A publicly accessible site (www.genome.ucsc.edu) was used to identify putative promoter regions of selected genes that exhibited circadian oscillation using the table browser function. Promoter sequences were then analyzed with the Tres analysis tool (http:// bioportal.bic.nus.edu.sg/tres/) using a matrix cutoff score of 95. Only transcriptional elements that were shared by at least 70% of the analyzed sequences were reported.

Statistical Analysis

The circadian gene expression data from Affymetrix chips and Q-PCR experiments were jointly analyzed using nonlinear mixed regression models as implemented in the function nlme7 from library nlme v3.1-56 for R 2.0-1 (www.cran.r-project.org). The Affymetrix data were estimated by use of a periodic (cosine) function with a 24-hour period⁸ in the baseline model, whereas the Q-PCR data were estimated by a constant value. The means and variances of the Q-PCR and Affymetrix data were allowed to differ. An alternative model estimated the Affymetrix and Q-PCR as synchronous cosine functions, allowing different variances, mean levels, and amplitudes but constraining the cycles to the same acrophase. Synchronous circadian rhythmicity in the Q-PCR and Affymetrix data were indicated by an improvement in fit, as measured by a negative change in the AIC statistic.9 Statistical significance in vascular leakage data was analyzed by 1-way ANOVA followed by Bonferroni's post hoc test.

Results

Global expression profiling performed in mouse aortae harvested at 4-hour intervals for 2 circadian cycles (48 hours) revealed that 330 genes exhibited a circadian pattern of gene expression, as identified through a cosine wave-fitting algorithm, COSOPT¹⁰ (Figure 1A). This is roughly comparable to the number identified in liver and SCN.3 Circadian gene transcription in the aorta is pervasive over the circadian day and does not display the phase consolidation seen in the SCN.3 More cycling genes were shared between aorta and liver than between aorta and SCN (Figure 1B). In a de novo set of circadian experiments, we validated the microarray results by Q-PCR. Five percent of the 330 genes were confirmed, including genes intrinsic to the "molecular clock" and putative outputs of the molecular clock (Figure 1C; Data Supplement Figure I, A and C, http://circ.ahajournals.org/cgi/content/full/CIRCULA-TIONAHA.105.568626/DC1). We also confirmed the known rhythms of Rev-erb α ,¹¹ Wee1,¹² and Npas2,¹³ which were either not present or not detected by probe sets in the gene chips (Data Supplement Figure IB).



Figure 1. Circadian variation in gene expression and function in mouse aorta. A, Three hundred seven genes exhibited a circadian pattern of expression in the aorta shown in the heat map. B, Individual oscillating genes in common in aorta and liver (left), aorta and SCN (middle), and liver and SCN (right) are represented by line graphs. Genes are grouped by common phase pattern (line color) by a phase clustering method. C, Q-PCR (red lines) confirms oscillations observed by expression profiling (black lines).

Molecular Clock Components Oscillate in Aorta

We supplemented manual inspections of our circadian data set with an informatic approach using a publicly available gene ontology (GO) database (see Methods) to identify functionally linked cassettes of genes. We identified 13 major functional classes among our 307 rhythmic genes, of which 8 were categorized as biological processes. As expected, "circadian rhythm" was a highly enriched function in our data set. Additional functions that were identified to exhibit pervasive circadian gene expression by this informatic method included "carbohydrate metabolism" and "lipid metabolism" (Figure 2A).

Essential components, including Bmal1, Per1, Per2, and Clock, of the autoregulatory loop that composes the molecular clock oscillated (Figure 2B). The oscillation of Bmal1 was antiphasic to the Period genes in the aorta (Figure 2B), as expected.¹⁴ The temporal expression of Per2 was aligned in liver, kidney, and aorta, but these peripheral tissues were out of phase with the SCN (Figure 2C), as has been described previously.¹⁵ In addition, genes with a modulatory role in circadian gene regulation also exhibited circadian rhythmicity, including nocturnin (Ccm41), interleukin-3–regulated nuclear factor (Nfil3), and D-site albumin promoter binding protein (Dbp)^{16–18} (Figure 2B).

