robust circadian expression of *Per2* in the cell-autonomous clock.

MATERIALS AND METHODS

Cell culture

Mouse fibroblast NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin and streptomycin at 37°C under a humidified 5% CO_2 atmosphere.

Small interfering RNA (siRNA)

We designed E4BP4 siRNA for knockdown experiments using BLOCK-iT™ RNAi Designer (https://rnaidesigner.invitrogen.com/rnaiexpress/), and BLOCK-iT Fluorescent Oligo (Invitrogen) served as a control. These oligonucleotides were introduced into NIH3T3 cells at a final concentration of 10 nM using X-treamGENE (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) according to the suppliers' protocols.

Western blotting

NIH3T3 cells were transfected with the expression vectors, Myc-tagged E4BP4 or Myc-tagged HLF after siRNA manipulation. After a 24 h incubation, proteins were separated on 10% SDS–PAGE gels (31) and transferred to nitrocellulose membranes (Bio-Rad). After blocking nonspecific binding with 3% dry milk in PBS, proteins were probed with anti-Myc monoclonal antibody (clone 9E10; Roche Diagnostics) and then incubated with a horseradish peroxidase-conjugated anti-mouse IgG (Upstate). Immunoreactive proteins were visualized using ECL (Amersham Biosciences) according to the manufacturer's instructions. The same membrane was reprobed with anti-actin antibody (clone C4, CHEMICOM).

Northern blotting

Total RNA was prepared using ISOGEN (Nippon Gene) and then poly(A)$^+$ RNA was purified using a GenElute mRNA Miniprep Kit (Sigma-Aldrich). Northern blotting proceeded as described (32). Probes labeled with ^{32}P were generated from cDNA fragments of *Per2* (bases 1123–1830; GenBank accession no. AF036893), *E4BP4* (bases 61–770; GenBank accession no. U83148), *Bmal1* (bases 231–910; GenBank accession no. AF015953) and β-actin. The relative expression level of each gene to that of β-actin was calculated using Image Gauge (FUJIFILM).

Real-time luciferase assay

Fragments of DNA containing the *Per2* promoter region and its derivatives were cloned into pGL3-dLuc that contains a rapid degradation domain modified from mouse ornithine decarboxylase at the carboxy-terminal end of firefly luciferase (33). After transfecting reporter plasmids using PolyFect (Qiagen), NIH3T3 cells were stimulated with 100 nM dexamethasone for 2 h and then incubated with DMEM containing 0.1 mM luciferin (Promega), 25 mM HEPES (pH 7.2) and 10% FBS. Bioluminescence was measured and integrated for 1 min at intervals of 10 min using Kronos AB-2500 (ATTO).

Transient luciferase assay

The *Per2* promoter region containing E4BP4-binding sites and its derivatives were cloned into pGL3-Basic vector (Promega). The constructs were co-transfected with phRG-TK (Promega) as an internal control into NIH3T3 cells. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a Luminometer Model TD-20/20 (Turner Designs). The transcriptional activities were normalized relative to *Renilla* luciferase activities.

Gel shift assay

Gel shifts were examined as described (34). Briefly, nuclear extracts were purified from NIH3T3 cells using CelLytic NuCLEAR EXTRACTION KIT (Sigma). Recombinant-E4BP4 was purchased from ABNOVA. The probes (A-site, from −251 to −92; B-site, from +112 to +332 of *mPer2*) were amplified using the following primer sets: A-site, 5′-GGAAGTGGACGCGCCTACTCG-3′ (forward) and 5′-CGGAACCTGAGAGCTACGCTC-3′ (reverse); B-site, 5′-TTGACGCGGCGAAGCGGTGAGTG-3′ (forward) and 5′-GGGACGCAGTGTGAACCTGG-3′ (reverse). Nucleotide sequences of the 16-bp oligonucleotide probes for the A- and B-sites were 5′-CGTCTTATGTAAAGAG-3′ and 5′-CGTCTTACGTAACCGG-3′, respectively. These probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England BioLabs). The DNA probes were suspended in 10 μl of 16 mM HEPES (pH 7.5), 150 mM KCl, 16% (v/v) glycerol, 1.6 mM $MgCl_2$, 0.8 mM dithiothreitol, 0.4 mM PMSF, 1 mM EDTA, 0.8 mg/ml BSA, 0.06 mg/ml poly(dI–dC) and 0.01% NP-40 in the presence of or absence of competitor oligonucleotides and incubated with the nuclear extracts or E4BP4 protein. The anti-E4BP4 antibody was also added for super-shift assays. The samples were resolved by electrophoresis on 4% polyacrylamide gels in 40 mM Tris–acetate, 1 mM EDTA and 5% glycerol at 110 V for 2 h.

Chromatin immunoprecipitation (ChIP) assay

Assays of ChIP proceeded as described (34). Briefly, NIH3T3 cells were cross-linked with 1% formaldehyde for 15 min at room temperature and then washed twice with ice-cold PBS. The cells were shattered with lysis buffer (25 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA and 1 mM PMSF) on ice for 30 min. Sonication to shear DNA into 100–300 bp fragments was followed by centrifugation and supernatants containing soluble chromatin were collected. The chromatin fraction was incubated with anti-E4BP4 antibody (E-16, Santa Cruz Biotechnology) overnight at 4°C, followed by salmon sperm DNA/protein G agarose (Upstate). Chromatin immunocomplexes were washed three times, once with wash buffer 1 (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), once with wash buffer 2 (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA) and once with wash buffer 3 (10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1% Nonidet P-40, 1%

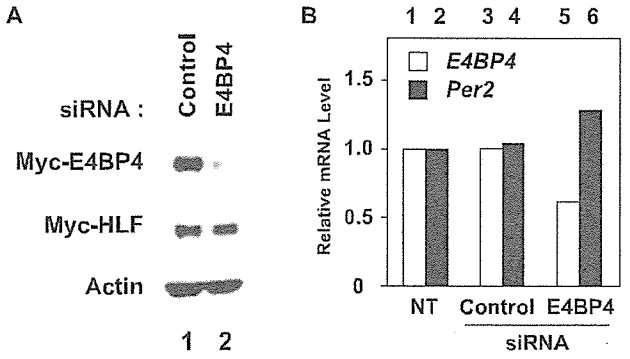


Figure 1. E4BP4 is a negative regulator of *Per2* transcription. (**A**) RNA interference with E4BP4. NIH3T3 cells were transfected with scrambled siRNA (Control siRNA) or specific siRNA for E4BP4 (E4BP4 siRNA). Myc-tagged E4BP4 was co-transfected with Myc-tagged HLF (as a negative control) 24 h later. At 48 h after siRNA transfection, the E4BP4 protein level was determined by Western blotting using anti-Myc antibody. The same membrane was stripped and reprobed with anti-actin antibody. (**B**) Knockdown of E4BP4 increased *Per2* transcription. NIH3T3 cells transfected with siRNAs (Control siRNA or E4BP4 siRNA) or non-transfected (NT) were analyzed for *E4BP4* and *Per2* mRNA by Northern blotting. Poly(A)$^+$ RNA was purified from total RNA of two independent experiments. Expression levels were normalized to those of β-actin. Values are relative to that of NT cells.

