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Circadian clock and steroidogenic-related gene expression profiles in mouse Leydig cells following dexamethasone stimulation

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ABSTRACT

Previous studies have shown that circadian clock genes are expressed in mammalian testes; however, it remains unclear if the expression patterns of these genes are cyclic. Furthermore, it is unknown whether Leydig cells, the primary androgen secreting cells in the testis, play a role in the rhythmicity of circadian clock and steroidogenic-related gene transcription. Here, we examine the circadian clock of mouse Leydig cells, and the link to steroidogenic-related gene transcription. We confirm, via sampling over a full circadian time (CT) period, a lack of circadian rhythmicity in mouse testes in comparison with the robust gene expression cycling of circadian clock genes in mouse livers. Immunofluorescence imaging of mouse testes collected at CT0 and CT12 show that the BMAL1 protein is exclusively expressed in mouse Leydig cells, and clearly linked to the circadian oscillation. Furthermore, dexamethasone treatment synchronized the expression of several of these canonical circadian clock and steroidogenic-related genes. Bioinformatic analyses revealed the presence of several circadian clock-related sequence motifs in the promoters of these steroidogenic-related genes. Our results suggest mouse Leydig cells may contain a functional circadian oscillator and the circadian clockwork in mouse Leydig cells regulates steroidogenic-related gene transcription by binding to the E-box, RORE, and D-box motifs in their promoters. However, additional research is required to determine the specific molecular mechanisms involved.

1. Introduction

In mammals, many physiological processes exhibit robust circadian rhythms, these include, sleep-wake cycles, endocrine secretions, lipid and glucose metabolism, and immune responses [1]. At the cellular level, the components of the endogenous circadian clockwork are responsible for the generation and maintenance of the diurnal rhythms that govern several physiological functions. These circadian regulatory mechanisms are not only found in the suprachiasmatic nucleus (SCN), considered the central pacemaker of the body, but also in most peripheral cells and tissues (peripheral oscillators) [2–4]. While the SCN controls the peripheral oscillators via humoral and neuronal cues in a hierarchical manner [5,6], at the molecular level, the circadian clockwork consists of interlocking transcriptional-translational feedback loops involving multiple circadian clock-regulated genes, including *Clock*, *Bmal1*, *Pers*, and *Crys*, and their protein products, which are required to generate endogenous circadian oscillations [7]. Several other associated proteins, including the nuclear receptors ROR and REV-ERBs, and DBP, make up additional regulatory loops that modulate these circadian rhythms [8–11].

Mammalian testes are heterogeneous organs comprised of several well-defined cell types, including germ cells of different stages, Sertoli cells, and Leydig cells. As a critical component of the male reproductive and endocrine tract, the primary functions of the mammalian testes are to produce sperm and androgens, primarily testosterone. In a previous study, using Northern analysis, *Per1* and *Per3* RNA levels were shown to undergo circadian oscillations in mouse testes [12]. However, attempts to find molecular oscillations

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in mammalian testes are inconsistent. Two studies published in 2003 showed non-cyclic expression patterns of the circadian clock genes *Per1*, *Cry1*, and *Bmal1* in mouse testes using an RNase protection assay [13,14]. Furthermore, a recent study, employing quantitative real-time PCR (qRT-PCR), confirmed that the expression of these circadian clock genes is not cyclic in mouse testes [15].

Mouse testes undergo dynamic spermatogenesis, and therefore, contain germ cells in various stages of differentiation. It has been hypothesized that the cyclic expression of circadian clock genes is suspended during cellular differentiation. Furthermore, it has been established that plasma testosterone concentrations exhibit diurnal fluctuations in healthy young men, laboratory rats, and mice [16–18]. Leydig cells are the primary androgen producing cells in the body. Despite the observed non-cyclic expression patterns of circadian clock genes in homogenates of mouse testes, it has yet to be determined if Leydig cells contain functional circadian regulatory mechanisms, and if there is a connection between these putative mechanisms and testosterone secretion.

Dexamethasone, a glucocorticoid hormone analog, has been shown to induce circadian clock gene expression in rat-1 fibroblasts, and transiently change the phase of circadian clock gene expression *in vivo* [19,20]. Previously, we have shown that a 2 h dexamethasone treatment synchronizes the expression of circadian clock-related genes in mature rat granulosa and endometrial stromal cells *in vitro* [21–24]. Therefore, we have concluded that dexamethasone is an ideal compound for the investigation of circadian rhythm regulation in cell culture.

In this study, we investigate the expression of various circadian clock genes in mouse testes using qRT-PCR and immunofluorescence techniques, and examine the expression patterns of several circadian clock and steroidogenic-related genes in mouse Leydig cells, *in vitro*, following dexamethasone synchronization.

