Human skeletal myotubes display a cell-autonomous circadian clock implicated in basal myokine secretion

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ABSTRACT

Objective: Circadian clocks are functional in all light-sensitive organisms, allowing an adaptation to the external world in anticipation of daily environmental changes. In view of the potential role of the skeletal muscle clock in the regulation of glucose metabolism, we aimed to characterize circadian rhythms in primary human skeletal myotubes and investigate their roles in myokine secretion.

Methods: We established a system for long-term bioluminescence recording in differentiated human myotubes, employing lentivector gene delivery of the Bmal1-luciferase and Per2-luciferase core clock reporters. Furthermore, we disrupted the circadian clock in skeletal muscle cells by transfecting siRNA targeting CLOCK. Next, we assessed the basal secretion of a large panel of myokines in a circadian manner in the presence or absence of a functional clock.

Results: Bioluminescence reporter assays revealed that human skeletal myotubes, synchronized in vitro, exhibit a self-sustained circadian rhythm, which was further confirmed by endogenous core clock transcript expression. Moreover, we demonstrate that the basal secretion of IL-6, IL-8 and MCP-1 by synchronized skeletal myotubes has a circadian profile. Importantly, the secretion of IL-6 and several additional myokines was strongly downregulated upon siClock-mediated clock disruption.

Conclusions: Our study provides for the first time evidence that primary human skeletal myotubes possess a high-amplitude cell-autonomous circadian clock, which could be attenuated. Furthermore, this oscillator plays an important role in the regulation of basal myokine secretion by skeletal myotubes.

Keywords Circadian clock; Human skeletal myotube; Myokine; Interleukin-6; Circadian bioluminescence recording

1. INTRODUCTION

Circadian oscillations are daily cycles in behavior and physiology that have been described from photosynthetic bacteria to vertebrates. They are reflected by the existence of underlying intrinsic biological clocks with near 24 h periods that generate self-sustained rhythms, influenced by environmental stimuli, such as light and feeding [1]. Under homeostatic conditions, the clock acts as a driver of metabolic processes with remarkable tissue specificity that reflects the unique demand of each tissue [2]. In peripheral organs, a large number of key metabolic functions are subject to daily oscillations, such as carbohydrate and lipid metabolism by the liver, and xenobiotic detoxification by the liver, kidney and small intestine [2,3]. In rodents, the presence of peripheral circadian oscillators and their impact on gene expression and organ function has been demonstrated in liver, whole pancreas, pancreatic islets, and adipose tissue (reviewed in [4]). Of note, feeding rhythms represent a potent synchronizing cue for peripheral oscillators. Elegant studies involving inverted or restricted feeding schedules convincingly demonstrate that feeding rhythms are powerful enough to uncouple liver and other organ clocks from the SCN [5]. Moreover, rhythmicity in a number of clock-deficient mouse models could be restored by feeding rhythms [6–8]. There is growing evidence for a tight reciprocal link between a number of metabolic diseases, including obesity and type 2 diabetes mellitus (T2D), and the circadian clockwork [3,4]. Mice with circadian clock ablation develop hyperphagia, obesity, hyperglycemia and hypo-insulinemia [9]. The adipocyte-specific Bmal1 knockout leads to obesity development [10], while the islet-specific Bmal1 ablation

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2. MATERIAL AND METHODS

2.1. Study participants, skeletal muscle tissue sampling and primary cell culture

Muscle biopsies were derived from non-obese or obese donors with the informed consent obtained from all participants (see Table 1 for the

| Table 1 — Characteristics of donors for skeletal muscle biopsies. |
|-------------------|-------|-------|----------------|
| Donor | Sex | Age (years) | BMI (kg/m²) | Biopsy source |
| Non-obese | M = 14, F = 5 | M 58 ± 18 | 24.88 ± 3.20 |
| 1<sub>°</sub> | M 48 | 21.7 | Rectus abdominis |
| 2<sub>°</sub> | M 45 | 21 | Rectus abdominis |
| 3<sub>°</sub> | F 58 | 20.5 | Rectus abdominis |
| 4<sub>°</sub> | F 42 | 19.5 | Rectus abdominis |
| 5<sub>°</sub> | M 23 | 29.34 | Rectus abdominis |
| 6<sub>°</sub> | M 62 | 24.3 | Rectus abdominis |
| 7<sub>°</sub> | F 77 | 25.6 | Rectus abdominis |
| 8<sub>°</sub> | M 57 | 26 | Rectus abdominis |
| 9<sub>°</sub> | M 60 | 24 | Rectus abdominis |
| 10<sub>°</sub> | F 88 | 29.64 | Rectus abdominis |
| 11<sub>°</sub> | F 65 | 25.8 | Rectus abdominis |
| 12<sub>°</sub> | M 25 | 19.27 | Rectus abdominis |
| 13<sub>°</sub> | M 64 | 28 | Rectus abdominis |
| 14<sub>°</sub> | M 87 | 25.51 | Rectus abdominis |
| 15<sub>°</sub> | M 85 | 25.54 | Rectus abdominis |
| 16<sub>°</sub> | M 72 | 26.5 | Rectus abdominis |
| 17<sub>°</sub> | M 48 | 24.3 | Rectus abdominis |
| 18<sub>°</sub> | M 45 | 29.1 | Rectus abdominis |
| 19<sub>°</sub> | M 57 | 28.1 | Rectus abdominis |
| Obese | M = 1, F = 4 | M 53 ± 15 | 39.21 ± 7.16 |
| 20<sub>°</sub> | M 70 | 30.1 | Gluteus maximus |
| 21<sub>°</sub> | F 70 | 32.9 | Gluteus maximus |
| 22<sub>°</sub> | F 43 | 43 | Gluteus maximus |
| 23<sub>°</sub> | F 39 | 45.48 | Rectus abdominis |
| 24<sub>°</sub> | F 45 | 44.58 | Rectus abdominis |
| All donors | M = 15, F = 9 | M 57 ± 17 | 27.87 ± 7.23 |