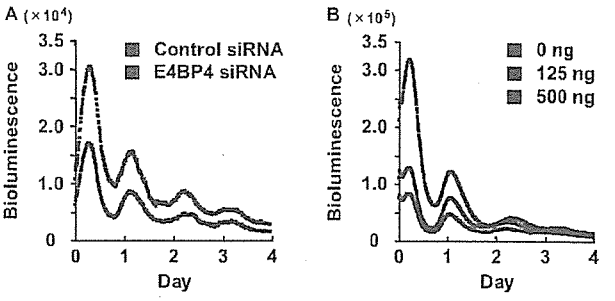


Figure 2. E4BP4 negatively regulates *Per2* oscillation in cell-autonomous clock. (**A**) Effect of E4BP4-knockdown by siRNA. B, Effect of E4BP4-overexpression. Promoter region of *mPer2* (−798 to +331 relative to the cap site) was examined in real-time reporter gene assays. After introduction of siRNAs (A) or E4BP4-expression vector (**B**), bioluminescence was measured and integrated for 1 min at intervals of 10 min. The results are representative of three independent experiments.

deoxycholate and 1 mM EDTA). All washes proceeded at 4°C for 5 min. The samples were then washed twice with TE buffer. The immunocomplexes were removed with 1% SDS and 0.1 M $NaHCO_3$ and then heated overnight at 65°C to reverse the crosslinks. The crosslinks of DNA input samples were similarly reversed. Sample DNA was purified and then putative E4BP4 target regions (A-site, from −251 to −92; B-site, from +112 to +332 of mPer2) were amplified by PCR using the following primer sets: A- site, 5′-GGAAGTGGACGCG-CCTACTCG-3′ (forward) and 5′-CGGAACCTGAGAGCT-ACGCTC-3′ (reverse); B-site, 5′-TTGACGCGGCGAAGCG-GTGAGTG-3′ (forward) and 5′-GGGACGCAGTGTGAAC-CTGG-3′ (reverse).

RESULTS

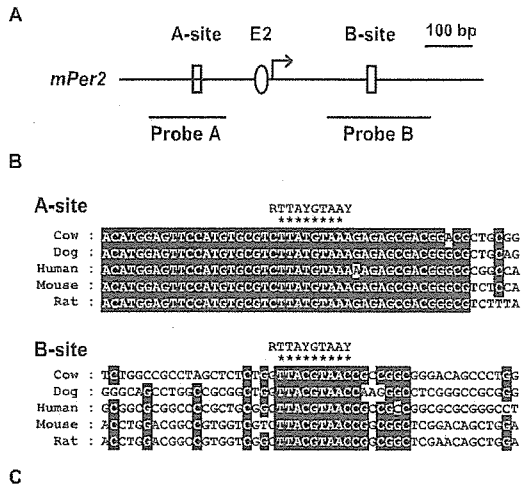
E4BP4 down-regulates *Per2* transcription

E4BP4 is a mammalian homologue of *vrille* (*vri*) that functions as a key negative component of the *Drosophila* circadian clock (12,26,27). However, whether or not E4BP4 is required for mammalian circadian clocks remains unclear. We initially examined the effect of E4BP4 upon *Per2* transcription. To determine whether E4BP4 regulates *Per2* gene expression, we performed knockdown experiments using small interfering RNA (siRNA) for *E4BP4* (E4BP4 siRNA). The induction of E4BP4 siRNA into NIH3T3 cells resulted in a significant decrease in the protein level of exogenously expressed Myc-tagged E4BP4, whereas the level of Myc-tagged Hepatic Leukemia Factor (HLF) was not affected (Figure 1A, lane 2), suggesting that the siRNA specifically knocked down E4BP4. We then examined the mRNA levels of *E4BP4* and *Per2* by Northern blotting. After introducing E4BP4 siRNA into NIH3T3 cells, the mRNA level of endogenous *E4BP4* decreased to ~60% of that in non-transfected (NT) cells (Figure 1B, lane 5). On the other hand, that of *Per2* increased to ~130% of that in NT cells (Figure 1B, lane 6). The levels of neither *E4BP4* mRNA (Figure 1B, lane 3) nor *Per2* mRNA (Figure 1B, lane 4) were affected by induction with scrambled siRNA (Control siRNA). These results suggest that E4BP4 suppresses *Per2* transcription.

The circadian clock is cell-autonomous (35,36). Circadian oscillators are located not only in the suprachiasmatic nucleus (SCN) of the brain, which is the central circadian pacemaker in mammals, but also in most peripheral tissues (19,37,38) and even in established cell lines (39). To examine the role of E4BP4 on the circadian expression of *Per2* in the cell-autonomous clock, we performed real-time luciferase assays (33,40) in NIH3T3 cells using the reporter plasmid containing the *mPer2* (−798 to +331) promoter to drive destabilized luciferase (*mPer2*-dLuc). The transcriptional start site (TSS) is indicated as +1 (24). After the introduction of E4BP4 siRNA, the cells were transfected with *mPer2*-dLuc and circadian gene expression was induced with 100 nM dexamethasone (41). We measured bioluminescence in the presence of luciferin and integrated signals for 1 min at intervals of 10 min. As reported (24,25,30), the transcriptional fluctuation from *mPer2*-dLuc showed robust circadian oscillation. The induction of E4BP4 siRNA caused a remarkable overall 1.86-fold increase in the transcriptional activity of *mPer2*-dLuc compared with Control siRNA (Figure 2A). Conversely, exogenously expressed E4BP4 resulted in a gradual reduction in the circadian expression of *Per2* (Figure 2B). These results suggested that E4BP4 functions as a negative regulator of *Per2* oscillation in the cell-autonomous clock.

Two putative E4BP4-binding sites on the *Per2* promoter region

Although many putative E4BP4-binding sites are located in clock and clock-related genes (30), whether the E4BP4-mediated negative regulation of *Per2* is direct or indirect remains unknown. To examine whether E4BP4 directly represses the transcriptional activity of *Per2*, we searched the mouse *Per2* promoter region and genomic gene sequences for the E4BP4-binding site, RT(G/T)AYGTAAY (where R is a purine and Y is a pyrimidine) (42). Sequence analysis



Distance from E2	Cow	Dog	Human	Mouse	Rat
to A-site (bp)	136	136	138	137	137
to B-site (bp)	236	204	203	226	226

Figure 3. Two putative E4BP4-binding sites on mammalian *Per2* promoter regions. (**A**) Maps of *mPer2* promoter region. Arrow and oval indicate transcription start site (TSS) and E2 enhancer (position at −20 relative to cap site) (24), respectively. Two putative E4BP4-binding sites are shown as open boxes. Positions of probes used for gel shift analysis are indicated as horizontal bars. (**B**) Sequence alignment of region around E4BP4-binding sites. Conserved sequences are shown as white characters on black. Consensus E4BP4-binding sequences are shown above and identical bases are indicated by asterisks. (**C**) Distance between putative E4BP4-binding sites and E2.

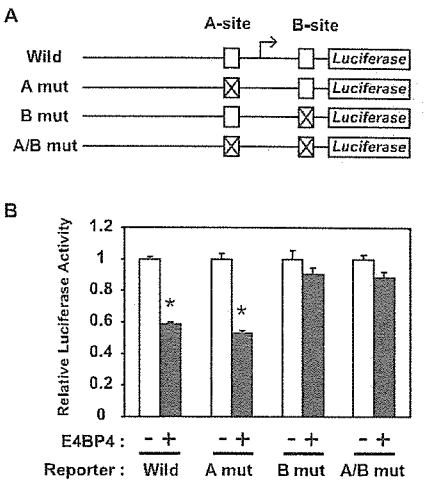


Figure 4. B-site is responsible for E4BP4-mediated transcriptional repression of *Per2*. (**A**) Schematic representation of mutant constructs of *mPer2* promoter. Arrow indicates TSS. Open boxes, putative E4BP4-binding sites. Each or both of the putative E4BP4-binding sites were mutated (A-site, 5′-CTTATGTAAA-3′ to 5′-CCAGTGTAAA-3′; B-site, 5′-CTTACGTAAC-3′ to 5′-CCAGCGTAAC-3′). (**B**) Analysis of E4BP4-binding sites on *Per2* promoter. Transcriptional assay was performed with indicated mutant constructs. E4BP4 expression plasmid is present (+) or absent (−). Normalized expression level was calculated relative to luciferase activity in absence of E4BP4. Values are means ± SEM of three replicates from a single assay. (*) Significant difference between presence versus absence of E4BP4 ($P < 0.001$). Results are representative of two independent experiments.

revealed two putative E4BP4-binding sites (termed A-site at −151 and B-site at +197) around the TSS (Figure 3A). The nucleotide sequences of the A- and B-sites matched 8/10 and 9/10 bp of the consensus E4BP4-binding sequence, respectively. Further analysis revealed that the nucleotide sequence around the A-site is highly conserved among mammalian *Per2* promoter regions (cow, dog, human, mouse and rat) (Figure 3B, upper panel). On the other hand, a B-site is located at a diverse region of intron 1 (Figure 3B, lower panel). Interestingly, the location of both sites with respect to the E2 enhancer was highly conserved beyond species (Figure 3C). These data suggest that the A- and B-sites are functionally important for the E4BP4-mediated negative regulation of *Per2*.

