HSG cells, a model in the submandibular clock
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Synopsis
Circadian rhythm of vital processes is essential to health, and various tissues show unique peripheral rhythms. HSG is the human submandibular gland cell line that has been used for analysing the effects of steroids and growth factors. In the present study, we analysed the transcriptional regulation of the Bmal1 gene, a critical component of the mammalian clock system to investigate the possibility of using HSG cells as a model system of the submandibular clock. The Bmal1 gene was expressed with circadian oscillation after stimulation with dexamethasone, and its regulatory region contained two recognition motifs for ROR (retinoic acid-receptor-related orphan receptor) and ROREs [RORα (ROR α-subunit)-binding elements] in hypomethylated CpG islands with an open chromatin structure. REV-ERBα was expressed with circadian oscillation, and knockdown experiments suggested that REV-ERBα is involved in circadian transcription of the Bmal1 gene in HSG cells. These results are similar to those in NIH 3T3 cells, a standard model for the circadian system, whereas RORα required for REV-ERBα antagonism was expressed very little in HSG cells. These findings show that in the salivary gland cell line HSG there is a rhythm in the core oscillator components Bmal1 and REV-ERBα, indicating that circadian-based transcriptional regulation can be modelled in this peripheral cell type.

Key words: Bmal1, circadian rhythm, HSG cell, Rev-ererbα, salivary gland, transcription

INTRODUCTION

Circadian rhythms in behaviour and physiology are regulated by the master clock located in the SCN (suprachiasmatic nuclei) of the hypothalamus [1]. In turn, peripheral clocks directly regulate many local rhythms that probably feed back to the SCN through hypothalamic integration [2]. Circadian rhythms of vital processes are essential to health, and disturbed circadian timing comprises an increasingly relevant factor in major systemic illnesses [3] and central nervous system diseases [4].

The molecular mechanism of the circadian oscillator consists of autoregulatory transcriptional and translational feedback loops that have both positive and negative elements [1]. The key transcription factors, CLOCK and BMAL1, form heterodimers that bind to E-box enhancer sequences and activate the transcription of the Per and Cry genes. The PER and CRY proteins subsequently repress transcription at their own promoters through negative feedback caused by acting on the CLOCK-BMAL1 complex [5]. This feedback loop system controls the central clock in the SCN and the peripheral clocks in most peripheral tissues.

Since the activity of Bmal1−/− mice immediately becomes completely arrhythmic under constant darkness, BMAL1 is apparently an essential and non-redundant component of the mammalian clock [6]. The level of Bmal1 transcripts robustly oscillates in the SCN and in peripheral clock cells, and the circadian regulation of Bmal1 transcription contributes to the formation of interconnected feedback loops [5]. The Bmal1 promoter contains two recognition motifs for ROR (retinoic acid-receptor-related orphan receptor) and ROREs [RORα (ROR α-subunit)-binding elements] (REV-ERB orphan nuclear receptors) [7]. REV-ERBα represses Bmal1 expression [8] and RORα activates Bmal1 transcription [9]. The opposing activities of these receptors are important in the maintenance of circadian clock function [10]. In addition, we found that the unique chromatin structure around ROREs is required for intracellular circadian transcriptional regulation of the Bmal1 gene in NIH 3T3 cells [11].

Non-invasive salivary sampling facilitates dynamic tests of hormone function and assessment of biological rhythms. This is because the amount of secreted saliva and its content of numerous enzymes, hormones, growth factors, immunoglobulins and other bioactive substances have circadian rhythms. The circadian expression of clock genes in salivary glands suggests that a peripheral clock system is active in these glands [12]. Despite the likelihood of easily obtaining information about circadian timing from saliva, the circadian clock mechanism in salivary glands has not yet been elucidated in detail.

Abbreviations used: ChIP-chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LM-PCR, ligation-mediated PCR; MNase, micrococcal nuclease; ROR, retinoic acid-receptor-related orphan receptor; RORE, ROR α-subunit-binding element; RT-PCR, reverse transcription–PCR; SCN, suprachiasmatic nuclei; siRNA, small interfering RNA.
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Here, we characterized BMAL1 transcripitional regulation in HSG cells [13] and showed that there is a rhythm in the core oscillator components BMAL1 and REV-ERBα in this peripheral cell type.