Circadian Control of Metabolism

Glucose Metabolism

A large cluster of genes implicated in the regulation of glucose metabolism underwent circadian expression in the aorta (Figure 2D, i and ii). Key rate-limiting enzymes in most of the pathways that respond to energy utilization and storage exhibited circadian rhythmicity in their steady-state mRNA levels. These included genes relevant to glycolysis, gluconeogenesis, glycogenolysis, fatty acid synthesis and degradation, triglyceride mobilization and storage, and cholesterol biosynthesis (Figure 2D, iii and iv). These 22 genes exhibit pathway-specific coordination. For example, glycolytic genes cycled out of phase with those relevant to gluconeogenesis and glycogenolysis, whereas genes implicated in cholesterol and fatty acid biosynthesis were out of phase with enzymes involved in lipolysis and β -oxidation of fatty acids. Circadian rhythms were pervasive among enzymes in the glycolytic pathway, including glucose phosphate isomerase-1 (Gpi1), phosphofructokinase (both platelet and liver forms), phosphoglycerate mutase-muscle (Pgam), and enolase-3 (Figure 3). Similarly, enzymes of the hexose monophosphate shunt (malic enzyme, transketolase), which links glycolysis and gluconeogenesis, also exhibited rhythms. Phosphoenolpyru-



Figure 2. Molecular clock components and metabolic pathways. A, Bioinformatic analysis of array data reveals abundant functions under putative circadian control. Enriched classes included adipose tissue, hematological system, and nervous system development and function in addition to classes that overlapped with the GO-based analyses (see Methods), such as circadian rhythm and lipid and carbohydrate metabolism. B, Depicted in the mini-heat map are core clock genes that exhibit circadian gene expression in the aorta. C, Rhythmic expression of Per2 rhythms is phaseadvanced in the SCN compared with aorta, liver, and skeletal muscle. Data are derived from 6 pooled aortae per time point, hybridized to 2 separate microarray chips per time point). D, Circadian gene expression pattern in aorta relevant to metabolic regulation. Aortic oscillators involved in glycolysis, gluconeogenesis, glycogenolysis, lipolysis and fatty acid β -oxidation, cholesterol and fatty acid biosynthesis, adipocyte differentiation, and obesity shown as clustergrams at left and listed at right, commonly identified by roman numerals and color.

vate carboxykinase (Pepck) and malic enzyme, both implicated in gluconeogenesis, and their transcriptional regulators (C/ebp- β , Atf-2, and Atf-3)¹⁹ exhibited rhythmicity.

Lipid Metabolism

Predominant cyclical expression of rate-limiting enzymes in a variety of lipid biosynthetic pathways was apparent early in the subjective night (Figure 2D, iii and iv), coinciding with energy intake. These included glyceronephosphate *O*-acyltransferase (Gnpat), which catalyzes the initial step in ether-phospholipid biosynthesis and 1-acylglycerol-3-phosphate *O*-acyltransferase 3 (Agpat3), which catalyzes the synthesis of phosphatidic acid. The rate-limiting enzyme in triglyceride biosynthesis, diacyl-glycerol *O*-acyltransferase 1 (Dgat1), as well as enzymes involved in fatty acid synthesis, stearoyl-Coenzyme A (CoA)-desaturase 2 (Scd2) and mitochondrial acyl-CoA thioesterase 1 (Mte-1), also peaked to coincide with supply of their respective substrates. Enzymes that catalyze the intermediate steps of isoprenoid and cholesterol biosynthesis also exhibited rhythmicity. These included squalene epoxidase, farnesyl pyrophosphate

synthase (Fpps), mevalonate decarboxylase (Mvd), isopentenyl pyrophosphate (Ipp) isomerase, and an enzyme similar to NAD(P)-dependent steroid dehydrogenase (Nsdh1). A similar pattern was observed in the liver.³

Genes encoding lipolytic enzymes, including the key enzymes involved in fatty acid oxidation, including carnitine palmitoyltransferase 1 (Cpt1) and fatty acid CoA ligase (Facl2) oscillated in the aortic tissue. These genes were phase-delayed relative to peak expression of the transcriptional regulators of fatty acid oxidation and energy balance Ppar- α and estrogen-related receptor- α (Err α). Other lipolytic genes that oscillated included stearoyl CoA desaturase, Cpt1, Dgat1, and Ppar- α . Remarkably, the subset of genes involved in metabolism within the aorta did not exhibit the pervasive oscillations in other "metabolic" tissues (Data Supplement Figure II).

