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Time- and exercise-dependent gene regulation in human skeletal muscle

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Abstract

Background: Skeletal muscle remodeling is a critical component of an organism's response to environmental changes. Exercise causes structural changes in muscle and can induce phase shifts in circadian rhythms, fluctuations in physiology and behavior with a period of around 24 hours that are maintained by a core clock mechanism. Both exercise-induced remodeling and circadian rhythms rely on the transcriptional regulation of key genes.

Results: We used DNA microarrays to determine the effects of resistance exercise (RE) on gene regulation in biopsy samples of human quadriceps muscle obtained 6 and 18 hours after an acute bout of isotonic exercise with one leg. We also profiled diurnal gene regulation at the same time points (2000 and 0800 hours) in the non-exercised leg. Comparison of our results with published circadian gene profiles in mice identified 44 putative genes that were regulated in a circadian fashion. We then used quantitative PCR to validate the circadian expression of selected gene orthologs in mouse skeletal muscle.

Conclusions: The coordinated regulation of the circadian clock genes *Cry1*, *Per2*, and *Bmal1* 6 hours after RE and diurnal genes 18 hours after RE in the exercised leg suggest that RE may directly modulate circadian rhythms in human skeletal muscle.

Background

Resistance exercise (RE) can improve the overall quality of life and reduce the symptoms of many clinical disorders, including obesity, type II diabetes mellitus [1], coronary heart disease, and stroke [2]. The effects of RE on skeletal muscle are mediated by activation of muscle-specific signaling cascades that increase muscle mass [3], cytoskeletal protein levels, and the force of contraction without increasing the number of myofibers [4]. Exercise-induced transcription of genes involved in growth, vascularization, and metabolism [5-8] indicates that large-scale changes in transcriptional regulation play a key role in muscle remodeling, inducing growth responses and metabolic shifts.

Exercise also appears to help reset circadian rhythms in shift-workers, travelers who have changed time zones, and people

with sleep disorders [9-13]. Circadian rhythms are approximately 24-hour fluctuations in gene regulation, physiology, and behavior that have evolved to optimize daily cycles of sleep, activity, feeding, and metabolism [14]. Exercise influences both the phase [15] and the amplitude [16] of circadian rhythms in mice, but this phenomenon is largely unexplored in human tissues.

Circadian rhythms are controlled by a clock mechanism located in central and peripheral tissues. The mechanism comprises an autoregulatory transcriptional feedback loop that includes the circadian-clock genes *Clock*, *Bmal1*, *Period* (*Per*) and *Cryptochrome* (*Cry*). Clock and Bmal1 constitute the positive arm of the feedback loop. These proteins form heterodimers, bind to specific DNA regulatory elements (E-boxes), and initiate transcription of *Per 1*, *2*, and *3* and *Cry 1* and *2*. In mammals, the *Per* and *Cry* gene products, which constitute the negative arm of the transcriptional feedback loop, form homo- and/or heteromeric complexes, translocate to the nucleus, and repress Clock/Bmal1-mediated transcription [17,18] in a temporal manner. Although most studies have focused on the rhythmic expression of these few core clock genes, recent evidence suggests that genes regulated in a circadian fashion (or circadian output genes) constitute 8-10% of all genes expressed in mouse tissues [19,20].

Classically, peripheral clocks are thought to be controlled by a central clock located in the suprachiasmatic nucleus (SCN), which is believed to also synchronize clocks in other brain regions [21]. However, under certain conditions (such as restricted feeding), peripheral tissue clocks can be regulated independently of the SCN [22,23]. The phase-shifting effects of exercise on mammalian circadian rhythms are thought to be mediated by inputs to SCN neurons (these include serotonin [24], neuropeptide Y [25], and melatonin [13,26]) and ultimately lead to acute changes in SCN *Per1* and *Per2* expression [27]. The extent to which RE may be able to directly affect circadian-regulated genes in peripheral tissues such as human muscle is not known, however.

To understand the global transcriptional effects of RE and time of day on gene expression in human skeletal muscle (hSkM), we used DNA microarrays to analyze biopsies of exercised and non-exercised hSkM collected at different times of day. This study was designed to answer three specific questions. What genes and biological processes are regulated in hSkM by RE and time of day? Which orthologs of genes that undergo temporal regulation in hSkM also undergo circadian regulation in mouse skeletal muscle (mSkM), liver (mLvr), heart (mHrt) or SCN (mSCN)? Are diurnally regulated genes expressed in skeletal muscle directly affected by exercise? To answer these questions, we compared gene expression in the exercised and non-exercised legs of four human subjects after an acute bout of RE. We then filtered and annotated (by biological process) the significantly changed genes and compared these genes to orthologs regulated in microarray studies of rodent models of exercise and circadian gene regulation. Finally, we used quantitative reverse-transcription polymerase chain reaction (RT-PCR) to validate the circadian regulation of selected diurnally regulated hSkM gene orthologs in mSkM.

Results and discussion

Genes regulated in the context of biological processes

Comparison of gene expression profiles of the exercised and non-exercised legs showed that 704 genes were differentially regulated at 6 hours and 1,479 genes at 18 hours after RE ($p < 0.05$). In the non-exercised leg, comparison of gene expression at 0800 and 2000 hours with the same statistical criteria showed that 608 genes were differentially regulated.

We used MAPPFinder [28] to link gene expression data to the Gene Ontology (GO) hierarchy (Table 1). The program computes a significance score (Z score) that is useful for ranking GO terms by their relative amounts of gene expression changes (see Materials and methods). With 40% of the circadian-rhythm genes classified by the GO hierarchy significantly changed at 6 hours after RE, circadian rhythm displayed the highest Z score (Z = 7.17) in this comparison, indicating that RE may regulate circadian genes directly in the exercised leg (see below). As expected, RE also upregulated a variety of genes involved in intracellular responses (nucleic acid metabolism, G2/M transition of mitotic cell cycle, anti-apoptosis, and transcription) and downregulated genes involved in oxygen and calcium transport, DNA repair, regulation of translational initiation, and glycogen metabolism (Table 1). Many of these processes were similarly regulated in a rat model of RE [8].

MAPPFinder analysis identified several biological processes influenced by diurnal gene regulation in hSkM, including transcription, cell differentiation, response to stress, hemopoiesis, oncogenesis, protein biosynthesis, and metabolism (carbohydrate metabolism and tricarboxylic acid cycle) (Table 1). These findings provide human gene targets that correlate with the circadian regulation of metabolism and cancer recently reported in mouse models [19,20,29-31].