MATERIALS AND METHODS

Cell culture
NIH 3T3 and HSG cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (fetal bovine serum) and a mixture of 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator at 37 °C under a 5% CO₂ atmosphere.

Real-time quantitative RT–PCR (reverse transcription–PCR)
Real-time quantitative RT–PCR was performed using LightCycler (Roche) with a LightCycler-FastStart DNA Master SYBR Green I kit (Roche) as described in [11]. The primer sequences used are as follows: Bmal1, 5′-GGCCGAATGA-TTGCTGAGGAAATCATGG-3′ and 5′-TTACAGGGGCATG-GCAAAGTCACATAAG-3′; Gapdh (glyceraldehyde-3-phosphate dehydrogenase), 5′-ACCACAGTCCATGGCCATAC-3′ and 5′-TTCCACACCTGTGCTGTA-3′; Revbera, 5′-TTGTA-GAGTTTGCGCCATCGC-3′ and 5′-TACGGGGCTCC-ACCAGGAAGAC-3′; Rora, 5′-TGACTCTCAGAACACACAGCGT-3′ and 5′-CGACAAATGCTGTTATATTGC-3′; Rorγ, 5′-GCTTCTATACCAATGGTGCC-3′ and 5′-TCTCCTGTAGGACTTCAGAC-3′. We constructed an authentic template containing PCR products cloned into the pGEM-T Easy vector (Promega). Relative expression levels were evaluated using LightCycler software, version 3.5.

Reporter gene assays
Reporter gene assays were performed as described in [11]. Reporter plasmids were constructed based on pGL3-dLuc [7]. Cells were transfected using HilyMax (Dojindo) and then stimulated with a mixture of 100 units/ml penicillin and 0.5 mM streptomycin, in a humidified incubator at 37 °C under a 5% CO₂ atmosphere.

sirNA (small interfering RNA)
Oligonucleotides of the siRNA for REV-ERBα [5′-rt(CU-GAAUCCUCUAAUAGUGA)d(TT)-3′ and 5′-rt(UCAUUA-AGAGGGGAUCAG)d(TT)-3′] and a negative control (target sequence, 5′-ATCCCGCGTATGACGTA-3′) (B-Bridge) were introduced into HSG cells using HilyMax (Dojindo).

RESULTS

Oscillatory BMAL1 transcription in HSG cells
We examined expression profiles of the BMAL1 gene, which is essential for the mammalian clock, in HSG cells. The BMAL1
This region (nt –201 to +24) showed promoter activity that was suppressed and enhanced at 24 and 36 h after stimulation respectively (Figure 2). The region (nt –201 to +24) corresponds to the minimal promoter region required for rhythmic expression of the Bmal1 gene in mouse NIH 3T3 cells [11], and nucleotide homology between them was 96.9%. The construct (nt –501 to –201) did not have distinct promoter activity. These results indicate that the BMAL1 gene is transcriptionally regulated in HSG cells.

Transcriptional regulation of the BMAL1 gene was analysed using transient reporter gene assays. The shortest construct (nt –201 to +24) showed promoter activity that was suppressed and enhanced at 24 and 36 h after stimulation respectively (Figure 2). This region (nt –201 to +24) corresponds to the minimal promoter region required for rhythmic expression of the Bmal1 gene in mouse NIH 3T3 cells [11], and nucleotide homology between them was 96.9%. The construct (nt –501 to –201) did not have distinct promoter activity. These results indicate that the BMAL1 gene is transcriptionally regulated in HSG cells.