E4BP4 directly represses the transcriptional activity of *Per2* through the B-site

To understand the functional importance of the A- and B-sites for E4BP4-mediated transcriptional repression of *Per2*, we performed luciferase assays with mutant constructs of the *mPer2* (−798 to +331) promoter (Figure 4A) to prevent E4BP4 binding (Supplementary Figure S1). The mutated sequences of the A- and B-sites were 5′-CCAGTGTAAA-3′ and 5′-CCAGCGTAAC-3′, respectively (43). E4BP4-mediated transcriptional repression of these mutant constructs was examined, and normalized expression level was calculated relative to the luciferase activity in the absence of E4BP4. Consistent with the observations in Figure 2B, exogenously expressed E4BP4 repressed the transcriptional activity of the wild type of *mPer2*(−798 to +331) promoter (Wild, % of repression by E4BP4 was 41.0%). A mutation of the A-site (A mut; 47.2%) resulted in the same transcriptional repression as with the Wild type. However, mutation of the B-site (B mut; 9.67%) and of both the A- and B-sites (A/B mut; 11.7%) recovered from the repression (Figure 4B). Similar results were obtained using deletion constructs of E4BP4-binding sites (data not shown). As the PAR transcription factors (DBP, HLF and TEF) are known to bind to the identical nucleotide sequence as E4BP4 *in vitro* (29,30), we also examined the effect of DBP, HLF and TEF on the *mPer2* promoter activity using same mutant and deletion constructs. However, neither the A- nor B-site was responsible for the transcriptional activation of *mPer2* by these PAR transcription factors (Supplementary Figure S2). These results suggest that the B-site is functionally important for the E4BP4-mediated transcriptional repression of *Per2*.

To clarify the importance of the B-site for E4BP4-mediated transcriptional repression of the *Per2* promoter through the DNA-binding activity of E4BP4, we performed gel shift assays using nuclear extracts from the NIH3T3 cells expressing Myc-tagged E4BP4 and the probes shown in Figure 3A. We used end-labeled DNA fragments of ~200 bp containing either the A- or the B-site probes (Figure 3A). We observed shifted bands for both probes and the bands disappeared in the presence of an unlabeled competitor containing the consensus E4BP4-binding sequence, indicating that the protein-DNA complexes were specific for the E4BP4-binding site (Figure 5A, asterisk in probe A and double asterisk in probe B). However, the

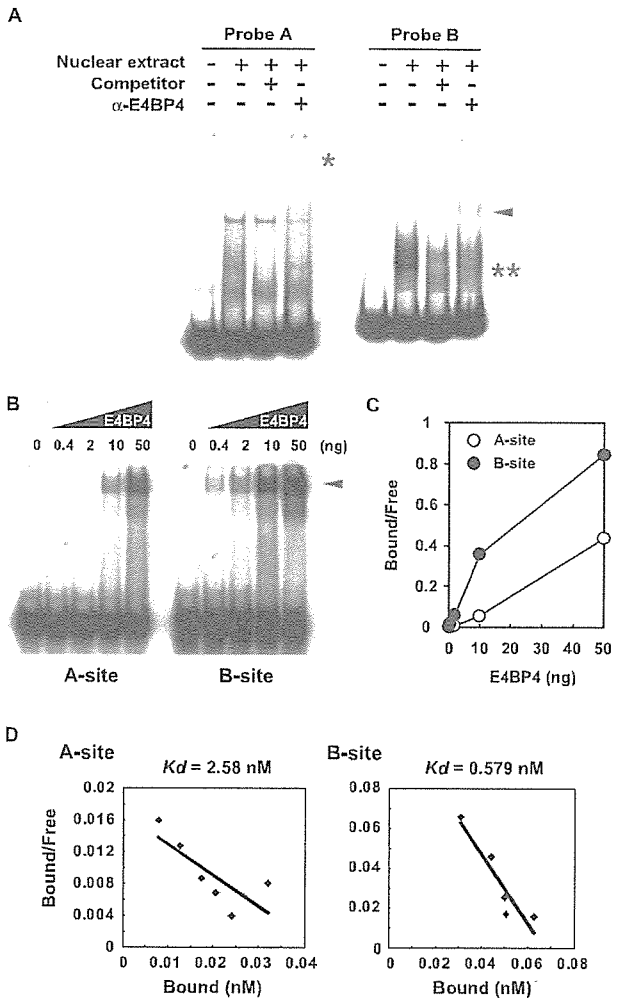


Figure 5. E4BP4 preferentially binds to B-site *in vitro*. (**A**) Gel shift analysis using nuclear extracts from NIH3T3 cells transfected with the E4BP4-expression vector. Positions of probes A and B are shown in Figure 3A and competing nucleotide sequence is 5′-TCGAGAAAAAATTATGT-AACGGTC-3′. * and **, band specifically bound to E4BP4-binding site; arrowhead, supershifted band. The band specifically bound to E4BP4-binding site in probe B (**) not but in probe A (*) was supershifted with an anti-E4BP4 antibody. (**B**) Gel shift analysis using recombinant-E4BP4. Oligonucleotide probes (A-site, 5′-CGTCTTATGTAAAGAG-3′; B-site, 5′-CGTCTTACGTAACCGG-3′) were incubated with increasing amounts of recombinant-E4BP4 (0, 0.4, 2, 10 and 50 ng). Arrowhead, band bound to E4BP4. (**C**) Quantitation of E4BP4-oligonucleotide probe complex. (**D**) Determination of *Kd*s for binding of recombinant-E4BP4 to A- and B-sites. Recombinant-E4BP4 (10 ng) was incubated with increasing amounts of radiolabeled 16-bp core probes for A- and B-sites. After gel electrophoresis and autoradiography, radioactive bands corresponding to the bound and free forms were quantified. Concentration of bound probe was plotted against total input probe to show saturation curves. *Kd* values were determined from these data on Scatchard plots. Slope of best-fit line is equal to $-1/Kd$.

supersifted band with an anti-E4BP4 antibody was observed in only probe B (Figure 5A, arrowhead). This was also confirmed using an anti-Myc antibody (data not shown). These results suggest that E4BP4 binds to the B-site and forms a DNA-protein complex. We questioned why only the probe B (double asterisk) showed a supershifted band. Figure 3B shows that the nucleotide sequences of A- and B-sites were not identical. Therefore, we examined the affinity of E4BP4 for the A- and B-sites using gel shift assays, with recombinant-E4BP4 protein and 16-bp core probes for each site. Figure 5B and C shows that the B-site had higher affinity than the A-site for E4BP4. Further analysis determined that the *Kd*s for binding of the recombinat-E4BP4 to the A- and B-sites were 2.58 and 0.579 nM, respectively (Figure 5D). These results indicated that E4BP4 preferentially binds to the B-site rather than to the A-site *in vitro*.

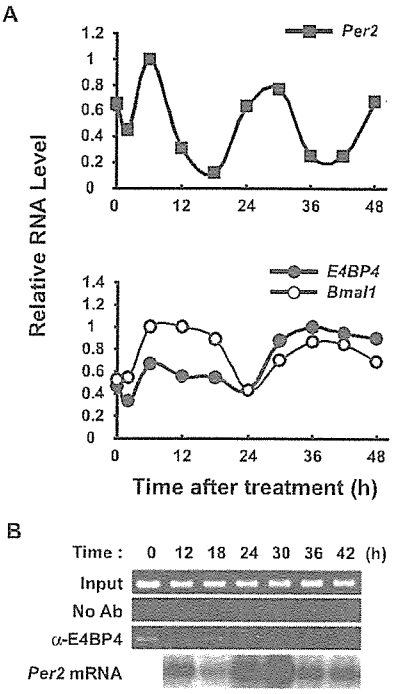


Figure 6. Binding of E4BP4 correlates with negative *Per2* regulation. (**A**) *E4BP4* transcript shows anti-phase to *Per2* oscillation. NIH3T3 cells were stimulated with 100 nM dexamethasone, and mRNA was analyzed by northern blotting. Levels of RNA were normalized to β-actin expression and peak values of individual curves were set to 1. (**B**) Oscillatory binding of E4BP4 to *Per2* promoter *in vivo*. NIH3T3 cells were stimulated with dexamethasone and ChIP assays of B-sites were applied. Bottom panel, *Per2* mRNA oscillation. No Ab, without antibody; α-E4BP4, with anti-E4BP4 antibody.