M, male; F, female.

Table 1, M = 15, F = 9.

Abbreviations

BMAL1: brain and muscle ARNT-like 1
CLOCK: circadian locomotor output cycles kaput
CRY: cryptochrome
DBP: D-albumin binding protein
GLP-1: glucagon-like peptide 1
HPRT: hypoxanthine-guanine phosphoribosyltransferase
IL-6: interleukin-6
IL-8: interleukin-8
Luc: luciferase
M-CSF: macrophage colony-stimulating factor
PER: period
ROR: retinoid-related orphan receptor
REV-ERB: reverse-erb alpha
SCN: suprachiasmatic nucleus
T2D: type 2 diabetes mellitus
VEGF: vascular endothelial growth factor
ZT: zeitgeber time

approximately 70% of insulin-stimulated glucose uptake and therefore representing the most important site of insulin resistance in T2D patients [12]. The primary and best-described function of skeletal muscles is their mechanical activity. During the last decade, however, skeletal muscle has been characterized also as a secretory organ, producing and releasing myokines that act on the muscle itself and display endocrine effects on distant organs [13,14]. Recently, it has been proposed that fully differentiated primary human skeletal muscle cells secrete over 300 potential myokines [15]. Interleukin-6 (IL-6) is one of the first identified myokines, which is produced by the contracting skeletal muscle [16]. Acute high plasma levels of IL-6 are associated with exercise, while chronically elevated IL-6 is observed upon obesity and T2D. Moreover, IL-6 promotes pancreatic alpha cell mass expansion [17] and stimulates GLP-1 production and secretion by alpha and L-cells upon metabolic syndrome, thus exerting beneficial effects in T2D mouse models [18]. Besides IL-6, skeletal muscle produces a number of additional myokines, such as MCP-1 and IL-8, which play a role in skeletal muscle homeostasis. It is responsible for 70–80% of insulin-stimulated glucose uptake and therefore representing the most important site of insulin resistance in T2D patients [12]. The primary and best-described function of skeletal muscles is their mechanical activity. During the last decade, however, skeletal muscle has been characterized also as a secretory organ, producing and releasing myokines that act on the muscle itself and display endocrine effects on distant organs [13,14]. Recently, it has been proposed that fully differentiated primary human skeletal muscle cells secrete over 300 potential myokines [15]. Interleukin-6 (IL-6) is one of the first identified myokines, which is produced by the contracting skeletal muscle [16]. Acute high plasma levels of IL-6 are associated with exercise, while chronically elevated IL-6 is observed upon obesity and T2D. Moreover, IL-6 promotes pancreatic alpha cell mass expansion [17] and stimulates GLP-1 production and secretion by alpha and L-cells upon metabolic syndrome, thus exerting beneficial effects in T2D mouse models [18]. Besides IL-6, skeletal muscle produces a number of additional myokines, such as MCP-1 and IL-8, which play a role in skeletal muscle homeostasis, recruitment of macrophages and insulin sensitivity [19,20]. In rodents, about 7% of the skeletal muscle transcriptome is expressed in a circadian manner [21]. Moreover, clock ablation (Bmal1<sup>−/−</sup>) leads to skeletal muscle pathologies [22]. MyoD, a master regulator of myogenesis, exhibits a robust circadian rhythm and was identified as a direct target of CLOCK and BMAL1. In addition, disruption of myofibril formation was detected in Bmal1<sup>−/−</sup>, Clock<sup>−/−</sup>, and in MyoD<sup>−/−</sup> mice, suggesting a direct link between the circadian clock and skeletal muscle function in rodents [22]. Furthermore, a recent study suggests that in mice muscle insulin sensitivity might be clock-dependent [23]. In view of the accumulating evidence in rodent models, it has been accepted that the skeletal muscle clock plays an essential role in maintaining proper metabolic functioning (reviewed in [24]), although the mechanism of this important regulation is not entirely clear, particularly in human subjects. Given that, the circadian clock in human skeletal muscle has remained largely unexplored, we aimed to characterize the circadian oscillator in primary human myotubes and explore its impact on the regulation of human skeletal muscle myokine secretion.
donor characteristics). The experimental protocol (‘DIOMEDE’) was approved by the Ethical Committee SUD EST IV (Agreement 12/111) and performed according to the French legislation (Huriet’s law). All donors had HbA1C levels inferior to 6.0% and fasting glycemia inferior to 7 mmol/L, were not diagnosed for T2D, neoplasia or chronic inflammatory diseases, and not doing shift work. Biopsies were taken from the Gluteus maximus (n = 18) or the Rectus abdominus (n = 6) muscles during the planned surgeries. Primary skeletal myoblasts were purified and differentiated into myotubes according to the previously described procedure [25]. Briefly, after removal of apparent connective and fat tissue contaminants, the muscle biopsy was minced with scissors and incubated successively at least 4 times for 20 min in Trypsin-EDTA (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C under agitation. Trypsin-EDTA digested extracts were pooled, and centrifuged (1500 g). The pellet was rinsed several times with PBS and cells were filtered on a 70-µm filter before being plated in a Primaria flask (Falcon; Becton Dickinson, Bedford, MA) containing growth medium composed of HAM F-10 supplemented with 2% Ultroser G (BioSepra SA, Cergy-Saint-Christophe, France), 2% fetal bovine serum (FBS, Invitrogen), and 1% antibiotics (Invitrogen). After 4 days, the myoblasts were immuno-selected using a monoclonal human CD56 antibody combined with paramagnetic beads (CD56 MicroBeads, Miltenyi Biotec, Germany), according to the manufacturer’s instructions. The selected cells were plated on Primaria plasticware at 4500 cells/cm² and cultured in growth medium at 37 °C. After reaching confluence, myoblasts were differentiated into myotubes during 7–10 days in DMEM supplemented with 2% FBS. Muscle differentiation was characterized by the fusion of myoblasts into polynucleated myotubes (Supplementary Figure 1).