Diurnal comparison

Diurnal gene regulation in hSkM: evidence of circadian genes

The significant upregulation of *Per1* and *Per2* in our diurnal comparison provided the first indication that the 608 genes that changed significantly with time contained known circadian-regulated genes. To filter out potential noise in this comparison and to identify genes with the highest likelihood of being regulated in a circadian fashion, we compared our results to published data on circadian gene regulation in mouse peripheral tissues [19,20]. These comparisons resulted in a list of 44 'putative circadian genes' that were significantly regulated in both our human diurnal dataset and in the mouse circadian studies (Figures 1, 2). We then used

Table 1

MAPPFinder analysis

Upregulated process		C	M	T	Z	Downregulated process	C	M	T	Z
6 hours after RE	Circadian rhythm	4	10	13	7.2	Oxygen transport	3	8	17	4.5
	Nucleic acid metabolism	59	1570	2905	2.7	Calcium ion transport	8	44	67	4.4
	Hearing	3	32	54	2.3	DNA repair	16	134	195	4.2
	G2/M transition of mitotic cell cycle	3	34	40	2.2	Regulation of translational initiation	5	26	30	3.6
	Anti-apoptosis	4	53	71	2.1	Di-, trivalent inorganic cation transport	8	66	102	3.0
	Transcription	38	1025	1992	2.0	Glycogen metabolism	4	24	25	2.9
18 hours after RE	Protein amino acid phosphorylation	60	383	735	4.7	Peripheral nervous system development	5	11	12	5.5
	Regulation of cell cycle	42	248	294	4.5	Glycosphingolipid biosynthesis	3	5	5	5.0
	Anti-apoptosis	12	53	71	3.5	Antigen processing, endogenous antigen via MHC class I	3	6	11	4.5
	Mitochondrion organization and biogenesis	5	15	23	3.3	Iron transport	4	10	21	4.5
	Protein-mitochondrial targeting	4	11	19	3.2	Glutamine family amino acid catabolism	4	13	19	3.7
	L-Amino-acid transport	3	7	9	3.1	Male gonad development	3	9	12	3.4
Diurnal	Regulation of transcription, DNA-dependent	46	938	1852	3.7	Regulation of protein biosynthesis	3	7	12	5.7
	Cell differentiation	7	68	109	3.6	Non-selective vesicle transport	7	57	63	3.7
	Response to stress	24	452	566	3.0	Actin cytoskeleton organization and biogenesis	3	15	23	3.5
	Hemopoiesis	3	22	28	2.9	Cell-cycle arrest	5	37	53	3.4
	Oncogenesis	14	253	282	2.4	Main pathways of carbohydrate metabolism	7	69	100	3.1
	Nucleic acid metabolism	61	1570	2905	2.3	Tricarboxylic acid cycle	3	20	23	2.8

Filtered genes ($p < 0.05$) were analyzed with MAPPFinder to determine the biological processes that were regulated in each comparison. C, number of genes changed; M, number of genes represented on chip in a process; T, number of genes in the Gene Ontology (GO) process: Z, significance Z-score value. The top six, non-redundant GO terms are shown for each comparison

quantitative RT-PCR to analyze mRNA expression levels of 12 mouse orthologs in mSkM isolated every 4 hours for 24 hours (Figure 3).

Circadian regulation of selected mouse orthologs of human diurnal genes and core circadian-clock genes

Quantitative RT-PCR indicated that the core circadian-clock genes *Bmal1*, *Per1*, and *Per2* and selected mouse orthologs of the 44 putative circadian genes exhibited circadian regulation in mSkM (Figure 3). Quantitative RT-PCR also provided a potential explanation for our inability to detect a significant change in the expression of the circadian-regulated core clock genes *Cry1* and *Bmal1* in hSkM [19,20], as these genes cycle out of phase with *Per1* and *Per2* in mSkM (Figure 3) and in many other tissues, including mLvr [19] and mHrt [19]. As only two time points were measured in the human samples, genes whose peak and trough times are out of phase with our sampling times (for example, *Cry1* and *Bmal1*) would not be detected.

An interesting finding is that *Per1* and *Per2* are upregulated in the morning in hSkM (Figure 2), and downregulated in the morning in mSkM (Figure 3b). We cannot determine the exact phase of the potentially circadian-regulated genes in hSkM with only two time points of sampling. However, the observed opposite regulation may reflect opposing phases of core clock gene expression between human and mouse peripheral tissues. Further examination of this phenomenon may provide a molecular explanation for the opposite activity/rest cycles in diurnal versus nocturnal mammals.

Discovery of three conserved putative circadian-regulated genes

To identify conserved circadian-regulated genes, three peripheral tissues were selected for comparison: mHrt [19],

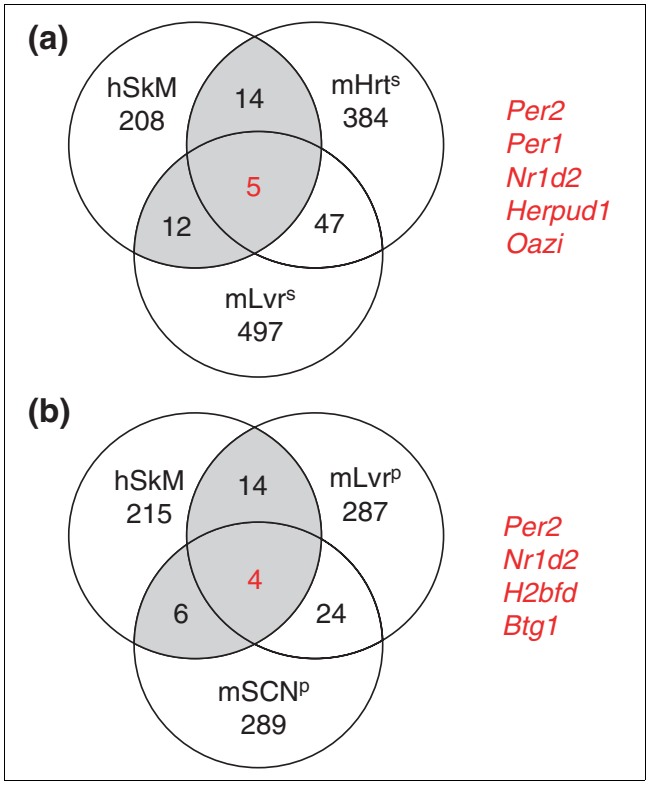

Figure 1
Venn diagrams of human genes undergoing diurnal regulation compared to their mouse circadian orthologs. **(a)** Comparison with mouse circadian gene orthologs reported in [19]; **(b)** comparison with mouse circadian gene orthologs reported in [20]. In the diurnal comparison (0800 vs 2000 h), 608 human genes were changed significantly ($p < 0.05$) in the non-exercised leg. An additional statistical filter was applied ($p < 0.05$ and absolute fold change > 20%) that resulted in a list of 239 diurnally regulated (0800 vs 2000 h) genes, that were compared with the mouse circadian orthologs. Gray shading indicates genes represented in Figure 2. Genes listed to the right of each Venn diagram are the intersection of all three tissues (red numbers). mHrt^{s}, mouse ortholog is circadian-regulated in heart [19] ($n = 462$); mLvr^{s}, mouse ortholog is circadian-regulated in liver [19] ($n = 575$); mLvr^{p}, mouse ortholog is circadian-regulated in liver [20] ($n = 335$); mSCN^{p}, mouse ortholog is circadian-regulated in the SCN [20] ($n = 337$).