We then performed ChIP assays in NIH3T3 cells expressing Myc-tagged E4BP4 to determine the situation *in vivo*. Consistent with the observations in Figure 5 *in vitro*, the ChIP assays also suggested that E4BP4 binds to the B-site much more than to the A-site on the *Per2* promoter *in vivo* (data not shown). Taken together, these results indicated that E4BP4 directly represses *Per2* transcription via the B-site on the promoter.

Binding of E4BP4 correlates with negative regulation of *Per2*

The circadian expression of E4BP4 is similar to that of the anti-phase to *Per2* oscillations in both the SCN and the liver (29,33). We therefore postulated that E4BP4 plays an important role in *Per2* oscillation in the cell-autonomous

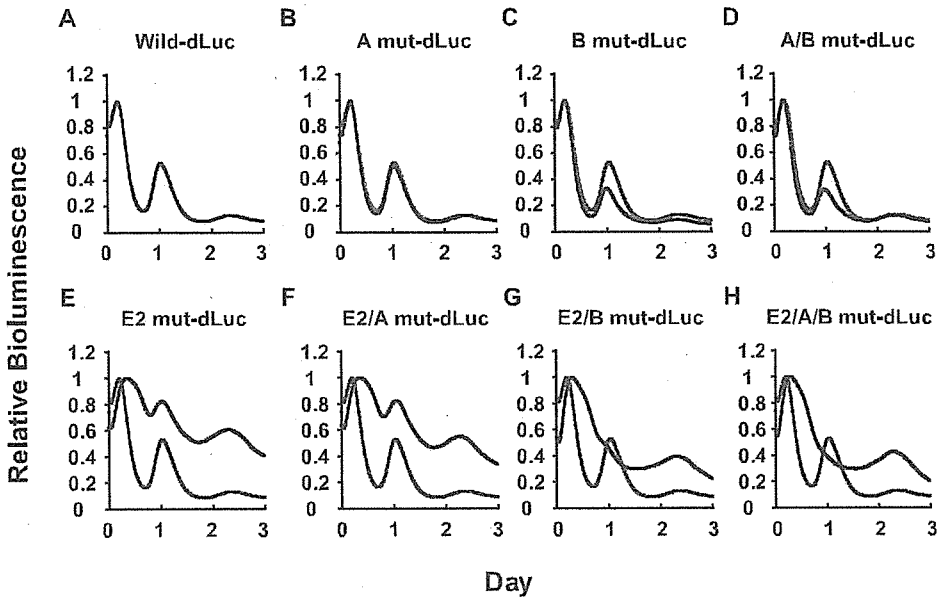


Figure 7. (A–H) B-site drives the circadian expression of *Per2*. NIH3T3 cells were transfected with indicated mutant constructs, incubated with dexamethasone and then bioluminescence was measured. For accurate comparison, light gray dots show bioluminescence from Wild-dLuc. Peak values of individual curves were set to 1. Results are representative of three independent experiments that generated similar results. Wild, wild type *mPer2* promoter; A mut, mutated A-site; B mut, mutated B-site; E2 mut, mutated E2 enhancer (5′-GCTAGT-3′).

core clock. To test this hypothesis, we analyzed the temporal expression profile of *E4BP4* mRNA in NIH3T3 cells after stimulation with 100 nM dexamethasone (44). Northern blots showed rhythmic *E4BP4* mRNA expression with a peak at 36 h and a trough at 24 h, an anti-phase to *Per2* oscillation and a similar phase to *Bmal1* oscillation (Figure 6A). We then examined the temporal binding of endogenous E4BP4 on the *Per2* promoter by ChIP analysis. The ChIP assay showed that the peak of endogenous E4BP4 binding to the *Per2* promoter almost matched the trough of *Per2* mRNA expression (Figure 6B; see 18 and 36 h). The correlation between the binding activity of E4BP4 and the transcriptional repression of *Per2* suggests that E4BP4 plays an important role as a negative regulator for the circadian expression of *Per2* in the cell-autonomous core clock.

B-site is responsible for the generation of high amplitude in *Per2* oscillation

To clarify the role of E4BP4 binding in *Per2* mRNA oscillation in the circadian clock, we performed real-time reporter assays using mutants of the A- and B-sites. Circadian oscillation of the construct with the wild type of *mPer2* promoter (Wild-dLuc) was obvious (Figure 7A). Superimposing the oscillation profile of the mutant construct of the A-site (A mut-dLuc) on that of the wild type, confirmed that both oscillation profiles were similar, indicating that the A-site is not prerequisite for the circadian expression of *Per2* (Figure 7B). On the other hand, the amplitude of oscillation by mutants of the B-site (B mut-dLuc) and of both the A- and B-sites (A/B mut-dLuc) was lower than that of Wild-dLuc (Figure 7C and D). These findings suggest that the B-site is responsible for generating the high amplitude of *Per2* oscillation.

Binding of E4BP4 to the B-site is required for circadian expression of *Per2*

The E2 enhancer is required for *Per2* oscillation, which is mediated by CLOCK:BMAL1 and it is located 226 bp upstream of the B-site (24,25). To understand the relationship between the E2 enhancer and the B-site for *Per2* oscillation, we performed real-time reporter assays using mutants of the E2 enhancer that lack transcriptional activation by CLOCK:BMAL1 (Supplementary Figure S3) (24). Surprisingly, the mutant of the E2 enhancer (E2 mut-dLuc), which contains both of the intact E4BP4 sites, retained the ability to potently drive the circadian oscillation of *Per2* (Figure 7E), whereas the circadian rhythmicity for the mutant of the E2 enhancer and the B-site (E2/B mut-dLuc) as well as the mutant of the E2 enhancer and the A/B-sites (E2/A/B mut-dLuc) was lost (Figure 7G and H). The mutant of both the E2 enhancer and the A-site (E2/A mut-dLuc) retained a clear oscillatory profile but it was subtly changed as compared with E2 mut-dLuc (Figure 7F). These results strengthened the notion that not only the E2 enhancer, but also the B-site for E4BP4 binding is critical for the circadian expression of *Per2* mRNA in the cell-autonomous core clock.

DISCUSSION

E4BP4 is a mammalian homologue of *vrille* (*vri*) that functions as a key negative component of the *Drosophila* circadian clock (12,26,27). E4BP4 in chickens probably plays an important role in the phase-delaying process as a light-dependent suppressor of *cPer2* (28). E4BP4 is rhythmically expressed in mammals with an anti-phase to *Period1* (*Per1*) oscillation in the liver and the SCN and exogenously expressed E4BP4 directly represses the *Per1* promoter

activity (29). These results indicated that E4BP4 functions as a key negative component of mammalian circadian clocks such as in *Drosophila*. However, no direct evidence has supported this notion until now. This study is the first to demonstrate that endogenous E4BP4 negatively regulates *Per2* transcription in mammals (Figure 1B), which is consistent with findings that E4BP4 represses the transcription of several genes (42,43,45). This was also confirmed in *mPer2* oscillatory transcription (Figure 2A). Figure 2 shows that the modulation of E4BP4 expression remarkably affected the amplitude, but not the period during *mPer2* oscillation. An *mPer2* mutant displays a short-circadian period followed by a loss of circadian rhythmicity in constant darkness (20), and our data also indicated a subtle elongation and shortening of the period induced by down- and up-regulated E4BP4 expression, respectively (Figure 2). The effect upon the period of oscillation might depend on the expression level and a distinguishing change in E4BP4 expression might be required. These results show that E4BP4 is involved in the circadian expression of *Per2*, which is one of the essential components of mammalian circadian clocks.

