Vascular circadian rhythms in a mouse vascular smooth muscle cell line (Movas-1)

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1Departments of Physiology, 2Medicine, and Ob/Gyn, University of Toronto, Medical Sciences Building; 3Heart and Stroke Richard Lewar Centre for Cardiovascular Excellence, and Toronto General Hospital Research Institute; and 4Centre for Biological Timing and Cognition, Department of Psychology, University of Toronto, Toronto, Ontario, Canada

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Chalmers JA, Martino TA, Tata N, Ralph MR, Sole MJ, Belsham DD. Vascular circadian rhythms in a mouse vascular smooth muscle cell line (Movas-1). Am J Physiol Regul Integr Comp Physiol 295: R1529–R1538, 2008. First published September 3, 2008; doi:10.1152/ajpregu.90572.2008.—The circadian system in mammals is a hierarchy of oscillators throughout the organism that are coordinated by the circadian clock in the hypothalamic suprachiasmatic nucleus. Peripheral clocks act to integrate time-of-day information from neural or hormonal signals, regulating gene expression, and, subsequently, organ physiology. However, the mechanisms by which the central clock communicates with peripheral oscillators are not understood and are likely tissue specific. In this study, we establish a mouse vascular cell model suitable for investigations of these mechanisms at a molecular level. Using the immortalized vascular smooth muscle cell line Movas-1, we determined that these cells express the circadian clock machinery with robust rhythms in mRNA expression over a 36-h period after serum shock synchronization. Furthermore, norepinephrine and forskolin were able to synchronize circadian rhythms in bml1. With synchronization, we observed cycling of specific genes, including the tissue inhibitor of metalloproteinase 1 and 3 (timp1, timp3), collagen 1α1 (col1α1), transgelin 1 (sm22α), and calponin 1 (cn1). Diurnal expression of these genes was also found in vivo in mouse aortic tissue, using microarray and real-time RT-PCR analysis. Both of these revealed ultradian rhythms in genes similar to the cycling observed in Movas-1 in vitro. These findings highlight the cyclical nature of structurally important genes in the vasculature that is similar both in vivo and in vitro. This study establishes the Movas-1 cells as a novel cell model from which to further investigate the molecular mechanisms of clock regulation in the vasculature.

real-time RT-PCR; ultradian; diurnal; microarray

THE CIRCADIAN SYSTEM IS AN integrated structure that operates through a hierarchy of oscillators in peripheral tissues and other brain regions with the suprachiasmatic nucleus (SCN) functioning as the primary pacemaker or master oscillator and is located in the SCN of the anterior hypothalamus (40). The clock is synchronized to the rhythms of the outside world primarily by photic signals received in the retina then transmitted to the SCN (43). Within the SCN, photic and nonphotic signals set and entrain molecular circadian rhythms generated within pacemaker cells via positive and negative transcriptional and translational feedback loops (44). This clock machinery is present not only in the SCN, but also in tissues throughout the body, including other brain regions (16, 17, 21).

The influence of the circadian clock on cardiovascular physiology is reflected in daily cyclic variations in systolic blood pressure, vasodilatation, metabolism, and basal heart rate (16, 50). For example, blood pressure rises in the early morning, peaks midday, and then starts to decline reaching a trough during the night (33). Peptides and hormones, including catecholamines and the renin-angiotensin aldosterone system, that regulate blood pressure and vasodilatation, are known to express robust circadian expression patterns, (39). Circadian regulation of these peptides combined with autonomic bias creates a “time of vulnerability” window of the cardiovascular system for initiation of adverse events such as myocardial infarction, angina, and ischemic stroke (26, 32).

Recently, in a rodent model of cardiomyopathic disease, desynchronization of the circadian clock was found to decrease longevity (38). We have shown that an abnormal 10:10-h light-dark schedule can exacerbate heart disease in mice, whereas a return to normal 12:12-h light-dark conditions can abate this effect (29). We have further demonstrated the essential role of internal circadian desynchrony in the development of heart disease using the tau mutant hamster model (28). The regulation of this multitude of circadian effects on the cardiovascular system by the SCN is only beginning to be understood from a circadian perspective. From this view, the heart and vasculature must be considered not only as organs with defined functions, but also as peripheral clocks that play a key role in maintaining the health of the organism, and consequently a part in disease as well.