Adipose Functions

Transcripts of many genes involved in energy balance as well as adipocyte maturation and obesity were detected oscillating



Figure 3. Circadian rhythms pervasive among enzymatic pathways in glucose metabolism. Pathway maps from KEG pathway enrichment analysis using EASE (see Methods) demonstrate a significant representation of genes observed by microarray analysis (green boxes) in the gluconeogenic, glycolytic, and pentose phosphate pathways.

in a circadian manner in aorta (Figure 2D, v). This most likely reflects inclusion of perivascular adipose tissue when the aortae were harvested. Global expression analysis confirmed circadian variation of the adipokine leptin and its receptor.²⁰ In addition, circadian expression of $Err\alpha$, hormone-sensitive lipase (Hsl), and β -3 adrenergic receptor (β 3AR) was observed. β 3AR is expressed on adipocytes and mediates inhibition of insulin-mediated leptin secretion.²¹ Interestingly, another adipokine, resistin, a potential candidate insulin resistance gene implicated as a molecular link between type II diabetes and obesity,22 cycled in phase with leptin, LepR, and β -3AR. In addition, we found circadian variation in the transcription factors C/ebp α and C/ebp β , which promote adipocyte differentiation and are important insulinsensitive targets,²³ and in Fatp1, which is upregulated during adipocyte differentiation and promotes adipocyte uptake of long-chain fatty acids in response to insulin.24,25

Protein Folding and Degradation

Some output genes oscillated in more than 1 tissue. For example, some heat-shock proteins oscillated prominently in both liver and aorta (HSP110, HSP90, HSP73). In aorta, a multitude of additional heat-shock proteins also oscillated (Figure 4A). Moreover, the temporal alignment of the heat-shock proteins was in phase (Figure 4B). In addition, genes related to intracellular degradation, particularly ubiquitination (ubiquitin-specific protease 2/USP-2, USP-4, α -proteasome subunit) oscillated in both liver and aorta. However, comparative analysis of aorta with SCN and liver indicates that the

majority of genes appear to oscillate in a tissue-specific manner, consistent with tissue-specific circadian functions.

Vascular Structure and Integrity

We found that transcripts of many genes involved in maintenance of vascular structure and integrity oscillated in a circadian manner (Figure 5, A and B). Constituent molecules of the contractile apparatus of vascular smooth muscle, such as α -actin, transgelin (Sm22), titin-cap, calponin, and tropomyosin, oscillated in the aortic data set (Figure 5B). Similarly, elastin and fibrillin, extracellular matrix genes relevant to elastic recoil in large arteries, displayed circadian variation.

The oscillation of constituents of tight junctions raised the additional possibility of circadian variation in vascular integrity of relevance to the inflammatory response and the propensity to edema. Junctional adhesion molecule-1 (Jam-1), claudin-5, intercellular adhesion molecule-2 (ICAM-2), and glycosylation-dependent cell adhesion molecule-1 (GLYCAM-1) oscillated with circadian expression pattern (Figure 5A). These endothelial cell-specific genes subserve a barrier function to regulate the paracellular flow of macromolecules through the endothelium. We confirmed the oscillations observed in the array analysis by performing real-time PCR for claudin-5 (Figure 1C). In addition, we demonstrated that vascular permeability to Evans blue dye was influenced by clock time (Figure 5C), with the greatest extravascular leakage of dye occurring with the onset of subjective night expressed in hours of circadian time, consistent with our microarray results.



Figure 4. Heat-shock proteins exhibit circadian gene expression. A, Heat-shock proteins that oscillated in aorta are shown according to the same time course as shown in B. The phase of heat-shock proteins was aligned. In B, data from A, overlaid, and relative expression are shown.