We identified two putative E4BP4-binding sites on the *Per2* promoter region (Figure 3). Ueda *et al.* described the A-site as an E4BP4-binding site that can oscillate SV40 promoter activity in a similar phase to *Per2* in established cell lines (30). Because the nucleotide sequence around the A-site is highly conserved, the A-site has simply been thought to play an important role. In this study, we showed that the novel E4BP4-binding site, the B-site, is required for the robust circadian expression of *Per2* (Figures 4 and 7). Furthermore, the importance of B-sites for E4BP4-mediated transcriptional repression of *Per2* is confirmed by the DNA-binding activity of E4BP4 *in vitro* and *in vivo* (Figure 5). Different nucleotides at the center of the consensus sequence, that is, 'T' and 'C' on the A- and B-sites, respectively, might explain the preference for the B-site.

Yoo *et al.* have recently identified a circadian enhancer (E2) with a non-canonical 5′-CACGTT-3′ E-box located 20 bp upstream of the *mPer2* transcription start site and demonstrated that a 210 bp promoter region with the E2 enhancer but without the B-site, is sufficient for *Per2* oscillation (24). Here, we performed real-time luciferase assays in NIH3T3 cells using the *mPer2* (−798 to +331) promoter containing the E2 enhancer as well as the novel E4BP4-binding site (B-site). Our findings showed that the novel *cis*-element for E4BP4 binding is required for robust circadian expression of *Per2* in the cell-autonomous core clock as well as the E2 enhancer, indicating that E4BP4 is a key negative regulator of the mammalian circadian clock.

The *Drosophila* circadian oscillator consists of interlocked *period/timeless* and *dClock* transcriptional/translational feedback loops (46–49). Within these loops, VRI negatively regulates *period* expression, which is activated by the dCLOCK:CYCLE complex (CYCLE is also known as dBMAL1), through the repression of *dClock* promoter activity (12,26,27). We showed that E4BP4, which is a mammalian homologue of *vri*, functions as a repressor of *Per2* transcription through the novel E4BP4-binding site (B-site) and that E4BP4 must bind to the B-site for the robust circadian expression of *Per2* in the cell-autonomous clock. These findings demonstrate the importance of negative regulation by the direct binding of E4BP4 as well as of positive regulation by CLOCK:BMAL1 in the mammalian circadian clock. Taken together, VRI/E4BP4 seems to function as a negative factor of *Period* oscillation in the circadian clock of *Drosophila* and mammals.

Missense mutations in clock genes have recently been linked to familial advanced (50,51) and delayed (52) sleep phase syndromes, an abnormality in the circadian timing system that affects the timing of sleep. Here we show the importance of negative regulation by E4BP4 for the circadian expression of *Per2*. As with these clock components, the identification of a nucleotide polymorphism in *E4BP4* should bring new insight into sleep disorders.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Review article

Circadian clock, cancer and lipid metabolism

Norio Ishida [a,b,*]

[a] *Clock Cell Biology, Department of Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), 6-5 Tsukuba Center, 1-1 Higashi, Tsukuba 305-8566, Japan*
[b] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8502, Japan*

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Abstract

Genetic analysis has revealed that mammalian circadian oscillator is driven by a cell autonomous transcription/translation-based negative feedback loop, wherein positive elements (CLOCK and BMAL1) induce the expression of negative regulators (Periods, CRY1 and CRY2) that inhibit the transactivation of positive regulators. Recent research reveals that this clock feedback loop affects many aspects of our physiology, such as cell cycle and lipid metabolism. In this review, I summarize the molecular links between the circadian clock mechanism and the cell cycle, and between the clock and lipid metabolism. Recent studies of clock mutants also suggest that clock molecules play a role as stress sensors. Lastly, we propose the importance of sterol for entraining peripheral clocks.

Keywords: Circadian; Lipid; Cancer

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1. Introduction

The behavior and physiology of most organisms are subject to circadian, 24-h rhythmicity. Circadian oscillators are controlled by negative feedback loops in clock gene expression from bacteria to mammals. CLOCK forms heterodimers with another bHLH-PAS transcription factor, BMAL1, and transactivates other clock genes such as *period1* (*Per1*), *Per2*, *cryptochrome1* (*Cry1*) and *Cry2* via E-box elements in their promoters (Dunlap, 1999; Ishida et al., 1999; Schibler and Sassone-corsi, 2002; Ishida et al., 2001). At least nine clock genes (clock, per1 and 2, Bmal1, cry1 and 2, Tim, casein kinase1 and glycogen synthase kinase 3b) generate circadian rhythms in mammals, where most of these genes were identified as homologues from *Drosophila* clock genes except for Clock (Table 1). Molecules generally of

* Correspondence address: Clock Cell Biology, Department of Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), 6-5 Tsukuba Center, 1-1 Higashi, Tsukuba 305-8566, Japan. Fax: +81 29 861 9499.
E-mail address: n.ishida@aist.go.jp.

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Table 1
A list of clock genes in *Drosophila* and mammals

Drosophila	Mouse
Period	mPeriod1 mPeriod2 mPeriod3
Timeless	ND
Timeout/timeless2	mTimeless
Cryptochrome	mCryptochrome1 mCryptochrome2
Clock(Jrk)	Clock NPAS2/MOP4
Cycle	Bmal1/MOP3 Bmal2/MOP9/CLIF
Doubletime	Casein kinase 1 epsilon Casein kinase 1 delta
Shaggy	GSK3β
Protein phosphatase 2A	Protein phosphatase 2A
Slimb	FWD1 (?)
Vrille	E4BP4

Most clock genes in mammals are identified from homologue to *Drosophila* clock genes except for mouse *Clock* gene. *Drosophila cry* have a role for input pathway in circadian rhythm. ND; not determined for real orthologue.

transcription factors that oscillate over a 24-h period control their own expression in a circadian fashion seem critical to the generation of circadian rhythm of most organisms (Dunlap, 1999; Ishida et al., 1999). However, some kinases that are involved in protein degradation (casein kinase 1) and nuclear translocation (glycogen synthase kinase 3b) are also important for defining the concentration of clock molecules at specific subcellular sites (Ishida et al., 2001; Iitaka et al., 2005).

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus in mammals is the central oscillator that controls approximately 24-h periodicity (circadian rhythms) in behavior and physiology (Schibler and Sassone-corsi, 2002). Neurons in the SCN receive light information via the retinohypothalamic tract and the phase of the circadian clock adapts to photoperiods (Hastings, 1997). Other peripheral tissues are also equipped with endogenous oscillators using clock gene products. In general, the expression of mRNA and proteins of mammalian clock genes including those involved in phosphorylation in the SCN and in other peripheral tissues oscillates in a robust circadian manner (Dunlap, 1999; Ishida et al., 1999; Schibler and Sassone-corsi, 2002) Thus, the oscillation of such clock genes is a useful marker for determining the phase and period of peripheral clocks and the central clock (Oishi et al., 2002; Sakamoto et al., 1998). Only recently the importance of circadian system has become apparent for the regulation of the cell cycle and metabolism because of a convergence of data from microarray analysis using clock mutants and clock gene expression as a marker. I will propose a role of clock gene products for stress sensor considering recent progress of many physiological phenomena from circadian clock.

1.1. Coupling of the circadian clock and the cell cycle

Circadian clock and cell cycles are global regulatory systems found in almost all organisms. Similarly, the both of cycles are periodic for ca. 24 h, and intrinsic to most cells. Circadian cycles of clock gene expression persists in every cell, in contrast the cell cycles where consists of Gap1 (G1), DNA synthesis (S), Gap2 (G2), and mitosis (M) phases are stopped at G1 or G0, which need a specific trigger to start such as liver regeneration (Nyberg et al., 2002). It is clear, further, that in mammals the circadian clock does not depend on cell cycle because adult neuronal cells do not divide yet, they exhibit robust circadian rhythm of gene expression. When cell division is inhibited, rhythmic clock gene expression continue (Nagoshi et al., 2004).