mLvr [19,20], and hSkM. Four genes were significantly regulated in all three tissues: *Per2*, *Nr1d2* (nuclear receptor subfamily 1, group D, member 2), *Herpud1* (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1), and *Oazi* (ornithine decarboxylase antizyme inhibitor) (Figures 1, 2). *Per2* is a well-characterized conserved core clock element [18]]. Human *Nr1d2* (*Rev-Erbβ*) is 90% identical to mouse *Rev-Erbα*, a newly identified component of the circadian clock that represses the transcription of *Bmal* [32]. Herpud1 appears to be a membrane-bound endoplasmic reticulum protein induced by stress [33]. It contains a ubiquitin-like domain at the amino terminus, indicating its involvement in a protein degradation pathway, a biological process important for maintaining circadian rhythms. The *Oazi* gene product is an inhibitor of antizyme, which inhibits ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis that is essential for normal cell growth [34]. The discovery of common circadian genes in multiple tissues suggests that these genes and their patterns of expression are important in a variety of tissue pathways.

Exercise comparisons

RE regulates circadian genes: evidence for RE-induced phase shifting

We hypothesized that RE affects circadian-regulated gene expression directly in skeletal muscle. In support of this, we found that three core circadian clock genes, *Cry1*, *Per2*, and *Bmal1*, were upregulated 6 hours after RE in the exercised leg (1.5-fold, 1.2-fold, and 1.2-fold, respectively; Figure 2b). Although the RE-induced changes of these circadian clock genes were modest in magnitude, they are statistically significant, coordinated, and precede RE-mediated changes in diurnal genes 18 hours after RE in the exercised leg.

RE appeared to shift the expression patterns of diurnal-regulated genes by upregulating genes ($n = 12$, $p < 0.1$) that normally were repressed in the morning ($n = 29$, $p < 0.05$) or by downregulating genes ($n = 5$, $p < 0.1$) normally induced in the morning ($n = 15$, $p < 0.05$) (Figure 2c). If we extend this analysis to all the diurnal genes that are significantly changed ($p < 0.05$) 18 hours after RE, we find all (64 genes) but one reflect this potential phase-shifting effect.

Figure 2
Human genes regulated in the diurnal comparison with orthologs that display circadian regulation in mouse heart and liver [19,20], and SCN [20]. The 608 significantly regulated ($p < 0.05$) hSkM genes identified in the diurnal comparison (0800 and 2000 hours) were subjected to an additional statistical filter of absolute fold change > 20% ($n = 239$) and linked to mouse circadian-regulated orthologs. The resultant 44 putative hSkM circadian-regulated genes are represented as boxes and colored in GenMAPP [57] using different filtering criteria. **(a)** The 44 putative hSkM circadian-regulated genes colored by *p* values and displaying fold changes from the diurnal comparison (0800 vs 2000 hours non-exercised leg). **(b)** The 44 putative hSkM circadian-regulated genes colored by *p* values and displaying fold changes from the comparison 6 hours after RE. **(c)** The 44 putative hSkM circadian-regulated genes colored by *p* values and displaying fold changes from the 18 hours after RE comparison. Red, blue, and gray boxes indicate significant upregulation, downregulation, and no significant regulation, respectively, using *p*-value stringencies defined in the key for each comparison. Numbers to the right of the gene boxes are the fold changes in the diurnal comparison. L, promoter for the light-responsive element; E, E-box (*Clock*/*Bmal1* promoter). Ortholog information is denoted to the left of the gene boxes: mHrt^{s} and mLvr^{s}, mouse ortholog was circadian-regulated as described [19] in mouse heart or liver, respectively; mLvr^{p} and mSCN^{p}, mouse ortholog was diurnally regulated as described [20] in mouse liver or SCN, respectively.