A variety of signals now have been shown to synchronize clocks in peripheral tissues, and/or moderate communication between the SCN and clocks in target tissues. Peripheral clocks in vivo can be synchronized by light and scheduled food availability (9, 17, 45). Rhythms also have been restored in cell cultures by temperature cycles (6) or treatment with serum plasma (FBS) (5, 19, 20), ANG II (35), glucocorticoids (i.e., cortisol) (4, 20), norepinephrine (NE), and the protein kinase A (PKA) activator, forskolin (10). Therefore, control is achieved via both sympathetic input and hormonal avenues. Though progress has been made in determining the hormonal mechanisms that control the circadian clock in peripheral tissues, the picture remains incomplete. This is largely due to the fact that...
only a small number of potential effectors that could alter circadian rhythms in the periphery have been investigated, and few different cell models exist with which to address such questions (21).

To investigate circadian regulation of genes that are important in vascular structure and function in detail, we used the mouse vascular smooth muscle cell line one (Movas-1) and studied the temporal expression of seven genes relevant to vascular function. We first determined whether Movas-1 expressed the components of the molecular clock and possessed a functional circadian clock. We tested the response of these cells to the known synchronizing factors NE and forskolin. We then examined the potential effect of the circadian clock on genes involved in maintaining the integrity and function of vascular cells.

MATERIALS AND METHODS

Maintenance of the Movas-1 cell line. The Movas-1 cell line was derived from aortic smooth muscle cells of male C57BL6 mice [Fig. 1A, (1)]. The cells were immortalized using a retrovirus containing the SV40 large-T antigen. These cells express the smooth muscle cell markers α-actin and sm22α. Movas-1 cells were grown in a tissue culture incubator set to 37°C and 5% CO2. Under regular conditions, Movas-1 cells are grown in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Hyclone; Fisher Scientific), and 1% penicillin-streptomycin (PenStrep; GIBCO-Invitrogen). To maintain Movas-1 in an immortalized state, the plasmid containing the T-antigen in these cells was also encoded with the gene conferring neomycin resistance. Therefore, in accordance with the established protocol (1), after 10 consecutive passages the Movas-1 cells were treated on alternate days for 3 days with 200 μg/ml Geneticin (G418; Gibco). Medium was refreshed before each treatment with G418. Replicate experiments were conducted with cells of a different passage number.

Experimental treatment of Movas-1 cells. Movas-1 cells were subjected to three time-course protocols in which they were either 1) left untreated for baseline experiments, 2) treated with FBS, or 3) treated with NE or forskolin alone. A summary of the treatment protocols can be found in supplemental Fig. 1 (The online version of this article contains supplemental data.). For all experiments, Movas-1 cells were grown to 100% confluency in 150-mm size tissue culture plates and then diluted 1:6 into 60-mm plates. Once diluted, the cells were grown overnight, again to 100% confluency and then used the next day. This was considered time 0, after which total ribonucleic acid (RNA) was collected every 4 h for 36 h. Confluent plates of Movas-1 cells were used for all experiments to exclude the cell cycle as a potential cause of any patterns in gene expression that might be observed over the time course.

To synchronize the clock in the Movas-1 cells in culture, a 20% (by volume) FBS serum shock was used. Serum shock synchronizes gene expression of individual cell to one another (10, 34). To serum shock the Movas-1 cells, the cells were washed with 1× PBS solution, placed in 3 ml serum free media (with 1% PenStrep) for 12 h, and the FBS was then administered for 30 min. The media was then removed and replaced with 3 ml of culture media. This was considered time 0, and total RNA was collected every 4 h for 36 h.

For experiments to determine whether the NE and forskolin could initiate clock gene cycling in the Movas-1 cells, a new protocol was established. NE (Sigma-Aldrich) and forskolin (Sigma-Aldrich) were each used at a concentration of 10−6 M (10, 25). NE was dissolved in water, and forskolin was dissolved in DMSO. For this study, Movas-1 cells were washed twice with 1× PBS, placed in 2.5 ml of 1% DMEM (low serum) medium for 12 h, and then treatments were added to 1% DMEM. Once the neuropeptides were added, this was considered time 0. Total RNA was then collected every 4 h for 36 h. For all experiments n = 3 replicates minimum. Each repeat was independently conducted on a separate day(s) using a different passage number of cells.