Although circadian and cell cycles consist of distinct molecular mechanisms, a recent review suggests that these cycles are linked in mammals (Lowrey and Takahashi, 2004). Surprisingly, recent two papers implicate the circadian clock in the regulation of cell division cycles because expression of key cell cycle regulators is altered by circadian clock mutations in mammals (Oishi et al., 2003; Matsuo et al., 2003). Matsuo et al. (2003) showed that liver regeneration is impaired in cry-deficient mice and that the expression profiles of cyclin B1, CDC2, cyclin D1 and wee1 differ from those of wild-type mice. The G2/M transition is a crucial point for the cell cycle which is controlled by the Cdc2 kinase/cyclin B complex. Another cell cycle regulator from G2 to M is WEE1. Matsuo et al. concluded that the negative feedback loop of circadian clock genes can initiate the cell cycle in the regenerating liver by regulating wee1 gene expression through a direct binding of CLOCK/Bmal1 to E-box in the promotor of wee1 gene. Oishi et al. (2003) also showed using Affymetrix DNA microarray analysis that hepatic wee1 mRNA is damped in Clock mutant mice whereas mRNA expression remains continuously high in cry-deficient mice. These data indicated that wee1 mRNA expression can be achieved by CLOCK/BMAL1 binding or repressed by CRY at E-boxes even in vivo. This microarray study also demonstrated damped circadian expression of growth-arrest and DNA damage-inducible (GADD) 45a and GADD45b mRNAs in clock mutant mice, but high levels of these mRNAs in cry-deficient mice (Oishi et al., 2003). The main role of *GADD*s is to block cell cycle at G1 and G2 checkpoints in response to DNA damage. The *GADD* genes are up-regulated in response to a variety of stressors including γ-radiation. In response to DNA-damage inducers including UV radiation, γ-radiation, and the alkylating agent methyl methanesulfonate (MMS), mammalian cells can prevent cell cycle progression by controlling critical regulators, the GADD genes. Furthermore, Fu et al. (2002) showed that Cyclin D1 and Gadd45a, both of which are themselves targets of c-myc activation, oscillate in wild type, but exhibit altered circadian rhythm in Per2 mutant mice. Thus, the cyclic expression of GADD45a and GADD45b suggests that peripheral clocks may control the checkpoint of cell cycle in the mouse liver. Taken together, G2/M transition is an target for the gating of circadian clock.

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1.2. Timeless couple circadian with cell cycle

The *Timeless* gene in *Drosophila* is necessary for the generation of circadian rhythms. Although we (Sakamoto et al., 2000) and others (Koike et al., 1998; Sangoram et al., 1998) have identified mammalian Timeless (Tim) by searching the dPERIOD-PAS binding protein from rat brain (the former) or in silico cloning using dTim homology (the latter), the role of TIMELESS protein has remained obscure because Tim knockout is lethal to embryos (Lowrey and Takahashi, 2004). However, Barnes et al. (2003) reported that the down-regulation of full-length mammalian Tim in SCN slices reduces the firing activity of the SCN and affects the expression levels of Per1, Per2, Per3, Cry1 and Cry2. Thus, mammalian Tim might function in the generation of circadian rhythms in the SCN. However, mammalian Tim has higher sequence similarity with drosophila(d)Tim2/dTimeout, which does not function for circadian clock. Studies in *Drosophila* have shown that high levels of dTim2 are expressed at larval stages (Benna et al., 2000). In mammals, mouse Tim appear to function in branching of the embryonic kidneys and lungs because of malformation of these organs in knock out mice, suggesting a developmental role of mTim (Li et al., 2000). Mammalian Tim has structural similarity to many cell-cycle related proteins from other species: budding yeast Tof1 (Park and Sternglanz, 1999) fission yeast DNA damage check points protein Swi1 (Noguchi et al., 2003) and *C. elegans* chromosome cohesion protein TIM-1 (Chan et al., 2003). A recent report by Sancar's group indicates that mammalian Tim is also involved in cell cycle-replication and intra-s check points (Keziban et al., 2005). They found that hTim expression is cell cycle regulated and that hTim binds to the checkpoint proteins Chk1 and ATR-ATRIP complex in a hydroxyurea damage-dependent manner. Furthermore, Tim siRNA application causes entry into mitosis before DNA replication is completed in Hela cells and increases hydroxyurea-induced premature chromatin condensation. These data indicate that Tim plays a role in the regulation of the mammalian cell cycle. Moreover, hTim binds to mCry2 and the down-regulation of Per2 protein by Tim knock-down through its siRNA application in HEK293Tcells suggests that hTim plays a role for the circadian clock. If this is the case, mammalian Tim protein seems to be a missing link protein coupling cell cycle and circadian cycle.

1.3. Light affect circadian clock and cell cycle gene expression

A common feature of clock and cell cycles is their sensing to light. Effect of light appeared to be very important for considering of cell cycle and circadian cycle. Light directly regulates circadian rhythmicity in most zebrafish tissues, because autonomous clocks in cultured cells and tissues have the ability to entrain to light. Hirayama et al. reported that light induces the expression of zebrafish(z)Wee1, which is involved in G2/M transition and the circadian clock genes, zCry1a and zPer2 (Hirayama et al., 2005). They found that the importance of light induced Fos binding to AP-1 sites in promoters of zWee1 and zCry1a genes through chromatin remodeling of histone H3. In zebrafish, light seems to be very important to connect cell cycle players and clock players by inducing FOS. In mammals, AP-1 tansiently inhibits G2/M progression through stimulation of Wee1 via an AP-1 element in its promoter (Kawasaki et al., 2003; Russell and Nurse, 1987). As noted above, Wee1 expression in the mouse liver is also dependent on CLOCK/BMAL1 transactivation through an E-box (Oishi et al., 2003; Matsuo et al., 2003). The similarity indicates that lights effect is quit old system. Even in the flagellate alga *Euglena*, the progression of G2/M is induced by light and its circadian control might be an integral part of the cell division cycle (Hagiwara et al., 2002). From an evolutionary viewpoint, light-induced change of players in both of clock and cell cycles might suggest that the missing molecules of cell and circadian cycle coupling may be a sensor for light. It is clear that these findings between divergent species bring new insights into the evolution of circadian and cell cycles.

1.4. Circadian clock genes and Cancer: a role of clock genes for environmental stress sensors

Although many clinical physicians noticed that chronotherapy is useful for the timing of application for anti-cancer drugs, the molecular mechanism underlying this phenomena is not understand. Surprisingly, recent studies have indicated a role of clock gene products as negative regulators for the generation of cancer. The incidence of radiation-induced lymphoma in Per2 mutant mice is increased and irradiated Per2 mutants die early (Fu et al., 2002). These results were explained by increased c-Myc expression and decreased p53 expression in mice with a Per2 loss-of-function mutation. They also showed that per2 mutants show increased sensitivity to γ-radiation and tumour development. Furthermore, they indicated that increased radio sensitivity in per2 mutants due to a role of clock genes for early responsive genes to γ-radiation, because the dysregulated mRNA induction of clock, cry1 and cry2 was detected in per2 mutant mice. A potential role of Per2 in this lymphoma seems to be as a gamma irradiation-responsive gene.

The notion that clock genes function as stress sensing factors is plausible, since sleep deprivation in *Drosophila* (Shaw et al., 2002) and food deprivation in mice (Kobayashi et al., 2004) alter the expression of circadian clock genes. In *Drosophila*, sleep deprivation in *clock* mutants have been shown to cause an early death that can be avoided by preheating the flies via the induction of heat-shock proteins (Shaw et al., 2002). Depriving mice of food for three days reduces peak levels of Per2 and DBP mRNA expression in the liver and heart and significantly induces Per1 expression in these tissues (Kobayashi et al., 2004). Another report indicated that CLOCK/BMAL1 activity modulates the effects of genotoxic stress such as cyclophosphamide-induced toxicity through altering B cell responses (Gorbacheva et al., 2005). Taken together, these studies suggest that clock genes play roles in stress sensing and releasing.