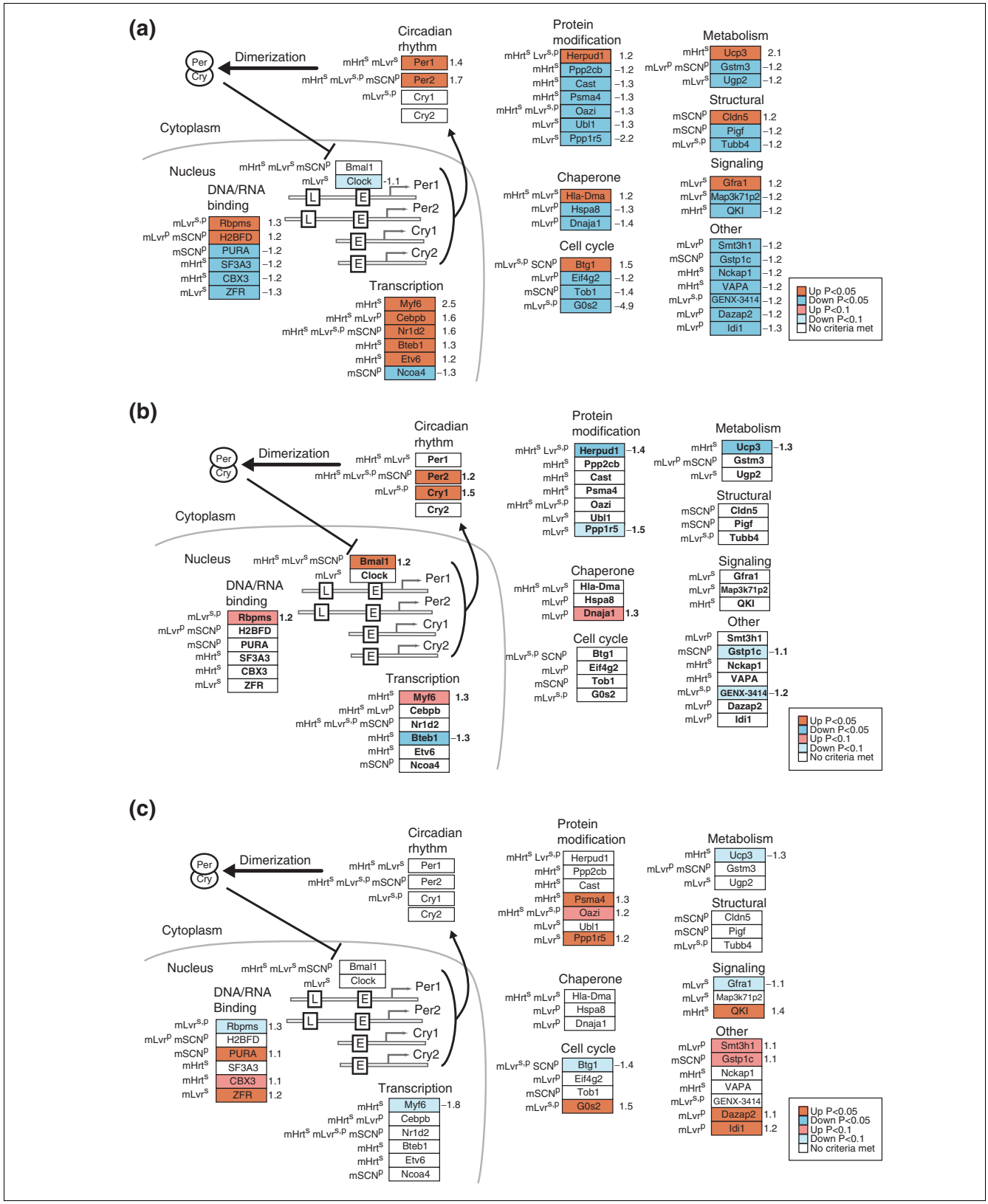

Figure 2 *(see legend on previous page)*

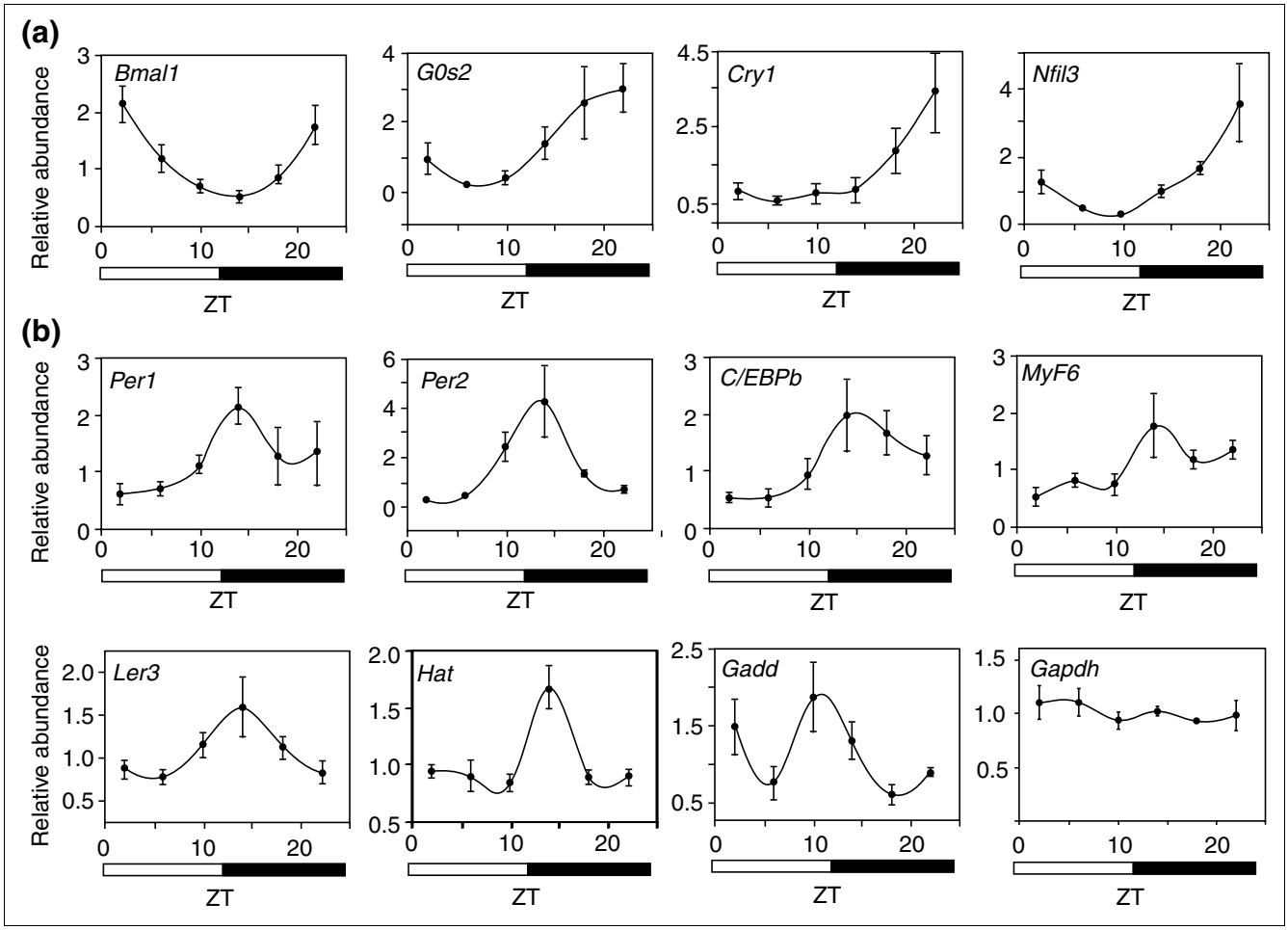

Figure 3
Confirmation of hSkM diurnal gene regulation by analysis of mSkM. Real-time RT-PCR (comparative C_T method) was performed on total RNA isolated from wild-type C57BL/6J mouse quadriceps muscle, collected at the indicated zeitgeber times (ZT). **(a)** Genes with a cycling phase similar to that of *Bmal1*, a key diurnal clock gene, are shown. **(b)** Genes with a cycling phase similar to that of *Per1* and *Per2*. One-way ANOVA was applied to all time points and confirmed a statistically significant effect of time on gene expression levels ($p < 0.001$ for *Bmal1* and *Hat*; $p < 0.05$ for all others). By normalizing average peak value to the average trough value (12-hour opposite peak), the following fold increases in gene expression were calculated: *Bmal1* = 4.1, *G0s2* = 7.0, *Cry1* = 4.5, *Nfil3* = 11.1, *Per1* = 3.5, *Per2* = 13.0, *C/EBPb* = 3.5, *MyF6* = 3.3, *Ier3* = 1.8, *Hat* = 1.8, and *Gadd* = 2.1. Values are mean 6 SEM. *Gapdh* was used as the reference gene and is included as the negative control.

Although we cannot determine whether RE induces a phase advance or phase delay in these genes, our data are consistent with previous studies that show that physical exercise during the day (similar to our study) can induce a circadian phase advance in humans [35] as measured by circulating hormone levels. Our studies now indicate that this phase advance may occur at the level of gene expression in muscle.