RNA isolation, cDNA synthesis, and semiquantitative and real-time RT-PCR. Movas-1 cells were collected from plates, and RNA was extracted in 0.5 ml of denaturing solution (Solution D: 4 M guanidinium thiocyanate, 0.75 M sodium citrate pH 7.0, 10% sarcosyl, 0.1 M β-mercaptoethanol), 0.5 ml of water-saturated phenol (Sigma), and 50 μl of 2 M sodium acetate (adapted from Ref. 8). All RNA samples were quantified by spectrophotometry to determine concentration and demonstrate high quality purification. Single-strand cDNA from the Movas-1 samples was synthesized using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA).
Two-step quantitative real-time PCR was used for Movas-1 samples; therefore cDNA was made separately as described in RNA isolation. cDNA synthesis, semiquantitative and real-time RT-PCR. Two-step real-time quantitative PCR was conducted using a master mix per sample containing: 10× SYBR Green (1:1,000 dilution), ROX (a passive reference dye), 10 mM dNTP, 50 mM MgCl2, 10× PCR buffer, and 5 U/μl platinum Taq enzyme, all purchased from Invitrogen. Samples were run in a 384-well 7900 ABI Prism machine using SDS 2.2.1 software. The PCR protocol used was RT at 50°C for 2 min and 95°C for 10 min (1 cycle) and then amplification at 95°C for 15 s and 60°C for 1 min (40 cycles). This was followed by a dissociation step set to 95°C for 15 s, 60°C for 15 s, and then 95°C for 15 s. See Table 1 for primer sequences.

One-step RT-PCR was used to screen Movas-1 cells for the circadian clock genes, neuropeptide receptors, and genes important in cardiovascular structure and function and was done using the Qiagen (Mississauga, ON, Canada) one-step RT-PCR Kit as per the manufacturer’s instructions. PCR was conducted according to the following cycles: 94°C for 30 s, 60°C for 30 s and 72°C for 1 min (45 cycles). A quantity of 200 ng of RNA was used for all samples. Real-time quantitative PCR was done using the one-step RT-PCR kit and SYBR Green PCR Master Mix (Applied Biosystems). Samples were loaded into a 96-well plate and run on ABI Prism 7000 Sequence Detection System. The RT protocol used for all primer sets (see Table 1) tested in the aortic tissue was 48°C for 30 min, 95°C for 10 min, followed by amplification using 95°C for 15 s, and 60°C for 1 min (40 repeats/cycles). Histone primers were used as control.

All primers were designed using mouse mRNA sequences, and were made to flank at least one intron. Newly designed primers and those taken from published work were tested in the Movas-1 cells (13, 18, 24, 29, 47) and sequenced for verification at The Centre for Applied Genomics (at The Hospital for Sick Children, MaRS Centre, Toronto, Ontario, Canada). See Table 2 for primer sequences. PCR samples were visualized on 2% agarose gels with 0.05 mg/ml ethidium bromide. A 1-kb ladder (Fermentas Life Sciences) was used to gauge product size. DNA bands were visualized under ultraviolet light on the Kodak Image Station 2000R, and 1D Image analysis software 3.6 (Eastman Kodak, Rochester, NY).

Animals. vascular RNA, microarray, and bioinformatics analysis. Vascular circadian gene rhythms in vivo were examined and compared with the MOVAS gene rhythm data generated in vitro. A total of 18 eight-week-old male C57BL/6 mice were used. All animal protocols were reviewed and approved by the University of Toronto Institutional Animal Care and Use Committee. Animals were maintained on a 12:12-h light-dark schedule [zeitgeber time 0 h (ZT0) and lights off at ZT12]. After 4 wk, animals were euthanized, and the ascending aorta was collected. The proximal end was dissected from the heart and the distal end, just past the subclavian artery. Aortae were collected every 4 h across the diurnal cycle. Vascular tissue was pooled at each time point, so as to generate enough RNA for subsequent microarray analysis. That is, six vascular samples were collected over 24 h, with three samples taken and pooled at each time point. When each vascular sample was collected, it was immediately placed on a thin layer of tinfoil resting on dry ice. Once all three samples at a given time point were collected and placed together, the foil was immediately folded, the sample packet quick frozen in dry ice, and then stored at −80°C until use.

Total RNA was prepared from the vascular tissue samples using Trizol reagent (Invitrogen, Burlington, Canada) following the manufacturer’s protocol. The quality of the RNA was determined on 1% agarose-formaldehyde gel electrophoresis and ethidium bromide stain.
BioAgilent spectrophotometry chips revealed a sample absorbance ratio of 260:280 > 1.8, and further confirmed the high quality of RNA being used.