2.2. siRNA transfection and lentiviral transduction

Human primary myoblasts were differentiated into myotubes as described above. Cells were transfected with 20 nM siRNA targeting CLOCK (siClock), or with non-targeting siControl (Dharmacon, GE Healthcare, Little Chalfont, UK), using HiPerFect transfection reagent (Qiagen). Cells were transfected according to the manufacturer’s protocol, 24 h prior to synchronization. To produce lentiviral particles, Bmal1-luc [26] or Per2-luc [27] lentivectors were transduced into 293T cells using the polyethylenimine method (for detailed procedure see [28]). Myoblasts were transduced with the indicated lentiviral particles with a multiplicity of infection (MOI) = 3 for each, grown to confluence, and subsequently differentiated into myotubes.

2.3. In vitro skeletal myotube synchronization and real-time bioluminescence recording

To synchronize primary myotubes, 10 µM forskolin (Sigma, Saint-Louis, MO, USA) or 100 nM dexamethasone (Alfa Aesar, Johnson Matthey, London, UK) were added to the culture medium, respectively. Following 60 min (forskolin) or 30 min (dexamethasone) incubation at 37 °C in cell culture incubator, the medium was changed to a phenol red-free recording medium containing 100 µM luciferin and cells were transferred to a 37 °C light-tight incubator (Pro-Lume LTD, Pinetop, AZ, USA), as previously described by us [28]. Bioluminescence from each dish was continuously monitored using a Hamamatsu photomultiplier tube (PMT) detector assembly. Photon counts were integrated over 1 min intervals. Bioluminescence traces are either shown as raw or detrended data. For detrended time series, bioluminescence signals were smoothed by a moving average with a window of 144 data points and detrended by an additional moving average with a window of 24 h [29]. For quantification of the circadian amplitude and period the first cycle was not taken into consideration.

2.4. mRNA extraction and quantitative PCR analysis

Differentiated myotubes were synchronized by forskolin or dexamethasone, collected every 4 h during 48 h (0 h–48 h), or during 24 h (12 h–36 h), deep-frozen in liquid nitrogen and kept at –80 °C. Total RNA was prepared using RNeasy Plus Micro Kit (Qiagen). 0.5 µg of total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) and random hexamers and PCR-amplified on a LightCycler 480 (Roche Diagnostics AG, Rotkreuz, Switzerland). Mean values for each sample were calculated from the technical duplicates of each qRT-PCR analysis, and normalized to the mean of two housekeeping genes (HPRT and 9S or GAPDH and 9S), which served as internal controls. Primers used for this study are listed in Supplementary Table 1.