Comparison of human and rat genes regulated by RE

To validate our experimental protocol and to identify key genes that may regulate the effects of RE in both humans and rodents, we compared human genes that were regulated by our isotonic RE protocol at 6 hours with rat orthologs that were regulated either transcriptionally or translationally in a published rat eccentric RE study [8] (Figure 4). The rat eccentric exercise protocol induced skeletal muscle hypertrophy [36] and consisted of titanic contractions that were electrically evoked with multistrand electrodes implanted on both sides of the sciatic nerve. Contractions consisted of 10 sets of six repetitions with each repetition lasting 3 seconds. Contractions were stimulated at a frequency of 100 Hz, 6-12 V, 1 msec duration, 9 msec delay. Rat muscles were then harvested 6 hours after the acute bout of exercise [8]. Lists of rat 6-hour RE genes were downloaded from the supplemental data at [37]. Human and rat gene orthologs that were regulated in the same directions are shown in Figure 4.

Grouping the orthologs by biological function revealed that two genes, *Rrad* (Ras associated with diabetes) and *G6pt1* (glucose-6-phosphatase, transport protein 1), were regulated

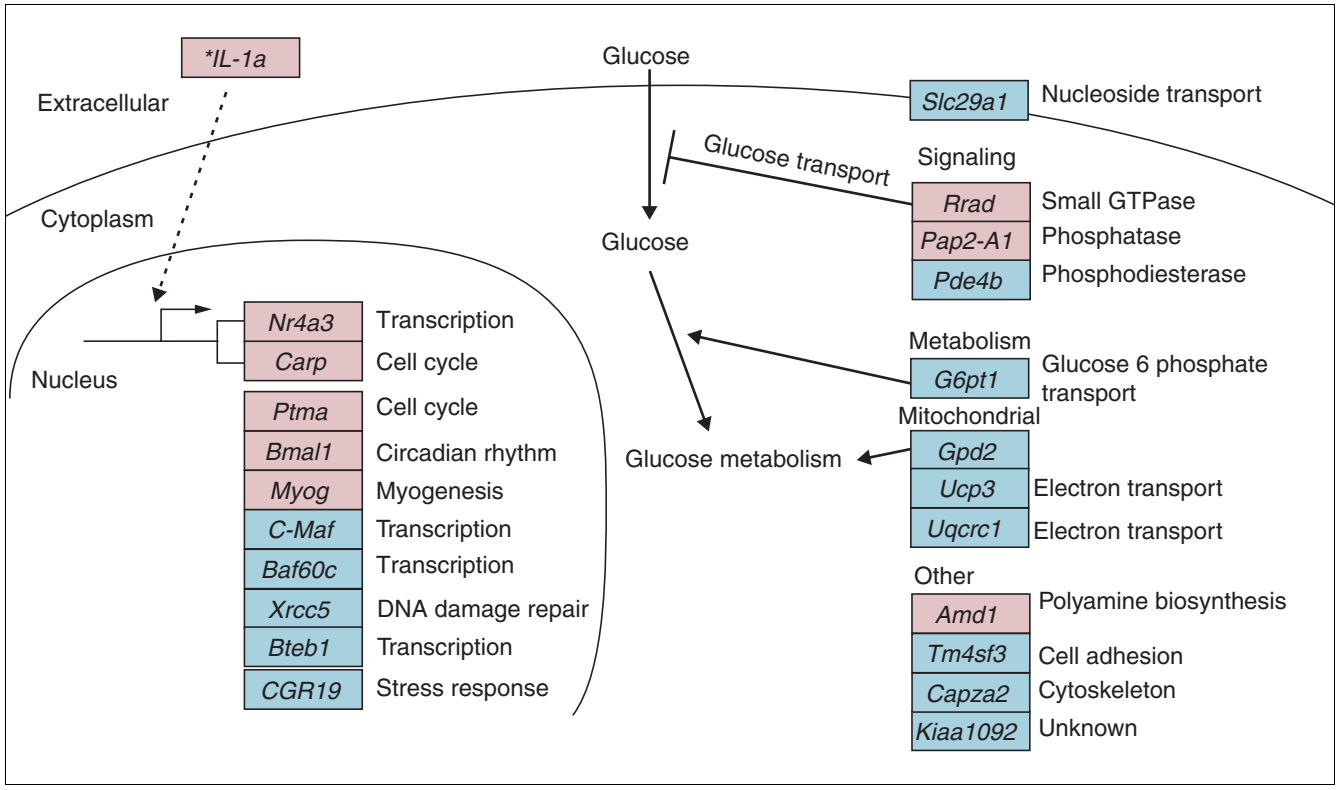

Figure 4
Gene-regulation model 6 hours after RE. The 144 human genes significantly changed ($p < 0.05$ and fold change > 20%) 6 hours after RE were compared to transcriptionally or translationally regulated rat orthologs ($p < 0.05$) 6 hours after RE in a rat model of RE [8]. Boxes represent individual human genes; red indicates upregulation and blue downregulation. **IL-1a* was not found in the rat exercise data but is included for discussion purposes.

in directions suggesting decreased glucose transport, and one gene, *Gpd2* (glycerol-3-phosphate dehydrogenase 2 (mitochondrial)) was regulated in a direction that suggested a decrease in glucose metabolism. Other genes involved in the metabolism of glycogen (the cellular storage form of glucose) were also downregulated (Table 1).

Interestingly, all three of these genes have been implicated in human diseases. *Rrad* was cloned because it was upregulated in patients with type II diabetes [38]. In cultured muscle and fat cells, overexpression of *Rrad* decreases insulin-stimulated glucose uptake [39]. The upregulation of *Rrad* provides a potential mechanism for previous reports that acute RE reduces insulin-stimulated glucose uptake [40-42]. Mutations in *Gpd2* were found in a family of type II diabetics [43], and mutations in *G6pt1* were found in patients with glycogen storage disease [44].

RE regulates potent myogenic genes

Two potent muscle-remodeling genes, myogenin and myostatin, were regulated in opposite directions in response to RE in humans, providing a potential mechanism for exercise-induced muscle hypertrophy. Myogenin was upregulated in the 6-hour comparison, and this observation was validated in the published [8] rat RE study (Figure 4). Myogenin, a muscle-specific transcription factor containing a basic helix-loop-helix domain, is important for muscle development and differentiation [45]. Myostatin, a member of the TGF-β family, is a negative regulator of skeletal muscle size and was downregulated in our study. It is a potent inhibitor of muscle development and proliferation [46]. Downregulation of myostatin may be associated with skeletal muscle growth [47] and is likely to have a major role in muscle remodeling in response to exercise.

Interleukin-1 signaling and exercise

The interleukin-1 gene (*IL-1*) was upregulated 6 hours after RE (Figure 4). The potential importance of *IL-1* regulation in response to RE is indicated by the fact that two genes regulated by *IL-1*, *Nr4a3* [48] and *Carp* [49], had the highest fold changes (3.5-fold and 2.3-fold, respectively, $p < 0.05$) 6 hours after RE. These two genes were also regulated in the rat exercise study (Figure 4). Furthermore, the vascular endothelial growth factor gene (*Vegf*) [50], which is also regulated by *IL-1*, had the highest fold change at 18 hours after RE (1.6-fold). *Vegf* is believed to be an important mediator of endurance exercise-induced angiogenesis in skeletal muscle [51]. However, resistance exercise protocols do not result in increases in capillaries per muscle area but do result in a redistribution of blood flow to hypertrophied muscle fiber types [4].