Gene expression was analyzed on the mouse MOE430A arrays (Affymetrix, Santa Clara, CA). Hybridization and scanning procedures were performed in accordance with manufacturers specifications. Prior to data analysis, the target signal expression intensities were scaled to 150 fluorescence units per gene chip using MicroArray Suite Version 5 (Affymetrix). MicroArray Suite statistical algorithms were also used to assign individual gene expression values. Data were then imported and standardized in GeneSpring GX version 5 (Agilent Technologies, Santa Clara, CA). Data were transformed such that values <0.1 were set to 0.1. Per chip normalizations were performed so that each measurement was divided by the 50th percentile of all measurements. Per gene normalizations divided each gene by the median of its measurements in all samples. The Cross-Gen Error Model was applied to estimate the precision of gene expression intensity by combining measurement variation and between-sample variation (GeneSpring). For genes of interest, results were confirmed using real-time PCR, as described above.

Statistical analysis. All Movas-1 data were analyzed using the Prism 4.0 for Macintosh (GraphPad Software). Movas-1 data are plotted with the means ± SE with an n ≥ 3 for all experiments and groups. Real-time PCR data for each gene were normalized to γ-actin (internal control for baseline cell activity) levels from the same sample. One- or two-way ANOVAs were conducted followed by Tukey’s multiple comparison posttest on real-time PCR results and were considered significant if P < 0.05. For analysis of circadian rhythms, the single cosinor method was used (31). Cosinor analysis was performed using CHRONOLAB for the Macintosh computer (Universidade de Vigo, Spain, Bioengineering and Chronobiology Laboratory, http://www.tsc.uvigo.es/BIO/). This inferential method involves fitting a curve of a predefined period(s) by the method of least squares. The rhythm characteristics and their dispersions SE and 95% confidence interval estimated by this method include the mesor (middle value of the fitted cosine representing a rhythm-adjusted mean), the amplitude (half the difference between the minimum and maximum), the phase (time at which the fitted cosine had a zero value), and the amplitude (half the difference between the minimum and maximum).
Cardiovascular Genes

sm22

Cells express the smooth muscle cell markers using a retrovirus containing the SV40 large-T antigen. These [1]. The cells were immortalized C57BL6 mice [Fig. 1]. A cell line was derived from aortic smooth muscle cells of male C57BL6 mice.

RESULTS

Clock Genes and Controls

mBmal1

S: gggagcccacagctcagatt
AS: gtacccacagaaaggccccattcgtc

mPer1

S: ctcaggattttggagactgcaaa
AS: tgtgtgacagccgatctttc

mPer2

S: tcatctttgagggacaaaa
AS: gccatcttgacgcttggatt

mClock

S: cacagccaaagactccacctgat
AS: tgagacattctgcttggctgt

mRev-erb alpha

S: tggaagacagcagccggtgt
AS: catatgggagcctgagccca

mG-actin

S: tctcccaccgcaatcttg
AS: ccgcgtcagtcaaggtcttcatcctcat

mHistone

S: gcaagatctgcgccccctactg
AS: gctctctgtgctgtcgtcaca

Cardiovascular Genes

mCol3a1

S: tggcaccagcatcacaagcta
AS: tgacatggcttcggctcc

mTimp1

S: ccacacagacagactcct
AS: tgtgtataaggtgttaccttggatt

mTimp3

S: gggagactgctgacctg
AS: gctccttggcaccacactttg

mCnr1

S: cgcctrggagcaactctattttatgt
AS: cccggtctcagcctttgtagaasat

mSm22alpha (tagln)