Chromatin structure of the BMAL1 promoter

The methylation status of the BMAL1 promoter is involved in BMAL1 transcription [15], and the results of bisulfite genomic sequencing from four individual clones indicated that the BMAL1 promoter in HSG cells exists in the extreme hypomethylated state in CpG islands (Figure 4). The BMAL1 promoter region around the ROREs was hypersensitive to MNase, suggesting that the region comprises an open chromatin structure (bracket in Figure 5A). ChIP assays of HSG cells expressing Myc-tagged REV-REBα showed that the transcription factor interacts with the ROREs (Figure 5B). Taken together, these results suggested that the structure around the ROREs in the BMAL1 promoter of HSG cells is open, which allows transcription factor access. Our analysis of local chromatin structure around the ROREs using LM-PCR showed a footprint profile at 36 h that was similar to that at 0 h, but not that at 24 h (the region between –80 and –50 in Figure 6). These data imply that the footprint profile around the ROREs is related to the BMAL1 expression level (Figure 1). The bases marked with circles are protected more at 24 h than at 0 and 36 h. On the other hand, the base marked by a triangle (at –72) is protected at 0 and 36 h but not at 24 h. These results suggest that transcription factors such as REV-ERB or ROR bind to the RORE at certain times of the circadian day. In addition, there are digested
bands indicated by a triangle around the –88 region at 24 h, which was similar to those observed previously as the result of the circadian changes of the chromatin structure around the ROREs in NIH 3T3 cells [11]. These analyses of the chromatin structure indicated that the mechanism of *BMAL1* transcription in HSG cells is similar to that in NIH 3T3 cells. The correlation between *BMAL1* expression and the altered chromatin structure around the ROREs, particularly the region adjacent to the upstream RORE, suggests that the ROREs participate in the regulation of circadian *BMAL1* expression in HSG cells through chromatin alterations.

**REV-ERBα** is an important factor for circadian *BMAL1* gene transcription in HSG cells

*REV-ERBα* mRNA expression in HSG cells showed a circadian rhythm, an antiphase to *BMAL1* oscillation, and a similar relationship between *REV-ERBα* and *BMAL1* expression in HSG cells to that in NIH 3T3 cells, suggesting that at least the circadian system functions in HSG cells (Figure 1). RT–PCR analysis revealed that the PCR product expression of *RORα* was very low in HSG cells, but obvious in NIH 3T3 cells. On the other hand, for another *ROR* member, *RORγ*, mRNA expression in HSG cells showed a circadian rhythm (Figure 1), whereas its expression level was very low, about a hundredth of that of *REV-ERBα*.

We performed knockdown experiments using siRNA for *REV-ERBα* to determine whether *REV-ERBα* is required for circadian *BMAL1* gene regulation in HSG cells. After introducing *REV-ERBα* siRNA into HSG cells, the mRNA level of endogenous *REV-ERBα* decreased to approx. 40% of that in the cells harbouring control siRNA (Figure 7A). Real-time quantitative RT–PCR with siRNA showed that the amplitude of the transcriptional rhythm in HSG cells harbouring *REV-ERBα* siRNA was smaller than that of the cells harbouring control siRNA, whereas the oscillatory profile of cells harbouring control or *REV-ERBα*...
BMAL1 transcription in HSG cells

Figure 6 Footprinting in vivo around the ROREs in HSG cells
The numbers and ‘N’ (naked DNA sample) above the Figure indicate time (h) after stimulation and naked DNA respectively. The triangle and circles indicate the nucleotides showing higher or lower intensity at 24 h after stimulation with dexamethasone respectively. Numbers beside markers indicate the position from the transcription start site.

Figure 7 REV-ERBα is important for BMAL1 gene oscillation
(A) RNA interference with REV-ERBα. HSG cells were transfected with siRNA (‘Cont’, 200 pmol of control siRNA; ‘100 and 200’, 100 and 200 pmol of REV-ERBα siRNA) and then transcripts were analysed by real-time quantitative RT–PCR. Levels of RNA were normalized to GAPDH expression, and the value of the control siRNA sample was set as 1. Values are means ± S.E.M. for triplicate assays. (B) Function of REV-ERBα in circadian BMAL1 gene transcription. HSG cells were transfected with siRNA (closed and open circles, 100 pmol of control and REV-ERBα siRNA respectively) and stimulated with 100 nM dexamethasone; then transcripts were analysed by real-time quantitative RT–PCR. The levels of RNA were normalized to GAPDH expression, and the peak value was set as 1. Values are means ± S.E.M. for triplicate assays.

siRNA was observed (Figure 7). These findings suggested that REV-ERBα affects circadian expression of the BMAL1 gene.