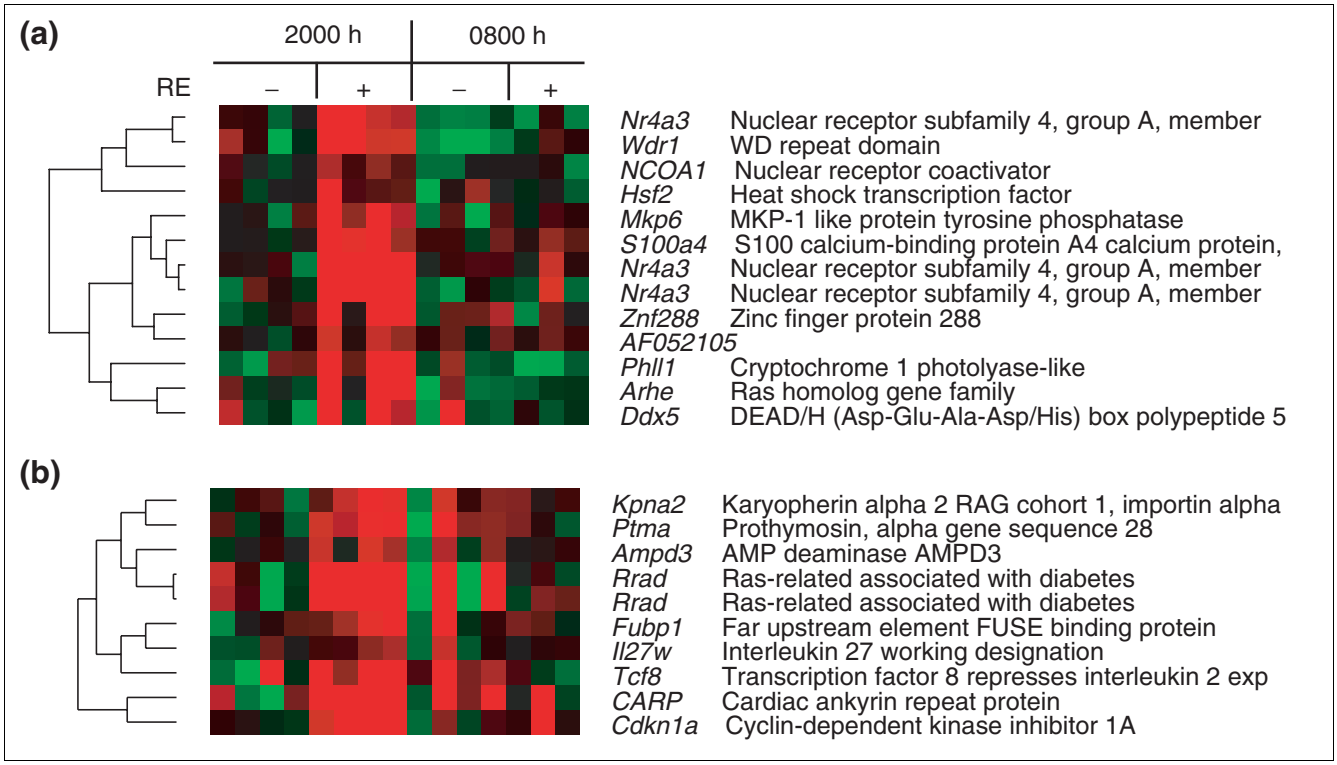

Figure 5
Cluster of genes upregulated 6 hours after RE. Columns indicate each subject and rows indicate individual genes. Each gene is represented by the difference of the genes expression value and the average expression value of the four non-exercised control legs 6 hours after RE. Red indicates upregulated genes, and green indicates downregulated genes. Gene names and descriptions (or GenBank IDs) appear to the right.

Hierarchical cluster analysis indicates potential co-regulated gene clusters

To identify potential co-regulated genes, we performed hierarchical clustering analysis [52] with expression values in the non-exercised leg at 2000 hours as the baseline. Two clusters included genes upregulated at 6 hours after RE (Figure 5), one centered on *Nr4a3* (Figure 5a) and the other on *Rrad* (Figure 5b). These two genes displayed the highest fold changes 6 hours after RE. *Cry1*, a member of the core clock mechanism (Figure 2), was found in the *Nr4a3* cluster.

Two clusters contained core circadian genes *Per1* (Figure 6a) and *Per2* (Figure 6b) which were upregulated at 0800 hours. A third cluster (Figure 6c) contained genes downregulated at 0800 but not at 2000 hours. The *Per2* cluster contained genes that were upregulated both at 0800 hours and in response to exercise (2000 hours + RE).

Conclusions

Analysis of our data in the context of biological processes and pathways allowed us to define physiologically the transcriptional basis of muscle remodeling induced by RE and its potential circadian gene regulation. This large-scale expression analysis of acute RE and circadian gene regulation in hSkM suggests that RE can directly regulate circadian clock genes (*Per2*, *Cry1*, and *Bmal1*) and circadian output genes. Our findings support the emerging idea that peripheral clocks can regulate themselves independently of the SCN [22,23]. If the SCN were responsible for the phase shifting, the same changes in gene expression would have occurred in both the control and exercised legs, which was not observed. However, acute phase advances in SCN *Per1* and *Per2* expression in response to exercise have been observed in hamsters [27]. Whereas the SCN is still probably involved in the long-term effects of clock phase shifting in peripheral tissue, our evidence suggests that the skeletal muscle clock responds quickly to RE by transcriptional regulation of specific clock genes.

Although circadian studies in human tissue are critical for understanding the effects of circadian gene regulation on human physiology, access to ample tissue samples is difficult, costly, and painful. We therefore found it valuable to validate and compare our results with those of similar experiments in rodents, in which tissue can be harvested at multiple time points to establish the circadian cycle. Such comparisons were also valuable in identifying gene orthologs regulated 6 hours after RE in both humans and rats. Cross-species, cross-tissue comparisons are essential for defining transcriptional