S: accgtggagatcacaacttgacat
AS: cattttagaagccagctctctgtcgtct

Table 2. Real-time RT-PCR primers

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<th>Genes</th>
<th>Oligo Sequence</th>
<th>Cross Intron</th>
<th>Annealing Temperature</th>
<th>Reference No.</th>
</tr>
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<tr>
<td>mBmal1</td>
<td>S: gggagcccacagctcagatt</td>
<td>Yes</td>
<td>60°C</td>
<td>*</td>
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<tr>
<td>mPer1</td>
<td>S: ctcaggattttggagactgcaaa</td>
<td>Yes</td>
<td>60°C</td>
<td>24</td>
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<tr>
<td>mPer2</td>
<td>S: tcatctttgagggacaaaa</td>
<td>Yes</td>
<td>60°C</td>
<td>*</td>
</tr>
<tr>
<td>mClock</td>
<td>S: cacagccaaagactccacctgat</td>
<td>Yes</td>
<td>60°C</td>
<td>24</td>
</tr>
<tr>
<td>mRev-erb alpha</td>
<td>S: tggaagacagcagccggtgt</td>
<td>Yes</td>
<td>65°C</td>
<td>18</td>
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<tr>
<td>mG-actin</td>
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<td>Yes</td>
<td>60°C</td>
<td>47</td>
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<tr>
<td>mHistone</td>
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<td>60°C</td>
<td>29</td>
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<tr>
<td>mCol3a1</td>
<td>S: tggcaccagcatcacaagcta</td>
<td>Yes</td>
<td>60°C</td>
<td>†</td>
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<tr>
<td>mTimp1</td>
<td>S: ccacacagacagactcct</td>
<td>Yes</td>
<td>60°C</td>
<td>†</td>
</tr>
<tr>
<td>mTimp3</td>
<td>S: gggagactgctgacctg</td>
<td>Yes</td>
<td>60°C</td>
<td>†</td>
</tr>
<tr>
<td>mCnr1</td>
<td>S: cgcctrggagcaactctattttatgt</td>
<td>Yes</td>
<td>60°C</td>
<td>†</td>
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<tr>
<td>mSm22alpha (tagln)</td>
<td>S: accgtggagatcacaacttgacat</td>
<td>Yes</td>
<td>60°C</td>
<td>†</td>
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</tbody>
</table>

Reference number indicates the source of the sequence. *F. Cai, unpublished observation; †J. Chalmers, unpublished observation.

maximum of the fitted cosine function), and the high point or acrophase (time of peak value in the fitted cosine function expressed as the lag in hours and minutes from midnight). Each time series was tested for a circadian rhythm by the fit of a 24-h single cosine. Detection of rhythm was achieved by rejection of the zero amplitude hypothesis with 95% certainty as reflected by the $P$ value resulting from a comparison of residuals before and after the cosine curve fit. Rhythm analysis is determined by testing a null hypothesis of zero amplitude with an $F$ test by the CHRONOLAB program.

Expression of other clock genes, per1, per2, and rev-erba, also vary significantly over time (Fig. 2, A–C) with periods of ~24 h. The period was determined by comparing the 4-h time point as the first trough in the curve with the second trough or the next time point that approximates that same level of mRNA expression for the gene. The radar diagram (Fig. 2D) compares the timing of peak mRNA expression following serum shock of members of the clock machinery over the course of 1 day (24 h). Both per genes peak at 1 h post-serum shock, rev-erba at 16 h postserum shock, and bmal1 at 12 h. Thus clock gene transcription in Movas-1 cell culture is coordinated with treatment of FBS as a synchronizing agent. These results indicate that the Movas-1 cells possess a functional circadian clock.

Circadian clock machinery in Movas-1 cells. The Movas-1 cell line was derived from aortic smooth muscle cells of male C57BL6 mice [Fig. 1A, (1)]. The cells were immortalized using a retrovirus containing the SV40 large-T antigen. These cells express the smooth muscle cell markers alpha-actin and sm22alpha. Movas-1 cells were screened for several genes critical to the core molecular circadian clock mechanism. As shown in Fig. 1B, bmal1, cry1, cry2, per1, per2, and clock mRNA transcripts were detected in Movas-1 cells. The gonadotropin-releasing hormone T-antigen 1-7 (GT1-7) cell line was previously used to study circadian rhythms in the hypothalamus (13). Sizes of PCR products are listed (Table 1). These results demonstrate that the core molecular clock elements are expressed in Movas-1, making them a possible candidate vascular cell line to study circadian rhythms in vitro.

Serum shock is traditionally used in cell culture to synchronize individual cells on the plate with respect to one another (5); otherwise a coherent pattern in mRNA cycling may not be observed. Synchronized cycling was not observed under normal culture conditions, as expected (Fig. 1C). However, mRNA cycling of bmal1 mRNA was observed following a 20% serum shock, consistent with the notion that serum shock reestablishes cellular synchrony (Fig. 1D).
significant (see Supplementary Fig. 2A) with the peak expression still occurring at 12 h. For rev-erbα the change in mRNA expression was significant with a peak expression level at 16 h (see Supplementary Fig. 2B). Therefore, the clock in the Movas-1 functions accordingly under low (1%) serum conditions when given an appropriate synchronizing stimulus.