Finally, many genes involved in endothelial activation oscillated. These included chemokines (stromal cell–derived factor 1 isoform β -precursor/Cxcl12, chemokine [C-X-C motif] ligand 13/Cxcl13), antigen recognition (H2-K/histo-compatibility 2, Ii/Ia-associated invariant chain, H2-q7, Igk-v28, H2-d1), the nuclear receptor Ppar α , and inflammatory response genes (GLYCAM-1, ICAM-2, platelet-activating factor acetylhydrolase).

Promoter Analysis

A preliminary analysis of promoter elements among the oscillating genes belonging to the heat-shock proteins, metabolism, and vascular function subsets revealed some common motifs (Table). Most common among these 3 groups was a consensus myoblast-determining factor (MyoD) element. Although the transcription factor MyoD did oscillate significantly in skeletal muscle, its apparent pattern of circadian oscillation in aortic tissue failed to attain significance (Data Supplement Figure III).

Discussion

Circadian rhythms are pervasive in mammalian biology. These rhythms are extended beyond the central nervous system into the periphery, affecting reproduction,²⁶ cell division,12,27 and metabolism.28-30 Many aspects of cardiovascular function are subject to diurnal variation, although the precise role of the molecular clock in these phenomena remains largely to be determined. Global gene expression analysis in discrete peripheral tissues, such as liver, heart, kidney, and skeletal muscle, has proved useful in generating hypotheses as to the role of the clock in tissue function.3,29,31-33 The core components of the molecular clock exhibited a circadian expression pattern in aorta, as demonstrated previously in other tissues in the periphery and SCN.1,3,29,31 Bmal1 and Clock, transcription factors at the core of the molecular clock, may also influence genes extrinsic to the circadian autoregulatory feedback loop by activating transcription on promoter sequences containing E-boxes. Moreover, circadian gene regulation involves other signaling mechanisms, including Rev-Erb α -responsive elements and posttranslational mechanisms that may also influence the expression of genes outside of the loop. Analysis of promoter motifs from a subset of the oscillating genes revealed a common MyoD regulatory element. In fact, MyoD is a basic helix-loop-helix protein that does bind E-box promoter-enhancing motifs, molecular features that are also characteristic of other molecular clock proteins (Bmal1, Clock, and NPAS2).34 However, the biological relevance of MyoD is most established in skeletal muscle,35,36 where it did, indeed, oscillate significantly. Surprisingly, a signal was detected in aortic tissue, but it exhibited only a trend toward a circadian rhythm. Future studies will be necessary to understand whether there is any significance to the consensus MyoD regulatory element among oscillating genes.

One functional gene cassette identified in the circadian subset was related to vascular structure and function. Many of the oscillating genes were constituents of the smooth muscle contractile machinery, such as tropomyosin, α -actin, and SM-22, or involved in actin binding such as Marcks, Titincap, and calponin. Other genes relevant to vascular function that oscillated were the angiopoietin receptors (Tie-1 and Tek), protein kinase B/Akt1, and platelet-activating factor acetylhydrolase (not shown). These oscillations may be relevant to the circadian variation in blood pressure³⁷ and the pressor response to infused contractile agonists in humans.³⁸ Others have described diurnal oscillation of endothelial barrier function: permeability of the aorta to albumin in rabbits³⁹ and permeability of arterioles to horseradish peroxidase in rats.⁴⁰ In the present studies, leakage of the Evans blue dye underwent a time-dependent variation in the mouse. Indeed, variation in genes relevant to barrier function may contribute not only to diurnal variation in edema accumulation^{41,42} but also to the diurnal variation in severity of inflammatory syndromes, such as bronchial asthma43 and arthritis.44 Inclusion of genes relevant to vascular integrity and response to injury in the oscillating subset raise the possibility that the dysplastic response to injury, which contributes to restenosis after percutaneous angioplasty, might be conditioned by the timing of the procedure. Similarly, circadian variation in





Figure 5. Oscillations in genes relevant to vascular function. Genes characteristic of endothelial cells (A) and smooth muscle cells (B) exhibited a circadian expression pattern. Significance is shown as *P* value shown above respective figure. C, Vascular leakage of Evans blue dye into the interstitium varied according to time of day in response to an ear injury in mice (n=13, *P < 0.01, CT11 versus CT23).

genes relevant to immune function and the inflammatory response raise the possibility that timing might also influence the incidence of graft atherosclerosis in patients undergoing arterial bypass.