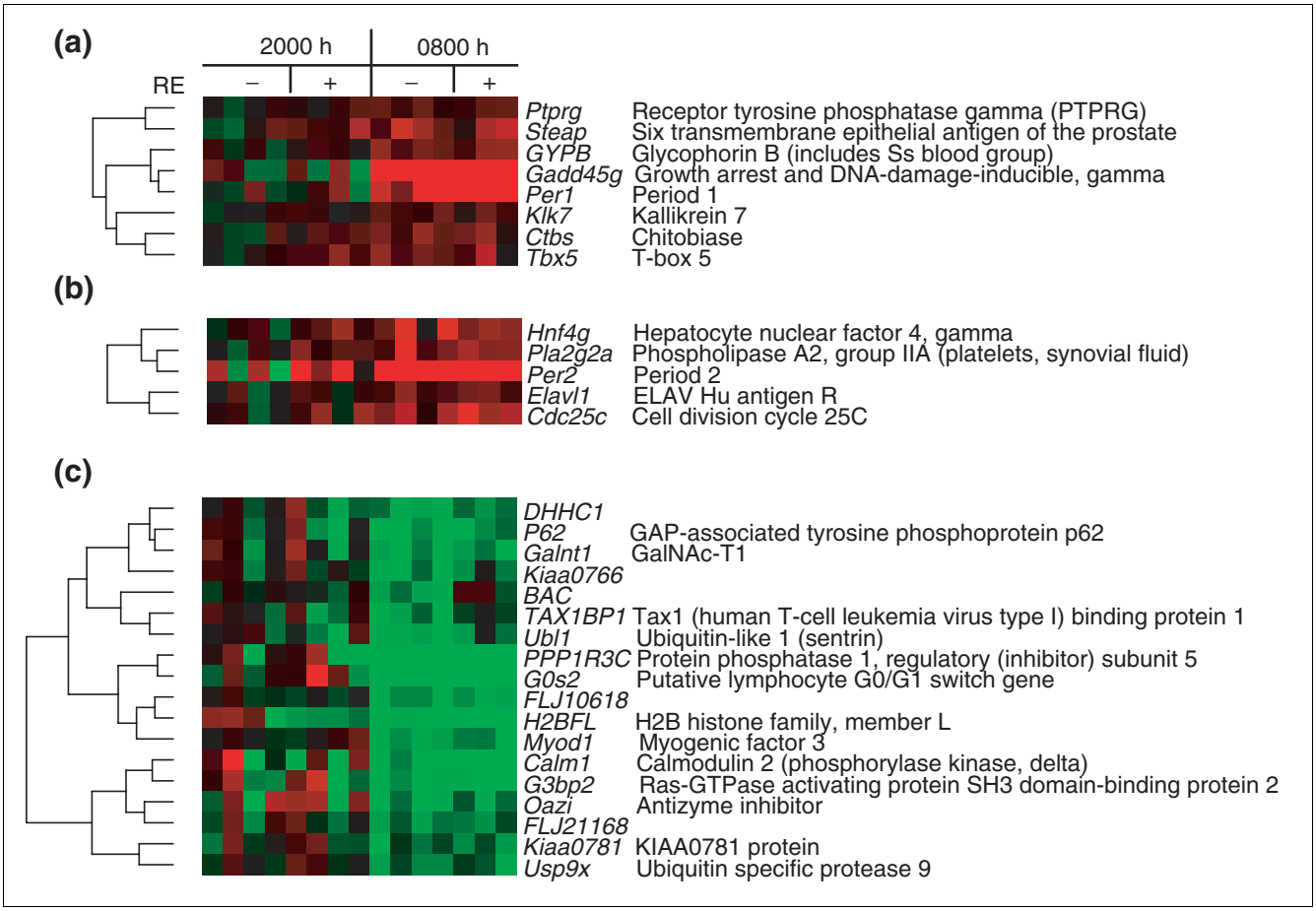

Figure 6
Cluster of genes regulated in the 0800 hours biopsies. Columns indicate each subject and rows indicate individual genes. Each gene is represented by the difference of the gene-expression value and the average expression value of the four non-exercised control legs 6 hours after RE. Red indicates upregulated genes; green indicates downregulated genes. Gene names and descriptions (or GenBank IDs) appear to the right. **(a)** *Per1* 0800 hours upregulated cluster. **(b)** *Per2* 0800 hours upregulated cluster. **(c)** 0800 hours downregulated cluster.

regulatory pathways of key genes and will allow us to define new genes and pathways responsible for tissue regulation and remodeling.

Materials and methods

Subjects

Four healthy men, 31-51 years old, were recruited for study. The subjects had not carried out RE training for at least 3 months before enrolment, had no history of chronic illnesses, and showed no abnormalities on the screening physical examination or routine hematology and chemistry tests. Subjects were admitted to the General Clinical Research Center at San Francisco General Hospital, where they were fed a constant metabolic diet that provided 1.2 g of protein/kg body weight and 35 kcal/kg body weight per day for nine days before the bout of exercise. Equilibration on the diet was evidenced by constancy of urine urea nitrogen excretion. The study protocol was approved by the Committee on Human Research of the University of California, San Francisco, and informed consent was obtained from each subject.

Exercise protocol

Maximum strength (one-repetition maximum) during isotonic knee extension from 90° to 170° was tested in the right leg 8 days before the study exercise session. Subjects did not perform any RE between the testing and the study session. On the ninth hospital day, beginning at 1330 h, subjects performed a vigorous bout of RE with the right leg. Exercise consisted of 10 sets of eight repetitions of isotonic knee extension (Cybex 4850 leg extension, Ronkonkoma, NY) at 80% of the predetermined one-repetition maximum, with 3-min rest periods between sets, over 30-45 min. The isotonic knee extensions include both concentric and eccentric phases.

Muscle biopsies

Muscle biopsies were performed 6 hours (between 1930 and 2000 hours) and 18 hours (between 0730 and 0800 hours the

next day) after RE in both the exercised and non-exercised leg. Tissue (200-300 mg) was obtained from the lateral portion of each vastus lateralis muscle approximately 20 cm above the knee with a 4-mm Bergstrom needle (Stille, Stockholm, Sweden) under local anesthesia with 1% lidocaine. Blood and visible fat were removed, and the tissue was immediately frozen in liquid nitrogen and stored at -80°C for later analysis. Although relatively homogeneous when compared to other tissues, skeletal muscle is a complex tissue consisting of many cell types, and thus our results must be interpreted in that context.

Sample preparation and analysis

Total RNA was extracted from frozen tissue with a polytron homogenizer and Trizol (Invitrogen, Carlsbad, CA). Fragmented, biotin-labeled cRNA samples were generated from 5-14 μg total RNA and hybridized to Affymetrix human U95Av2 arrays. For each array, the .cel files were generated with Affymetrix Microarray Suite 5.0 and analyzed with Robust Microarray Analysis (RMA) [53].

Preparation of expression array samples

Total RNA was extracted from 5-100 mg frozen tissue with a polytron homogenizer and Trizol (Invitrogen) and purified with an RNEasy kit (Qiagen, Santa Clara, CA). Depending on the amount of starting material, 5-14 μg total RNA was reverse transcribed with an oligo-dT primer containing a T7 RNA polymerase promoter (Affymetrix) and then converted into double-stranded cDNA (ds cDNA) with a ds cDNA synthesis kit (Invitrogen). After the second-strand synthesis, ds cDNA was extracted with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation. Biotinylated cRNA was generated from ds cDNA by *in vitro* transcription (IVT) with an Enzo BioArray high-yield RNA transcript labeling kit (Affymetrix). After a further round of purification with the Qiagen RNEasy kit, IVT reactions yielded 30-70 μg biotinylated cRNA, which was fragmented into lengths of around 100 base-pairs (bp) before hybridization.