Treatment with NE and forskolin synchronizes bmal1 mRNA rhythm expression in Movas-1 cells. We next examined the potential for NE and forskolin to act as synchronizing agents in this model. Both NE (10⁻⁶ M) and forskolin (10⁻⁶ M) have been shown previously to reinstate circadian clock machinery rhythms in cell culture on primary myocyte and vascular smooth muscle tissue, respectively (10, 25). However, their effects have not been demonstrated in smooth muscle cells in the case of NE or in an immortalized cell line in the case of forskolin. As shown in Fig. 3A, NE increases bmal1 expression in Movas-1 cells from 4 – 8 h, whereas forskolin elevates bmal1 mRNA expression from 4 – 12 h (Fig. 3B). No significant pattern in bmal1 mRNA expression was observed over time in the untreated control samples for both experiments (P < 0.05). Therefore, both the NE and forskolin treatment reinstates cycling of bmal1 mRNA expression in Movas-1 cells.

Movas-1 cells express cardiovascular cell-type specific, remodeling, structural-related genes. Our group recently demonstrated that diurnal rhythmicity is critical to cardiovascular structure and function in health and disease (29). We are now interested in incorporating an in vitro model to further study the transcriptional mechanisms involved, and postulate that the Movas-1 cells are ideal for this purpose. We used the Movas-1 cells to determine whether the transcription of several genes involved in the maintaining the health of the cardiovascular system is under the control of the circadian clock. Results from one-step RT-PCR screening revealed that the Movas-1 cells express the tissue inhibitor of metalloproteinase 1 and 3 (timp1, timp3), collagen 3a1 (col3a1), transgelin 1 (sm22α), and calponin 1 (cunl) mRNA transcripts (see Supplementary Fig. 3A). The presence of these transcripts indicates that the Movas-1 cells may serve as a model for examining cell-specific gene regulation.

Given these results we next conducted an in silico analysis to determine whether there were potential similar binding sites.
for circadian transcription factors within the promoter region of these genes. In general, E-boxes are defined by sequence CANNNG (where N can be any base pair), among the transcription factors that bind to these sequences are the BMAL1: CRY heterodimers. One-thousand base pairs of the promoter region upstream of the transcriptional start site of the: timp1, timp3, col3al, sm22α, and cnm1 genes were scanned for E-boxes; timp1 was the only gene examined found not to have any E-boxes within the specified region of the promoter (see Supplementary Fig. 3B). These results show that there are potential binding sites for circadian transcription factors in the promoter region of these genes.

Profile of cardiovascular structural, remodeling, and smooth muscle cell-type specific genes over time in Movas-1 cells, and mouse aortic tissue. We next focused on the potential for these genes to be under temporal regulation by the circadian clock. Of the cardiovascular genes found to be present in the Movas-1 cells, several displayed a robust, as well as significant, variation over time, post-serum shock (Fig. 4), compared with no serum shock (data not shown; \( P > 0.05 \)). These results were confirmed using the cosinor analysis program CHRONOLAB. These data suggest that the circadian clock plays a role in mRNA expression of important cardiovascular genes.

To corroborate the data gathered on cardiovascular gene expression from the Movas-1 cells in vitro, with gene expression in vivo, we performed microarray analyses. Aortae collected from male C57BL6 mice were entrained to a normal 12:12-h light-dark schedule. One-step real-time PCR validated the microarray observations (Fig. 4). Statistical analysis of the real-time PCR data from the mouse aortic tissue revealed a significant change in expression level across the day/night cycle for all the genes examined. Interestingly, the profiles of genes that appear similar to one another in vivo, for example, timp1, and sm22α also parallel one another in vitro, in the Movas-1 cells with serum shock. Overall, these results confirm that the temporal changes in gene expression observed in our in vitro model.

DISCUSSION

The SCN plays an essential role in the coordination of circadian rhythms in peripheral tissues (16). However, the means by which this coordination is achieved largely remains a mystery. This gap is due in part to the complexity of the circadian system and the relatively recent development of the technical and molecular tools required to fully answer questions of this nature. These tools have helped tease apart fundamental clues as to how the SCN works in the hypothalamus including the method that SCN neurons use to communicate among each other and the properties that invest these cells with their inherent rhythmicity (3, 7, 23). Given that many important aspects of the circadian system in the SCN are becoming better understood, much more work needs to be done to increase our knowledge of the communication of this master clock with peripheral tissues. The primary method(s) through which the SCN coordinates rhythms in peripheral tissues is currently debated (16). Evidence exists for both neural and hormonal control of the circadian clock in the cardiovascular system (2, 4, 14, 15, 30). The purpose of this study was to investigate the interactions between the circadian rhythms in aortic smooth muscle, as well as the potential clock control of output genes important in vasculature.