2.5. Circadian analysis of basal myokine secretion by ELISA and multiplex assay

In vitro synchronized differentiated myotubes, transduced with Bmal1-luc reporter, were placed into an in-house developed two-well horizontal perifusion chamber, connected to the LumiCycle. Cells were continuously perfused for 48 h with culture medium containing 100 µM luciferin. Bioluminescence recordings were performed in parallel to the automated collection of outflow medium in 4 h intervals. Basal IL-6 levels were quantified in the outflow medium using the Human IL-6 Instant ELISA kit (eBioscience, Affymetrix, Santa Clara, CA, USA) following the manufacturer’s instructions. Data were normalized to the genomic DNA content, extracted using the QIAamp DNA Blood Mini Kit (Qiagen). Perifusion medium samples were further concentrated using Amicon Ultra 2 ml centrifugal filters (Ultracl-3K, Merck Millipore, Darmstadt, Germany). The evaluation of myokine release from human primary skeletal muscle cells was carried out using a multiplex bead assay system (R&D Systems, Minneapolis, MN, USA). Custom-made luminex screening plates (CD44, CHI3L1, FABP3, galectin-3, GRO-alpha, IGFBP-3, IL-7, IL-13, IL-17A, M-CSF, MCP-1, MMP-2, Serpin C1, Serpin E1, TIMP-1) and high sensitivity perifusion plates (IL-1 beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 p70, IFN-gamma, TNF-alpha and VEGF) were analyzed according to the manufacturer’s instructions. Plate analysis was performed on a Bio-Plex 200 array reader (Bio-Rad Laboratories, Hercules, CA, USA), with the Bio-Plex software (Bio-Rad) used for data analysis.

2.6. Data analysis

Actimetrics LumiCycle analysis software (Actimetrics LTD) and the JTK_CYCLE algorithm [30] were used for bioluminescence and myokine secretion profile data analyses, respectively. For the ELISA and multiplex data analysis, 2 technical duplicates from 3 biological samples were analyzed for each myokine. From these values the average ± the SEM was calculated for each time point. For JTK_CYCLE analysis the period width was set at 20–24 h. Statistical analyses were performed using a paired student’s t test. Differences were considered significant for p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***)

3. RESULTS

3.1. High-amplitude self-sustained clocks are functional in primary human skeletal myotubes

Circadian bioluminescence recordings in living cells allow for the study of molecular clocks in human peripheral tissues, as previously demonstrated by us [28,31] and others [32]. We applied this powerful methodology to assess clock properties in human primary skeletal myotubes established from human donor biopsies and differentiated in vitro (see Supplementary Figure 1 and Table 1 for donor

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characteristics). Multiple in vitro stimuli have previously been demonstrated to efficiently synchronize cultured cells, among them forskolin and dexamethasone [28,33]. As shown in Supplementary Figure 2, short pulses of dexamethasone or forskolin were able to strongly synchronize human myotubes bearing the Bmal1-luc lentivirus. Both forskolin and dexamethasone induced oscillations with comparable period length: 25.29 ± 0.13 h, n = 19 for forskolin (Table 2), and 24.48 ± 0.24 h, n = 8 for dexamethasone (data not shown). However, the forskolin pulse induced more sustained cycles with higher circadian amplitude compared to dexamethasone-synchronized cells (Supplementary Figure 2) and was therefore mainly used in this study as the in vitro synchronization stimulus. The here established experimental settings allowed for continuous recording of oscillation profiles in human primary myotubes for several days with high resolution (Figure 1A, B). As expected, the profiles of the Bmal1-luc and Per2-luc reporters were antiphasic (Figure 1A, B; [3]). High-amplitude self-sustained oscillations were reproducibly recorded from human primary myotubes for Bmal1-luc and Per2-luc reporters with an average period length of 25.29 ± 0.13 h and 25.20 ± 0.19 h, respectively (Figure 1C). Of note, no significant difference in period length of the Bmal1-luc or Per2-luc reporter was observed between myotubes established from non-obese and obese donors (Table 2), which might reflect the resistance of the core clock to metabolic changes.

To further validate the results obtained by circadian bioluminescence reporter studies, we examined endogenous core clock gene expression profiles in forskolin-synchronized myotubes (Figure 2C, closed circles). mRNA accumulation patterns from synchronized skeletal myotubes were monitored every 4 h during 48 h by quantitative RT-PCR, using amplicons for BMAL1, REV-ERBα, PER3 and DBP. The values were normalized to the mean of the housekeeping genes HPRT and 9S. In agreement with our Bmal1-luc reporter experiments, endogenous BMAL1 transcript levels exhibited circadian oscillations over 48 h in synchronize myotubes (compare Figure 2C BMAL1 panel to Figure 1A), clearly antiphasic to the profiles of the endogenous REV-ERBα, PER3, and DBP transcripts (Figure 2C). Similar experiments were conducted in dexamethasone-synchronized myotubes (Supplementary Figure 3). BMAL1 andCRY1 mRNAs exhibited oscillatory profiles antiphasic to those of REV-ERBα and PER2-3, consistent with the dexamethasone-induced oscillations of the Bmal1-luc reporter (Supplementary Figure 2).