Our analysis of aortic tissue revealed that circadian oscillations of genes encoding key enzymes involved in glucose and lipid homeostasis oscillated, functions not traditionally ascribed to the vasculature. This may reflect our purposeful

Analysis of Promoter Elements

Response Element	Consensus Sequence	Heat Shock	Vascular	Metabolism
Myoblast determining factor	NNCANCTGNY	7/10	8/8	16/18
Bicoid	SGGATTAN			12/18
GC box elements	NRGGGGCGGGGCNK	7/10		
GATA binding factor-2	NNWGATAASA		5/8	
Stimulating protein 1	NGGGGGCGGGGYN	7/10		

Putative promoter elements of genes were analyzed using Tres (transcription regulatory element search). Analyzed were heat shock, vascular, and metabolism group sets. Shown is the number of promoters containing the respective element relative to the total number analyzed.

inclusion of perivascular fat, mindful of its influence on vascular function.^{45,46} Recently, we demonstrated that mice with a disrupted (Bmal1^{-/-}) or mutated (Clockmut) molecular clock lose rhythmic oscillations in aspects of glucose homeostasis.³⁰ Among these, gluconeogenesis and the circadian variation in Pepck activity were dramatically impaired in these mice. This is consistent with our present observations that Pepck exhibits circadian variation by global gene expression analysis.

Surprisingly, there was little overlap among the metabolic oscillators detected in the aorta and other tissues/organs that control metabolic homeostasis. Indeed, metabolic enzymes oscillating in aortic tissue may reflect local energy demands of vasculature tissue but may also reflect an underappreciated role of vascular tissue in systemic metabolic homeostasis. Recent work has challenged traditional assumptions with regard to a tissue-specific mechanism of homeostasis.⁴⁷ For example, mice with liver-specific deletion of Pepck sustain normal glucose levels during fasting through extrahepatic mechanisms of gluconeogenesis.⁴⁸ Moreover, deletion of the insulin receptor in the endothelium of blood vessels results in insulin resistance in mice on a low-salt diet.⁴⁹ Given the

emerging importance of other "unconventional tissues" in glucose homeostasis,⁵⁰ it is premature to dismiss the possibility that circadian oscillation of metabolic genes in the vasculature has functional relevance.

Diurnal variation in many aspects of cardiovascular function—blood pressure, coagulation, and contractile function—is well established, as is diurnal variation in important clinical events, such as myocardial infarction and stroke. We describe functional cassettes of vascular genes that exhibit circadian regulation in the mouse. This will facilitate elucidation of the mechanisms by which the molecular clock may interact with environmental variables to modulate cardiovascular function and the response to therapeutic interventions.

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CLINICAL PERSPECTIVE

Many aspects of circulatory physiology vary according to the time of day. Blood pressure, heart rate, and vascular function exhibit rhythmic changes over a 24-hour period—the essence of a circadian rhythm. The incidence of significant cardiovascular events, such as MI and stroke, also undergoes a diurnal variation. However, the relevance of the molecular clock to these phenomena remains to be established. As an initial approach to this issue, we identified the genes subject to circadian variation in vasculature. Aortic arteries and surrounding perivascular adipose tissue from normal mice were isolated at different times of day over a 48-hour period and analyzed for gene expression by transcriptional profiling. Bioinformatic analysis of the more than 300 oscillating genes identified several functionally integrated cassettes, such as clusters related to glucose and lipid metabolism, protein folding, protein degradation, adipocyte maturation, vascular function and integrity, and the response to injury. These studies suggest that timing may influence the response to vascular interventions, such as angioplasty and coronary bypass grafting, and that the molecular clock may have unexpected relevance to complex, multisystemic disorders, such as metabolic syndrome.