The circadian system is thought to have evolved as an adaptation to daily changes in the environmental such as light,

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temperature, food and toxic factors. When stress such as γ irradiation or sleep deprivation fails to inform the circadian clock in mouse Per2 mutants or *Drosophila* clock mutants, tumors form and death occur at early ontogeny. Thus, circadian clock gene products have a role as environmental stress sensors.

1.5. Cancer and clock genes—the unusual rhythmic expression of growth factor genes

Surprisingly, recent two papers reported that the unusual rhythmic expression of growth factor genes were observed in implanted tumors in mice. Koyanagi et al. (2003) reported that vascular endothelial growth factors (VEGF) in implanted sarcoma cells are expressed in an unusual high amplitude of circadian manner, in contrast, no significant rhythmic expression of VEGF was detected in normal tissues. Furthermore, the rhythmic expression of VEGF in Sarcoma 180 cells has been positively regulated mainly through the hypoxia-inducible factor, HIF-1a, binding to its promoter, and negatively, through PER2-HIF-1a binding during different circadian time in mice. This also showed a role of PER2 for suppression of tumor generation (Fu et al., 2002) through the down regulation of VEGF gene expression. This is the first study to show that tumor cells use the unusual high amplitude of circadian expression of growth factors as a strategy for growing tumors. The same group also found that the circadian rhythms of the protein and mRNA for methionine aminopeptidase 2, which is involved in the growth of endothelial cells during tumor angiogenesis, are also unusually rhythmic with high amplitude in implanted tumor cells (Nakagawa et al., 2004). Their data suggested that the 24-h rhythm of methionine aminopeptidase 2 activity is regulated by the transcription factors of clock gene products, CLOCK/BMAL1 in the positive limb of clock feedback loop. If this is indeed a strategy through which tumor cells can grow in a normal environment, then it can be applied to the timing of anti-tumor drugs to minimize side-effects and increase drug efficacy as shown by Ohdo group (Koyanagi et al., 2003).

Another group showed different data that the daily ordering of tumor cell clock gene expression might not be correlated to the daily gating of DNA synthesis by breast cancer cells, because cyclin E protein peaks 2 times each day,whereas the Bmal1 gene peaks once a day in cancer cells (You et al., 2005) This group did not find any significant daily rhythms associated with mPer1 and mPer2 gene expression in transplanted syngeneic mammary tumor cells. Mammary epithelial cells might be a different mechanism with sarcoma. But, in vitro, per2-luciferase real-time reporter assay demonstrate the rhythm of clock genes appeared circadian after dexamethasone stimulation in Sarcoma 180 (sarcoma) cells as well as NIH3T3 cells (Ohno, T., Onishi, Y., Hara, Y., Ishida, N., unpublished data). The data indicate that the circadian clock is still working even in sarcoma cells.

Taken together, the coupling between circadian clock genes and cancer appeared to be depending on specific developing times in various tumor tissues in vivo. In our hands, clock mutations seem to affect the susceptibility of normal cells to become tumors because the lack of stress sensing and stress releasing in clock mutants might cause altered response to cell immunity (Oishi et al., 2006c). The elucidation of this connection between the feedback loop of circadian clock genes and abnormal cell cycles in cancer should bring new insights into the mechanisms of cancer and the strategies for its treatment and prevention as well as the development of novel anti-tumor drugs.

1.6. Mouse clock mutants and obesity

One new area of investigation in chronobiology is the relationship between obesity and clock mutation. Clock mutant mice had the Clock allele originally on a BALB/c and C57BL/6J background (Vitaterna et al., 1994). The circadian periods of drinking behavior of homozygous Clock mutant mice on a BALB/c and C57BL/6J background were abnormally long, eventually becoming arrhythmic in constant darkness. A breeding colony was established by further backcrossing to ICR mice, and the new congenic strain was subsequently maintained by interbreeding for 10 generations. Interestingly, *clock* on ICR mice exerted a longer phenotype (28 h period) of behavior and body temperature with different periods in males and females (Oishi et al., 2002; Ochi et al., 2003). Thus, the background strain of *Clock* mutants affects behavioral phenotypes (Vitaterna et al., 1994; Oishi et al., 2002).

Turek's group reported that Clock mutants on a BALB/c and C57BL/6J become obese after 6 weeks of age even after being fed with regular chow (Turek et al., 2005). Furthermore, they showed that appetite regulating peptides, orexin and ghrelin as well as cocaine- and amphetamine-regulated transcript (CART) mRNA in the mediobasal hypothalamus of the brains of clock mutant mice. This finding could explain why the mutant mice have such a large appetite during day and night. However, some questions unanswered in this report. Firstly, orexin and ghrelin play roles in the induction of feeding, whereas CART reduces feeding when centrally administered. Secondly, they showed that serum leptin levels increase during the light phase in Clock mutants, and leptin inhibits food intake. However, the authors showed that food intake in Clock mutants is increased during the light phase (resting time for nocturnal animals) and suggested that this phenotype is one explanation for obesity. Although their data could not explain how obesity and a metabolic syndrome develop in Clock mutant mice, the notion is very important because several genome-wide analyses have already shown that lipid metabolism is altered in circadian clock mutant mice (Schibler and Sassone-corsi, 2002; Lowrey et al., 2004; Oishi et al., 2003, 2005a,b).

In contrast to results in BALB/c and C57BL/6J mice, Oishi et al. (2006a) found that clock mutant mice with ICR background and their sibling controls weigh the same when fed with a normal diet. Furthermore, this ICR clock mutants on a high-fat diet do not gain weight like their sibling controls and orally administered fatty acids are not absorbed from the intestines of clock mutants. These investigators also reported that reduced levels of the cholecystokinin-A receptor and of lipase in the pancreas disrupt fat absorption and thus reduced

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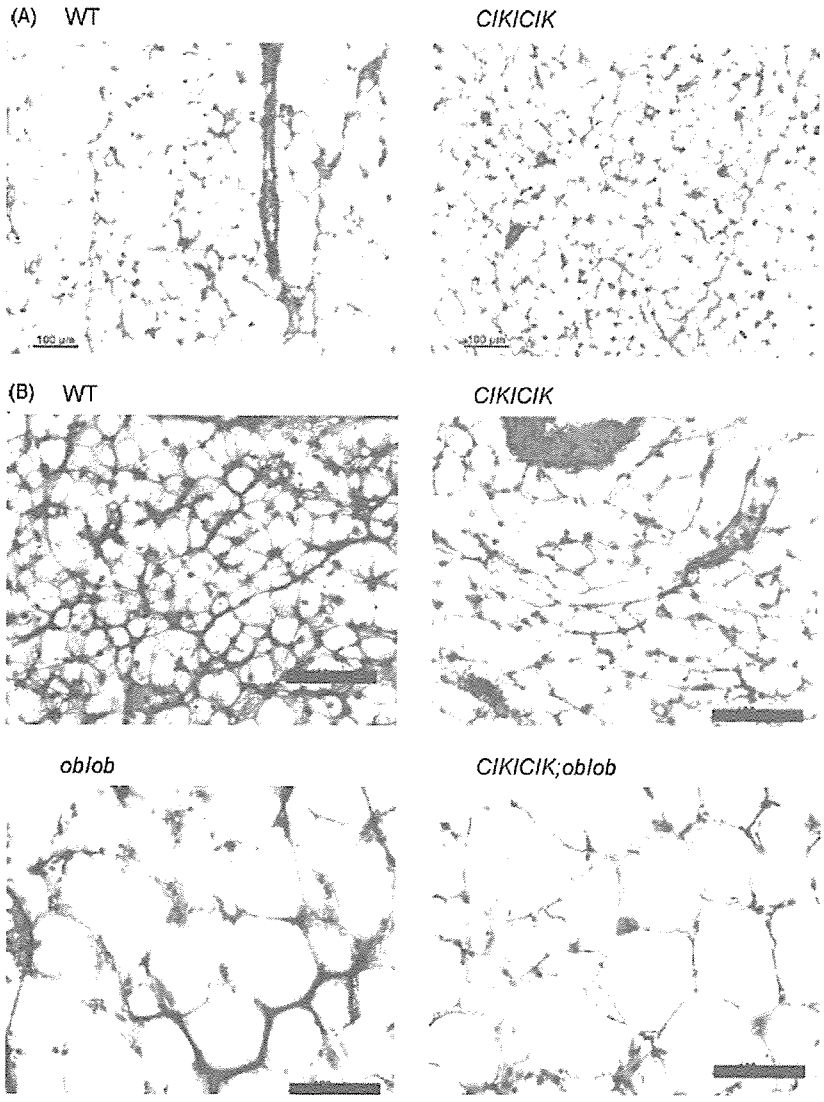