### 3.2. Human myocyte clock disruption by siRNA-mediated CLOCK knockdown

In order to disrupt the circadian clock in cultured human myotubes, we set up an efficient siClock transfection protocol, resulting in more than 80% knockdown of CLOCK transcript levels (Figure 2A, Supplementary Figure 4A). Circadian expression of the Bmal1-luc reporter was blunted in siClock-transfected myotubes upon forskolin or dexamethasone synchronization, if compared to cells transfected with non-targeting sequences (siControl) or to non-transfected counterparts (Figure 2B, Supplementary Figure 4B), thus validating circadian clock disruption. Moreover, the amplitudes of endogenous REV-ERBα, PER3 and DBP transcript profiles were strongly blunted in siClock-transfected cells, in comparison to siControl cells (Figure 2C, Supplementary Table 2). By contrast, BMAL1 transcript levels were slightly upregulated (Figure 2C, Supplementary Table 2).

### 3.3. Regulation of basal IL-6 secretion by the circadian clock in human primary myotubes

Given the accumulating evidence on the essential role of IL-6 secretion by skeletal muscle under physiological conditions and upon metabolic diseases [14,17,18], we next monitored basal circadian IL-6 secretion by human primary myotubes established from non-obese and obese donors. To this end, we developed an in-house perfusion system connected to the LumiCycle chamber that allows for parallel cell perfusion and bioluminescence profile recordings. Basal IL-6 secretion from in vitro synchronized skeletal myotubes was monitored in “around-the-clock” experimental settings, with a continuous flow of culture medium (see Material and methods for details). The perfusion experiments suggested that forskolin-synchronized myotubes exhibited a circadian profile of basal IL-6 secretion over 48 h, with a Zenith around 8 h—12 h and 32 h—36 h and a Nadir of 20 h—24 h (Figure 3A). JTK_CYCLE analysis [30] revealed that average profile of basal IL-6 secretion did not reach significance to be qualified as circadian over the entire time span of 48 h. However it was significantly circadian over the first 36 h of perfusion following in vitro synchronization (**p < 0.01, n = 6). Similar basal IL-6 secretion profiles were observed from dexamethasone-synchronized myotubes (Supplementary Figure 5).

We next tested the effect of CLOCK depletion on IL-6 secretion. Similar to previous experiments (Figure 2), CLOCK transcript expression was at least 80% downregulated in siClock-transfected myotubes (***p < 0.001, paired t-test, Table 3) compared to siControl-transfected cells. The achieved clock disruption was also confirmed by parallel Bmal1-luc bioluminescence recording in perfused cells (Figure 3B). Importantly, IL-6 secretion decreased on average 64% in siClock-transfected myotubes compared to siControl-transfected counterparts (*p < 0.05, Table 3). Furthermore, the profile of basal IL-6 secretion became flat upon clock disruption if compared to the siControl-transfected profile (see red line vs. black line, Figure 3A). Of note, overall basal IL-6 secretion was on average higher in obese siControl-transfected subjects (n = 3) compared to non-obese siControl-expressing counterparts (n = 3), although values did not reach statistical significance (Table 3).

To get an insight into the regulation of basal IL-6 secretion by the circadian clock, we assessed I6L6 transcription levels following in vitro synchronization with forskolin. No clear circadian pattern was observed (Figure 3C). The strong immediate early peak of IL6 transcription induced by the forskolin pulse might be attributed to the presence of a CAMP response element previously identified in IL-6 promoter region [34]. Moreover, CLOCK depletion by siClock had no effect on basal IL6 transcription (Figure 3C).

### 3.4. Multiplex screen identifies additional clock-regulated myokines

Since we obtained convincing evidence on the requirement of a functional circadian clock for basal IL-6 secretion by skeletal myotubes (Figure 3A,B), we selected an additional panel of myokines for analysis by multiplex assay. The around-the-clock secretory profiles of IL-6 and 24 other myokines were assessed in perfusion samples obtained from
primary human myotubes as described in Figure 3, and further concentrated to allow for the detection of basal myokine secretion in siControl and siClock-transfected myotubes. Selected myokines were chosen based on their presence in the secretome of differentiated primary skeletal muscle cells [15], their function and implication in metabolic diseases, and their availability and compatibility for multiplex array analysis. Out of the selected panel of 25 myokines, 15 myokines were detected in the concentrated perifusion samples, while 10 myokines remained undetectable, due to their low levels of basal secretion (Table 4).

Importantly, the profile and concentration levels of around-the-clock IL-6 secretion measured by multiplex analysis were very similar to those obtained by ELISA (compare Figure 3D—A). JTK_CYCLE analysis [30] confirmed that the average profile of secreted IL-6 measured by multiplex analysis was significantly circadian within 48 h (Figure 3D, Table 4). In addition to IL-6, MCP-1 and IL-8 were secreted in a circadian manner according to JTK_CYCLE analysis (Figure 4A, Table 4). M-CSF and GRO-alpha exhibited a secretion profile that might be clock-controlled; however, the adjusted minimal p-value did not reach significance according to JTK_CYCLE analysis (Figure 4B, Table 4). Furthermore, although the temporal secretion patterns of VEGF, CD44, FABP3, Galectin-3 and TIMP-1 were not identified as circadian, the overall secretion levels of these myokines were significantly reduced upon CLOCK depletion (Figure 4C, Table 4).