DISCUSSION

The present study showed that HSG cells have an endogenous clock system that can be reset by stimulation with dexamethasone and that BMAL1, a critical component of the clock system, is transcriptionally regulated with circadian oscillation (Figures 1–3). These findings are consistent with those of previous reports showing that the structure and functions of HSG cells are similar to those of the salivary glands [13] and that a Clock- and Cry-dependent molecular clock system is active in salivary glands [12]. The regulatory region of the BMAL1 promoter for oscillatory transcription in HSG cells contains two ROREs in an open chromatin structure (Figure 5), which might be related to the intrinsic characteristics of the DNA sequence of the BMAL1 promoter such as CpG islands [16]. The unique chromatin structure around the ROREs that is hypomethylated to allow REV-ERBα access (Figures 4 and 5B) was altered in parallel with BMAL1 transcription (Figure 6). These common features of the chromatin structure are also found in NIH 3T3 cells [11], implying that the clock systems of HSG and NIH 3T3 cells are similar and cell-autonomous.

The ROREs in the Bmal1 promoter of NIH 3T3 cells are essential for the circadian oscillation of Bmal1 transcription [7], and the opposing activities of RORα and REV-ERBα are important in the maintenance of circadian clock function [10]. The circadian expression of Rev-erba in NIH 3T3 cells peaked at 24 h and that of Rorα reached a nadir at 24 h, which is essentially antiphase to Rev-erba expression (Figure 1). Surprisingly, very little RORα was detected in HSG cells, although the phase of REV-ERBα expression was similar to that in NIH 3T3 cells (Figure 1). The relationships between the circadian profiles of BMAL1 and REV-ERBα expression in HSG cells are consistent with those in mouse salivary glands [12], indicating functional similarity between HSG cells and salivary glands.
Knockdown of REV-ERBa damped the circadian oscillation of BMAL1 transcription (Figure 7) in accordance with the report that circadian Bmal1 expression in peripheral tissues is severely blunted in mice deficient in Rev-erba [8]. These data suggest that REV-ERBα is involved in circadian transcription of the BMAL1 gene in HSG cells.

All REV-ERB (α and β) and ROR (α, β and γ) family members are crucial components of the molecular circadian clock, with functional differences among various peripheral tissues [17]. Both REV-ERBα and β are functionally redundant and are required for rhythmic Bmal1 transcription, whereas RORs contribute to Bmal1 amplitude, but are dispensable for Bmal1 rhythms [18]. We found that RORγ (Figure 1) but not RORβ (results not shown) is expressed in HSG cells. However, the phase of the RORγ expression profile was similar to that of REV-ERBα oscillation, although the function of RORγ is thought to oppose that of REV-ERBα. It is possible that the co-ordinated expression of REV-ERBα and RORγ can generate BMAL1 oscillatory transcription in HSG cells by the variety of ligands to REV-ERBα and RORγ by modulating the time delay in the function of REV-ERBα and RORγ. There is still the gap of the expression levels between REV-ERBα and RORγ, and the exact mechanism of the co-ordination for the circadian regulation of BMAL1 transcription remains obscure. Then, the involvement of other REV-ERB and ROR molecules in the salivary regulation of BMAL1 circadian transcription should be elucidated.

Here, we showed that circadian oscillation of the BMAL1 gene is induced by dexamethasone stimulation in HSG cells and is regulated through the ROREs in the promoter when the chromatin environment is altered, a process in which REV-ERBα plays a key role. In the salivary gland cell line HSG, there is a rhythm in the core oscillator components, indicating that circadian-based key role. In the salivary gland cell line HSG, there is a rhythm in

**References**


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