Studies by Nagoshi et al. (34) illustrate that when fibroblast cells are left untreated and total RNA is collected, no coherent pattern in clock gene expression can be observed. In Movas-1 cells under unsynchronized conditions, bmal1 mRNA did not vary significantly over time. This finding supports the conclusion drawn from evidence collected from fibroblast cells, in which the individual clocks of each cell in a tissue culture plate of cells cycle, but out of phase with one another, unless they are synchronized via some external stimulus (34, 49). The only immortalized cell line that is able to demonstrate coherent circadian oscillation in clock genes without such a signal is the SCN 2.2 cell line (19). Therefore, to observe cycling of the circadian clock machinery in Movas-1 cells, a synchronization protocol was established using exposure to a 20% FBS shock for 30 min. Under these conditions, most clock components bmal1, rev-erba, per1, and per2, cyclized significantly, whereas clock was expressed at similar levels throughout the time course (data not shown). This constitutive expression profile of clock mRNA as opposed to robust cyclical change is consistent with reports from cultured hypothalamic cells [GT1-7; (5, 13)], primary SCN tissue (12), and primary retinal tissue (22), although data collected from whole heart tissue in mice from our group and others suggest a slight (2-fold) change in clock gene expression over time (27, 51). Cycling members of the circadian clock machinery had a period of ~24 h, as expected (41). Interestingly, the cycling of bmal1 and per2 mRNA in the Movas-1 cells was remarkably comparable to the pattern of those genes in serum-shocked primary human smooth muscle cells, including the times of maximal and minimal expression (25). The timing of peak clock gene expression is illustrated in a radar diagram in Fig. 2D: per1 and per2 peak at the 1 h time point, whereas bmal1 peaks at roughly 12 h and rev-erba at 16 h post-serum shock. This indicates that the clock machinery members are in proper phase alignment to one another, as bmal1 peaks after its negative transcriptional regulators per1, per2, and rev-erba have declined and vice versa. The phase difference between bmal1 and the period genes of 11 h is comparable to phase difference of 9–10 h observed in whole heart similar to that observed in primary cardiomyocytes and whole heart tissue (10, 27, 36). Also the phase difference between rev-erba and bmal1 is ~4 h, which is nearly identical to that observed in myocytes and primary heart tissue of 3–5 h (10).

Fig. 4. In vitro vs. in vivo comparison of gene expression in Movas-1 compared with microarray and real-time PCR data of murine aortic tissue. Real-time PCR data for timp1 (A), timp3 (B), col3al (C), cnm1 (D), sm22α (E). All genes examined in the Movas-1 cells vary significantly over time with a serum shock; \( P < 0.0001 \) (one-way ANOVA; \( n = 3 \) means ± SE). The gene col3al has period of ~24 h, cnm1 remains high until 12 h post-serum shock, and sm22α peaks at 8 h and then dampens. Zetageber time (ZT), which is the time of day according to the lighting schedule the animals were kept, in this case 12:12-h light-dark, is indicated by the light and dark bars below the graphs. Each time point represents 3 mice. One-way ANOVA indicates a significant variation in all genes shown (\( P < 0.0001 \)). Time points are relative to the 4-h time point for Movas-1 and 3-h time point for aortic tissue, using Tukey’s multiple comparison test, where *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \). Significant rhythmic expression patterns were confirmed using the cosinor analysis program CHRONOLAB.
A principal function of the circadian system is to appropriately time physiology and behavior with the rhythmic changes in the environment. The circadian clock achieves this by directly regulating the production of mRNA in a rhythmic manner, such that the peak expression of a particular gene precedes the time of day when the protein product of that gene is required for the cell to function properly (Fig. 5). This coordination at the cellular level combined with synchrony in