4. DISCUSSION

4.1. Molecular makeup of the circadian oscillator operative in human skeletal muscle

Our study provides for the first time evidence for cell-autonomous self-sustained circadian oscillators, operative in human primary skeletal myotubes. Molecular characteristics of the human myotube clock were assessed by two complementary approaches. Pronounced circadian oscillations were recorded with high temporal resolution for at least 4–5 consecutive days from in vitro synchronized primary skeletal myotubes, transfused with Bmal1-luciferase or Per2-luciferase lentivectors (Figure 1A,B). The circadian characteristics of human skeletal myotubes were in accordance with those reported for human primary thyrocytes, pancreatic islets, skin fibroblasts [28,31,35], and for mouse skeletal muscle assessed in vivo [21,22]. Sustained circadian oscillations were efficiently induced in our system by both forskolin (Figure 1) and dexamethasone (Supplementary Figure 2) pulses, suggesting that these oscillators are functional irrespective of the synchronization stimulus or entrainment pathway. It might be of interest to further explore whether other physiologically relevant stimuli like glucose, insulin, or myokine-induced signaling pathways play a role in human myotube synchronization.

In line with the outcome of our reporter experiments, endogenous around-the-clock gene expression analyses suggested that the core clock genes BMAL1, REV-ERBα, and PER3, as well as the clock output gene DBP, exhibit circadian oscillatory patterns in forskolin and dexamethasone-synchronized myotubes (Figure 2C) and for endogenous BMAL1 oscillations induced in vitro is typically lower if compared to in vivo oscillations from the same tissue, as demonstrated, for instance, for mouse islets synchronized in vitro or collected in vivo [11]. Thus, identifying clock-controlled genes in vitro by RT-qPCR represents a significant challenge due to rather low amplitudes [21,28]. More accurate methods like RNA sequencing of samples collected with higher temporal resolution might represent a solution to this problem.

4.2. Experimental model for circadian clock disruption in human primary myotubes

Here, we have established experimental settings for a highly reproducible siRNA-mediated CLOCK transcript knockdown of more than 80% in human primary muscle cells (Figure 2A, Supplementary Figure 4A). Upon such CLOCK silencing, significant flattening of the circadian amplitude was observed for the Bmal1-luc reporter (Figure 2B, Figure 3B, Supplementary Figure 4B) and for endogenous REV-ERBα, PER3 and DBP transcripts (Figure 2C), confirming circadian core clock disruption. The discrepancy between Bmal1-luc reporter data and endogenous BMAL1 expression upon siClock might be related to the fact that the knockdown effect of CLOCK on the Bmal1-luc reporter is evident primarily after 48 h, and that the promoter length of the Bmal1 reporter is different from the endogenous gene. While REV-ERBα, PER3 and DBP transcript profiles only exhibited residual circadian oscillations, which might be explained by incomplete clock ablation, the amplitude of the endogenous BMAL1 oscillatory profile was not reduced and even slightly increased (Figure 2C). Similarly, siRNA-mediated depletion of CLOCK in U2OS cells was reported to increase BMAL1 expression levels [36]. Moreover, Clock-deficient mice continue to exhibit circadian patterns of behavioral and molecular

Figure 1: High-amplitude cell autonomous oscillators are functional in differentiated human primary myotubes. Human primary myoblasts were transduced with lentiviral particles expressing Bmal1-luc (black line) or Per2-luc (blue line). Cells were differentiated into myotubes, synchronized with forskolin, and transferred to the Actimetrics LumiCycle for bioluminescence recording. Raw (A) and detrended (B) oscillation profiles are representative of 19 and 14 independent experiments, respectively (one donor per experiment). (C) The period length of Bmal1-luc or Per2-luc was on average 25.29 ± 0.13 h, (n = 19) or 25.20 ± 0.19 h, (n = 14), respectively. Data represent the mean ± SEM.
rhythms. In the SCN and liver of Clock-deficient mice, Bmal1 mRNA was elevated, as well as in pancreatic islets of Clock-mutant mice, which was attributed to reduced REV-ERBα expression and a compensatory effect of NPAS2 [11,37].

Clockwork perturbations may develop in humans with ageing and upon a number of disorders [3,4]. In this respect, our experimental model, which allows for reproducible clock disruption mediated by CLOCK depletion in differentiated skeletal myotubes, represents a valuable