Analysis of biotinylated cRNA with HG-U95Av2 microarray

IVT reaction products (5 μg) were hybridized to Affymetrix Test2 arrays before hybridization to the HG-U95Av2 chip (Affymetrix) to ensure full-length transcript representation of *GAPDH*. Each chip was hybridized to 15 μg fragmented cRNA. Arrays were hybridized and scanned with a GeneArray Scanner (Hewlett-Packard/Affymetrix) at the Genomics Core Facility of the General Clinical Research Center at San Francisco General Hospital.

Statistical analysis and comparisons

Three comparisons were made (Figure 7). Gene expression in the exercised leg was compared with that in the non-exercised leg at 6 and 18 hours after RE. The diurnal effects on gene expression were assessed by comparing gene transcript levels in the non-exercised leg at 2000 and 0800 hours. Two-tailed paired *t* tests were used to compare each sample with its respective baseline value. These *t* tests were validated using permutation *t* tests. Human U95Av2 chip probe set to gene annotations were obtained from NetAffx [54].

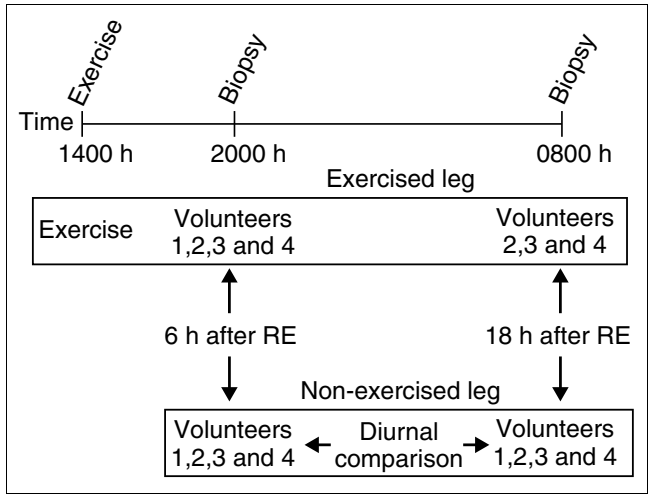

Figure 7
Diagram of the experimental protocol. Each volunteer performed a bout of resistance exercise (RE) consisting of 10 sets of eight repetitions of isometric knee extension at 80% of the predetermined one-repetition maximum with a single leg. Biopsies were obtained 6 and 18 hours after RE in both the exercised and non-exercised (contralateral) leg. Arrows denote the three comparisons of gene expression in the biopsy samples.

Gene Ontology (GO) analysis with MAPPFinder

Genes that were significantly upregulated or downregulated ($p < 0.05$) were annotated with GO [55] information with the MAPPFinder 1.0 program [28] (Table 1). MAPPFinder is an accessory program to GenMAPP [56], a freely available software tool that colors biological pathways with gene expression data [57]. MAPPFinder Z-scores, a statistical measure of significance for gene expression in a given group, were calculated by subtracting the number of genes expected to be randomly changed in a GO term from the observed number of changed genes in that GO term. This value was then divided by the standard deviation of the observed number of genes under a hypergeometric distribution. Output from the MAPPFinder analysis was manually filtered to remove processes that represented the same genes (typically parent-child processes). The top six biological processes for each comparison are listed in Table 1. For a biological process to be included in the table, it was required that at least three genes changed significantly (nested results) and the Z-score was > 2.

Gene expression analysis in mSkM

Adult male C57BL/6J mice were subjected to a 12-hour light/12-hour dark cycle for 2 weeks before tissue collection ($n = 3$ per time point). Mice were sacrificed every 4 hours for 24 hours, and quadriceps muscle was dissected and rapidly frozen on dry ice. Total RNA was extracted from frozen samples with Trizol (Sigma) and diluted to 0.1 mg/ml. TaqMan real-

time RT-PCR assays were performed using the comparative amplification detection threshold of target gene expression (C_T) method, an ABI 7700 Sequence Detector, and TaqMan EZ RT-PCR kit reagents (Applied Biosystems, Foster City, CA). Probe and primer sets were designed with Primer Express software (Applied Biosystems). mRNA levels were measured by determining the cycle number at which C_T was reached. In each sample, C_T was normalized to *GAPDH* expression, performed in parallel (ΔC_T). Normalized ΔC_T values from each time point were then subtracted from the average ΔC_T value at all time points ($\Delta\Delta C_T$) to determine the relative abundance values ($2^{-\Delta\Delta CT}$).

Linking databases for determining rat and mouse orthologs

To relate diurnally regulated hSkM genes to known circadian-regulated genes (potential hSkM circadian genes), we linked human U95Av2 probe sets to mouse U74Av2 ortholog probe sets using three public databases: NCBI Homologene [58], TIGR Resourcerer [59], and NetAffx. A similar approach was used to link the human and rat orthologs regulated 6 hours after RE (U95Av2 to U34A probe sets). 'Overlap genes' were defined as those exhibiting a fold change greater than 20% at a significance level of $p < 0.05$ that corresponded to significantly changed rodent orthologs based on the statistical filters used in the published studies [8,19,20].

Hierarchical clustering analysis

For hierarchical clustering, we used data from the 3,260 genes (of 12,626 examined) that resulted from a $p < 0.05$ in any of three comparisons. The analysis was performed with Cluster and TreeView [52]. As input for Cluster, the average (N = 4) $\log_2$ truncated value of the unexercised 2000-hour biopsies (control leg) was used as the baseline. All 3,260 genes were clustered by correlation uncentered similarity metric, using complete linking clustering. Figures were generated with TreeView.

Additional data files

The following additional data files are available with the online version of this article: two Excel sheets containing the Affymetrix data (MAS 4.0, target intensity 800) from rat 6 hours after exercise [8] total and polysomal (Additional data file 1); two Excel sheets containing $\log_2$ RMA signal values, fold changes, *P* values and probe level annotation, and descriptions of column titles, respectively (Additional data file 2); two sheets with information for linking orthologous probe sets between Affymetrix Hs. U95A, Mm. U74A and Rn. U34A arrays (Additional data file 3); two sheets (Hs RE/rat total RE and Hs RE/rat polysomal RE) of 6 h after exercise-regulated gene orthologs regulated in this study and those published by Chen *et al.* [8] (Additional data file 4); four Excel sheets containing the putative circadian overlap genes (Additional data file 5); and, finally, five sheets each with the unfiltered output results from the MAPPFinder analysis (Additional data file 6). All of the data, including RMA expression values, annotated chip information, GenMAPP expression dataset [57], MAPPFinder results [28], lists of links between the human and rodent probe sets, and full lists of ortholog matches, are also available for download from the GenMAPP site [60].

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