2. Materials and methods

2.1. Animal treatment and sample collection

Male Kunming white outbred mice (2–3 months old) were purchased from the Laboratory Animal Center of the Fourth Military Medical University, Shaanxi Province, PR China. The mice were housed individually in light-tight, ventilated closets in a temperature and humidity controlled facility with *ad libitum* access to food and water. The animals were entrained on a 12:12 h light-dark cycle (zeitgeber time, ZT0: 0800 light on; ZT12: 2000 light off) for at least two weeks to synchronize the circadian clocks of the mice to the ambient light-dark cycle. The mice were then exposed to constant darkness (circadian time condition: CT). Beginning on the second day of the CT condition, the mice were euthanized every 4 h ($n = 3$), and testis and liver samples were collected at six time points (CT0, CT4, CT8, CT12, CT16, CT20). All procedures were approved and performed under the control of the Guidelines for Animal Experiments by the Committee for the Ethics on Animal Care and Experiments of Northwest A&F University.

2.2. Immunofluorescence

Immunofluorescence was used to determine the localization of BMAL1, a core circadian clock protein, in the mouse testes. The testes were collected at CT0 and CT12 ($n = 3$), and immediately fixed in Bouin's solution overnight at 4 °C, washed in 70% ethanol for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm thick) were spread on gelatin-coated glass slides, deparaffinized, hydrated, washed with PBS, permeabilized for 30 min with 0.1% Triton X-100 in PBS (PBST) at room temperature, and blocked for 30 min with 5% normal goat serum in PBS at 37 °C. Sections were incubated in anti-BMAL1 (ab93806, Abcam, Cambridge, UK) diluted 1:200 in PBS overnight at 4 °C. Control sections were incubated in rabbit serum (ab7487, Abcam, Cambridge, UK) diluted 1:250 in PBS overnight at 4 °C. All sections were then washed in PBS, incubated for 2 h at 37 °C in AlexaFluor 488 donkey anti-rabbit IgG (H + L; Life Technologies, California, USA) diluted 1:200 in PBS, and re-washed with PBST; nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, 2 μg/ml) for 8 min and fluorescence signals were imaged using a Nikon A1R si confocal microscope system (Nikon Corporation, Tokyo, Japan).

2.3. Leydig cell isolation and culture

Leydig cells were isolated from the testes of 2–3 month old Kunming White mice and cultured as previously described [25]. Briefly, the testes were de-capsulated and digested using 0.05% type I collagenase in DMEM/F-12 (Invitrogen, Carlsbad, CA) for 15 min at 34 °C. The suspended Leydig cells were collected by filtration using a 200-μm nylon cell strainer, and purified over a discontinuous Percoll gradient (5%, 30%, 58%, and 70%). After centrifugation at 800 × g at 4 °C for 30 min, the majority of the purified Leydig cells were concentrated in the third layer. These cells were then re-suspended in DMEM/-F12 containing 10% fetal bovine serum (FBS, Invitrogen), (1 × antibiotic-antimycotic; AA). Approximately 5×10^5 Leydig cells were seeded on a 35 mm collagen-coated dish in 2 ml DMEM/-F12 containing 10% FBS supplemented with antibiotics. Cell culture was performed in a humidified atmosphere of 95% air-5% CO_2 at 37 °C, for 2 days until reaching 70–80% confluence. The purity of the Leydig cells was assessed using 3β-hydroxysteroid dehydrogenase (3β-HSD) staining via the modified Wiebe method [26].

2.4. Leydig cell synchronization

Confluent Leydig cells were synchronized using 100 nM dexamethasone (Sigma-Aldrich) for 2 h in serum-free DMEM/-F12 medium containing 1 × AA. The cells were then exposed to serum-free DMEM/-F12 medium supplemented with 0.1% BSA, 1 × AA, and 1 × Insulin-Transferrin-Selenium (ITS). Cultured cell samples were collected after 12 h at 4 h intervals (12 h, 16 h, 20 h, 24 h, 28 h, and 32 h).

2.5. RNA extraction and qRT-PCR

Liver, testis, and Leydig cell samples were harvested at the aforementioned time points, and total RNA was extracted using TRIzol reagent (TaKaRa Bio, Dalian, China). The RNA samples were treated with RNase-free DNase (TianGen, Beijing, China), and cDNA was synthesized using the primeScript ™ RT Reagent Kit (TaKaRa Bio). Primer sets used for qRT-PCR are listed in Supplemental Table 1. All primer sets were designed to span introns to prevent the amplification of genomic DNA. qRT-PCR reactions were performed in 20 μl volumes containing 10 ng cDNA using the SYBR Premix Ex Taq II kit (TaKaRa), with 300 nM specific primers, on the CFX96™ RT-qPCR system (Bio-Rad, California, USA) as previously described [27]. Melting peaks were determined by melting curve analysis to ensure amplification of only a single product. All reactions were performed in triplicate and had an amplification efficiency between 80% and 120%. The $2^{-\triangle\triangle Ct}$ method was used for quantitative analysis of relative gene expression. The relative expression of each sample was normalized to the average level of the constitutively expressed housekeeping gene *Gapdh*. Gene expression was further normalized to the level of the gene of interest in the control samples.

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