Figure 2: Silencing of CLOCK expression attenuates circadian oscillations in human skeletal myotubes. (A) CLOCK mRNA was measured in human myotubes transfected with siControl or siClock by RT-qPCR and normalized to the mean of βS and HPRT. CLOCK expression was reduced by 83.5 ± 3.4% (mean ± SEM, n = 8; ***p < 0.001) in siClock-transfected cells. (B) Amplitude of the Bmal1-luc reporter is strongly reduced in siClock-transfected myotubes. Representative Bmal1-luc oscillation profiles are shown for non-transfected (black line), siControl (blue line), and siClock (red line) transfected myotubes. Bmal1-luc oscillation profiles were recorded in duplicates (3 experiments, one donor per experiment). (C) RT-qPCR was performed for BMAL1, REV-ERBα, PER3 and DBP on RNA samples extracted from forskolin-synchronized human myotubes, transfected with siClock (open circles) or siControl (closed circles). Samples were collected every 4 h and normalized to the mean of βS/HPRT. Profiles (mean ± SEM) are representative of 3 experiments (2 donors for time points 0 h–48 h and 3 donors for time points 12 h–36 h) with duplicates per time point.
tool for getting significant insights into the roles of clock perturbations in different aspects of human skeletal muscle function. In order to detect oscillatory alterations caused by metabolic changes it might be necessary to develop further readouts involving clock output genes, as the here assessed Bmal1-luc and Per2-luc reporter profiles, although definitely informative regarding the core clock, might be of limited value as readout for metabolic perturbations.

4.3. Basal IL-6 secretion by human skeletal muscle is regulated by the circadian clock

Importantly, our study demonstrates that human skeletal myotubes, synchronized in vitro by forskolin or dexamethasone, secrete basal IL-6 in a circadian manner (Figure 3A, Supplementary Figure 5). Furthermore, this circadian pattern is flattened under siClock-mediated clockwork disruption, with overall basal IL-6 secretion being strongly downregulated by oscillator perturbation (Figure 3A, Table 3). The experimental settings we have developed, combining continuous perfusion and bioluminescence recording, allow for the direct assessment of IL-6 secretion by cultured human myotubes. Studies in healthy adults have suggested that IL-6 levels in the cerebrospinal fluid exhibit a 24 h oscillatory profile, and plasma levels of IL-6 have a biphasic 12 h component [38,39]. However, it has to be taken into account that plasma levels are originating from different sources of IL-6 and are also influenced by absorption in effector organs. Our finding that basal IL-6 secretion is regulated locally by the skeletal muscle clock is in line with accumulating evidence that endocrine body rhythms are tightly regulated in humans by the circadian system at several levels (reviewed in [40]). Insulin secretion by beta cells in rodents was suggested to be a subject for clock regulation [11]. Moreover, a number of proinflammatory cytokines exhibit pronounced circadian alterations in the magnitude of their response to an endotoxin challenge at different times of the day [41], among them IL-6. Such circadian gating of the inflammatory response was lost for IL-6 in REV-ERBα knockout mice. Moreover, attenuation of REV-ERBα levels in human macrophages implied a direct link between REV-ERBα and IL-6 secretion [41]. Given a general controversy around the roles of IL-6 level alterations in the etiology of metabolic diseases [42], such downregulation might have a positive or negative impact on skeletal muscle function and body metabolism. A recent in vivo study in humans suggested that injection of IL-6 to T2D patients did not affect insulin-stimulated glucose uptake [43], while it was reported to have beneficiary effects on the glucose uptake in young, healthy subjects [44]. Indeed,
Overall basal IL-6 secretion after forskolin synchronization in human myotubes.

Hypothesis beyond transcription could be that the circadian profile of IL-6 production in the skeletal muscle might give an advantage for the rhythmicity of their levels [40,49], the oscillatory pattern of IL-6 with other key hormones, such as glucose, insulin, and other key hormones, which exhibit circadian oscillations [48]. We speculate that similar to cortisol, thyroid hormones, and insulin, other key hormones, which exhibit circadian rhythmicity of their levels [55]. The following limitations of this part of the study should be taken into account: the low number of around-the-clock perfusion experiments analyzed by multiplex, due to the high experimental complexity of the automated perfusion system, and the low basal concentration levels of many myokines. Therefore, although the technical reproducibility for the duplicates in each around-the-clock experiment was high, in view of the large variability among the human donors with respect to their myokine secretion levels, these experiments must be interpreted with caution. More experimental repetitions will therefore be required to claim the circadian regulation of the myokines identified in our screen. Furthermore, in order to allow for the detection and quantification of myokines with low concentration levels in a circadian manner, more sensitive tools need to be developed.

### Table 3 — Overall basal IL-6 secretion after forskolin synchronization in human myotubes.

| Condition | Non-obese (n = 3) | Obese (n = 3) | All donors (n = 6) |
|-----------|-------------------|---------------|-------------------|
| IL6/48h in siControl samples (pg) | 4386.54 ± 411.71 | 5888.25 ± 1547.86 | 5137.40 ± 796.43 |
| IL6/48h in siClock samples (pg) | 1424.12 ± 408.83 | 2395.52 ± 625.60 | 1909.82 ± 365.20 |
| Inhibition of secretion (%) | 69.30 ± 10.61 | 59.66 ± 7.04 | 64.48 ± 6.09 |
| Clock mRNA silencing (%) | 91.32 ± 2.67 | 78.57 ± 8.15 | 84.94 ± 4.57 |