**Fig. 5**

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mRNA synthesis among cells that comprise a particular tissue, for example the heart, effectively prepare the organ to function in a specific manner according to the external environment (27, 37, 46). Our group has found via microarray analysis of the normal mouse heart, that genes that comprise the extracellular matrix (ECM; collagen), as well as those that mediate reconstruction of the ECM, including inhibitors of metalloproteinases, show a distinct profile over the day/night cycle. For instance, *timp3* increases and peaks during the night, the active phase of the cycle for mice, which are nocturnal, and troughs during the day when asleep (27). Therefore the next objective of this project was to determine whether the circadian clock is involved in controlling the mRNA expression of several cardiovascular genes, including those involved in matrix remodeling (inhibitors of metalloproteinases), structural organization (collagen), and smooth muscle cell specificity (calponin, transgelin) in vascular smooth muscle cells using the Movas-1 model. The Movas-1 cells were initially screened and found to express several cardiovascular genes: *timp1*, *timp3*, *col3a1*, *sm22α*, and *cnn1*.

To determine whether cycling in these genes in the in vitro setting was a reflection of the in vivo situation, we scanned and analyzed microarray data generated from mouse aortic tissue for profiles of each gene under normal day/night conditions. All of the cardiovascular gene profiles generated from the aortic tissue samples changed significantly over time. The dampening of the pattern in *timp1*, *cnn1*, and *sm22α* within the Movas-1 cells, compared with the mouse aorta, may be due to the fact that there is a cue missing from the serum that is present in the whole animal that maintains the rhythm, or perhaps these genes are controlled indirectly by a transcription factor that itself is directly clock regulated. Such indirect regulation may be altered more readily as the cells drift out of synchrony with one another over the time course. Our ability to detect true circadian cycling in our in vivo model and in vitro for these genes is supported by the fact that microarray analysis of the mouse aorta (42).

Radar diagrams of the microarray data and real-time PCR data from mouse aortic tissue are presented in Fig. 6. The microarray data are graphed according to mean fluorescence level (Fig. 6A) and the aortic tissue samples by relative mRNA expression (Fig. 6B). The timing of the first and second peaks in mRNA expression levels from the aorta was virtually identical when compared with the microarray for each of the genes investigated. Interestingly, both *timp1* and *timp3* peak together in both the Movas-1 and aortic tissue. Previous data gathered from the same animals show that *bmal1* mRNA peaks at ZT23 and *per2* mRNA peaks at ZT11 (29). The peaks in *bmal1* and *per2* occur 12 h apart, as was also observed in the Movas-1 cells. Taken together, these findings suggest that there is a specific coordination in the timing of peak gene expression in both the Movas-1 cells as well as in aortic tissue with respect...
to the circadian clock genes. Unlike the Movas-1, however, the peak expression in vivo is clearly divided largely into two groups, such that most genes peak at ZT7 and ZT19; once during the day and once during the night. The occurrence of two peaks in gene expression over the course of 1 day or every 12 h is considered to be an ultradian rhythm. These ultradian rhythms may serve important physiological functions and further analysis of these rhythms is necessary (48).

Regardless of the manner in which the clock regulates gene expression over time, its coordination appears to serve a physiologic purpose. For example, microarray and real-time PCR data from the mouse aorta indicate that the major peak of sm22a occurs during sleep, when we postulate that the ECM is being repaired, which is fitting, considering its role in mediating actin stability (52). Microarray data also placed the peak of cnn1 mRNA expression during the active time of day for the mouse, which is appropriate given the proposed function of cnn1 in mediating smooth muscle contractility and consistent with the notion of when these are best required for various physiologic processes (11). This has implications for the maintenance of health of the vasculature given the specific expression of these genes over the day-night cycle and the consequences of their disruption given their roles in the development in maintaining tissue health.

Perspectives and Significance

The cardiovascular gene expression profiles from Movas-1 cells exhibit remarkable similarity to that from the aortic tissue and microarray data. This finding is notable for several reasons: the harvested tissue contains a mixture of the various cell types found in the vasculature, whereas the Movas-1 are a pure smooth muscle cell line and the Movas-1 were synthesized using serum, whereas the animals are controlled by the LD schedule. Data from the microarray and mouse aorta thus generally support the idea that these genes are under circadian, or possibly ultradian, transcriptional regulation within vascular smooth muscle. This work, therefore, creates a new avenue of investigation into the control of cardiovascular genes via the circadian clock system, which may have longer-term implications for the understanding and treatment of cardiovascular disease. Therefore, this study highlights the value of using an immortalized cell line to address mechanistic questions regarding the circadian clock in peripheral tissues, which is more difficult to accomplish in vivo, and lays the foundation for future investigations using this model.

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