| Myokines with clock-regulated basal secretion. |

| Myokine | siControl | siClock | Fold change of secretion |
|---------|-----------|---------|-------------------------|
| CD44² | 0.777 | 0.692 | 1.00 ± 0.06 |
| CHI3L1/FKL40³ | 1.00 | 1.00 | 1.00 |
| FABP3/H-FABPa | 1.00 | 0.231 | 1.00 |
| Galectin-3³ | 1.00 | 0.096 | 1.00 |
| GRO-alpha/CXCL1³ | 0.096 | 1.00 | 1.00 |
| IGFBP-3³ | 1.00 | 1.00 | 1.00 |
| IL-6b | 0.01* | 1.00 | 1.00 |
| IL-8b | 0.020* | 1.00 | 1.00 |
| MCP-1/CCL2| 0.020* | 0.096 | 1.00 |
| M-CSF/CSF1³ | 0.059 | 0.777 | 1.00 |
| MMP-2³ | 1.00 | 1.00 | 1.00 |
| Serpin E1/PAI-1³ | 1.00 | 1.00 | 1.00 |
| Serpin C1³ | 0.492 | 1.00 | 1.00 |
| TIMP-1³ | 1.00 | 1.00 | 1.00 |
| VEGF³ | 0.949 | 0.231 | 1.00 |

*IFN-gamma, IL-1 beta, IL-2, IL-4, IL-7, IL-10, IL-12p70, IL-13, IL-17A, TNF alpha were below detection level.

* p < 0.05, ** p < 0.01, *** p < 0.001.

+a custom-made luminex screening plate.

b high sensitivity luminex performance plate.

Visualizing IL-6 secretion at the single cell level in synchronized human myotubes would be necessary to provide an insight into the mechanism of this phenomenon.
Of note, myokines identified in our multiplex screen as potentially clock-regulated for their basal secretion (Table 4, Figure 4) have been previously linked to obesity or T2D. For instance, serum IL-8 levels are increased in T2D patients, with a significant diurnal variation observed for IL-8 in blood upon LPS-stimulation [56,57]. Furthermore, insulin-resistant human myotubes secrete higher levels of IL-8 [19]. MCP-1, which mediates skeletal muscle macrophage recruitment, was also suggested to play a role in the etiology of T2D [20]. Interestingly, this proinflammatory cytokine has been previously shown to exhibit pronounced circadian alterations in the magnitude of its response to endotoxin challenge at different times of the day [41]. M-CSF has been shown to play an important role in inflammatory diseases including obesity [58]. VEGF, known for its angiogenic properties, is transcriptionally regulated by the circadian clock [59], and has also been implicated in T2D and insulin resistance [60,61]. Plasma levels of CD44 were positively correlated with insulin resistance in humans [62,63]. Furthermore, genetic association studies have linked CD44 with T2D [64,65]. Taken together, our data suggest that disruption of the circadian clock might affect the level and temporal profile of basal secretion for a number of myokines that play a role in the etiology of T2D and obesity. Inevitably, our findings raise the question on the physiological relevance of basal myokine secretion. One plausible argument is that while induced myokine secretion is regulated acutely (for instance by exercise), circadian regulation of basal myokine secretion might represent a fine-tuning mechanism, allowing adaptation of the skeletal muscle function to the rest—activity cycle. Given the major role of the circadian clock in allowing organisms to anticipate daily environmental changes rather than react to them, circadian regulation of basal myokine secretion might represent such an anticipatory mechanism that coordinates skeletal muscle “availability”. Furthermore, this newly discovered link between the functional skeletal muscle clock and basal secretion of a number of myokines might bear potential consequences for the development of chronic diseases, such as obesity and T2D.

5. CONCLUSION

Skeletal muscle represents the most important site of insulin resistance in T2D patients [12]. Moreover, the emerging critical role of inflammation in the etiology of T2D makes inflammatory cytokines plausible candidates for developing new therapeutic approaches for the treatment of this disease [65]. It is therefore of highest scientific and clinical importance to provide further insight into the emerging connection between circadian oscillator function, metabolic regulation, and T2D. Given that human primary myotubes, established from T2D patient biopsies and cultured in vitro, have been demonstrated to maintain their in vivo phenotypes of inflammation and insulin resistance [66], our model of synchronized cultured primary myotubes represents a valuable experimental tool that allows for studying the role of the circadian oscillator in human skeletal muscle function upon metabolic diseases. This work is the first detailed characterization of the human skeletal myotube circadian oscillator and its critical impact on basal myokine secretion. It opens the way for future studies that may link defects in these pathways with insulin resistance, obesity, and T2D. Given obvious obstacles for studying the human circadian oscillator in vivo, our model of synchronized myotubes represents a valuable experimental tool that allows for studying the role of the circadian oscillator in human skeletal muscle function upon metabolic diseases.
oscillator in vivo, our experimental approach, using human primary cell cultures established from biopsies that express a luciferase reporter driven from a circadian promoter, constitutes a powerful model system for human skeletal muscle clock studies.

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**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.07.009.

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