Fig. 1. (A) Cell size in adipose tissue of *Clock* mutant on Jcl mice is smaller than that of the sibling control. Epididymal adipose tissues from male wild-type (WT), homozygous *Clock* mutant (*Clk/Clk*) on Jcl. (B) Enlargement of cell size in adipose tissue of *Clock* mutated leptin-deficient *ob/ob* mice. Epididymal adipose tissues from male wild-type (WT), homozygous *Clock* mutant (*Clk/Clk*), leptin-deficient (*ob/ob*), and *Clk/Clk;ob/ob* mice were stained with hematoxylin and eosin. All four genotypes comes from the brothers after crossing *Clock* mutants on Jcl with *ob/ob* on C57/BL6. Bars indicate 100 μm.

weight induced by a high-fat diet in the ICR Clock mutant mice. One reason for the apparently contradictory effect of the clock gene on obesity might be the background of the clock mutants; the former were C57/BL6 (long and arrhythmic behavior after constant dark), whereas the latter were ICR (long phenotype, 28 h; Vitaterna et al., 1994; Oishi et al., 2002; Ochi et al., 2003). Recent data of the latter ICR Clock mutants crossed with ob/ob mice on C57/BL6 indicated the importance of background. Interestingly, this clock mutated ob/ob mice with mixed backgrounds showed gain weight, high triglycerol and high cholesterol to wild in contrast to the slender phenotype of Clock mutants on ICR (Oishi et al., 2006b,a). Furthermore, this clock mutated ob/ob mice showed a phenotype of adipocyte hypertrophy adding to ob/ob mice, but the ICR Clock mutants showed hypotrophy (Fig. 1). Taken together, these data indicate that normal CLOCK protein are required for the maintenance of homeostatic balance of lipid metabolism.

1.7. The circadian clock and cholesterol metabolism

The circadian peak of blood glucocorticoids is controlled by the SCN via the paraventricular nucleus (PVN) of the hypothalamus, where neurons containing corticotropin-releasing hormone (CRH) regulate the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (Buijs et al., 2003). This pathway is called the hypothalamic–pituitary–adrenal (HPA) axis. A recent study has revealed that light induces gene expression in the adrenal gland via the SCN-sympathetic nervous system (Ishida et al., 2005). Glucocorticoid signaling is thought to be an important time cue for peripheral clocks via

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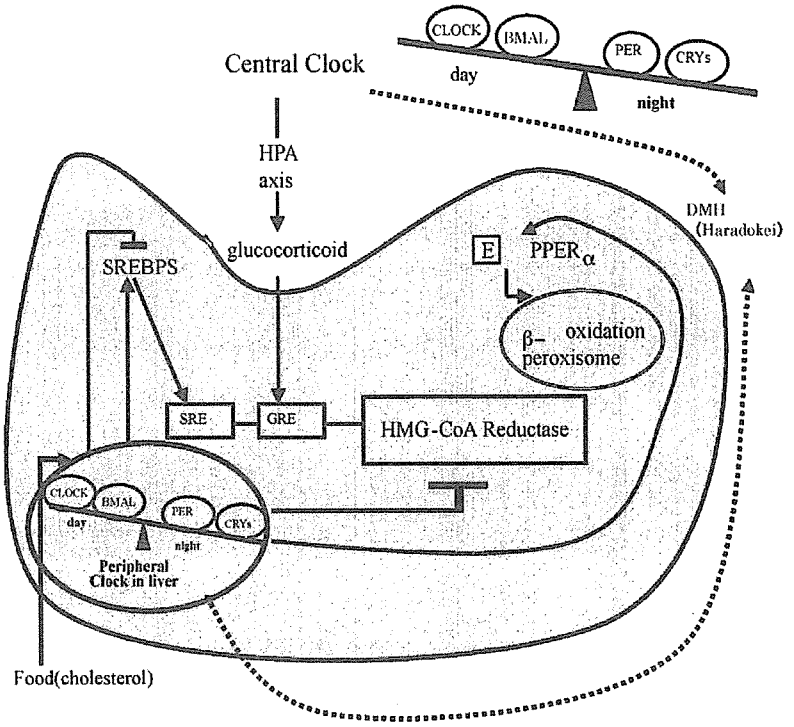

Fig. 2. Model of circadian sterol metabolism in peripheral clocks. Dietary cholesterol inhibit the expression of SREBPs and HMG-CoA reductase through a sterol sensing domain. The clock feedback loop regulate rhythmic PPARa expression through E-boy. SRE; sterol response element, GRE; glucocorticoid response element. DMH; Dorsomedial hypothalamic nucleus. Dashed line means hypothetical. "Haradokei" in Japanese means the phenomena that we can tell about time when we feel hungry without watch. My hypothesis is that peripheral clock can feed back the information to the brain-site like DMH (so-called Haradokei) when food is available.

humoral pathway, the HPA axis or via neuronal pathway, sympathetic nerves from the SCN (Balsalobre et al., 2000).

The key enzyme in the cholesterol biosynthetic pathway is 3-hydroxy-3 methylglutaryl-Coenzyme A (HMG-CoA) reductase, and daily variations in its hepatic activity have been recognized since 1969 (Hamprecht et al., 1969). However, the molecular mechanism of circadian mRNA of HMG-CoA regulation has remained unknown until recently. A DNA microarray study has shown that the adrenal gland is involved in the circadian regulation of HMG-CoA reductase mRNA expression in the mouse liver (Oishi et al., 2005a). Low levels of dietary cholesterol significantly induce HMG-CoA reductase mRNA in the rat liver and adrenal glands (food entrainable peripheral clocks; Jurevics et al., 2000). Such induction might be regulated by the selective binding of sterol regulatory element-binding protein family of transcription factors (SREBPs) to SRE elements or E-boxes in vivo by sensing dietary cholesterol levels. The SREBPs also regulate acetyl-CoA carboxylase, fatty acid synthase and squalene synthase, suggesting roles for SREBPs in cholesterol synthesis as well as fatty acid metabolism (Patel et al., 2001). A sterol-sensing domain in SREBPs is a candidate molecule in the pathway for food-entrainable peripheral clock (Schibler et al., 2003), because HMG-CoA reductase expression does not increase in peroxisome proliferator-activated receptor-a (PPARa)-null mice during feeding (Patel et al., 2001). Exposing mice that are usually nocturnal feeders to restricted feeding during the day shifts the peak of SREBP-1 activation and HMG-CoA reductase expression in the liver (Schibler et al., 2003). These data suggest that cholesterol in food might be a role for entraining peripheral clocks (Fig. 2). Restricted feeding does not affect the central clock SCN (Schibler et al., 2003), but ad libitum feeding during a light:dark reversal protocol seems to be shift the putative central clock more effectively than fasting because behavior reversed effectively (Kobayashi et al., 2004). In the restricted feeding, the central clock which affect behavior might be another site from the SCN, one possible site is dorsomedial hypothalamic nucleus (DMH) which is considered for monitoring food availability (Gooley et al., 2006). Since daily variations in fatty acid synthase and HMG-CoA reductase gene expression are attenuated or abolished in PPARa-null mice (Patel et al., 2001), PPARa seems to play an important role in the food entrainable expression of these sterol-regulated genes. Interestingly, a recent report demonstrated that the circadian clock gene product CLOCK is involved in the circadian expression of PPARa in mice (Oishi et al., 2005b). The molecular mechanism of the transcriptional regulation of HMG-CoA reductase by glucocorticoids will bring new insights into sterol metabolism and clock molecules. Preliminary data from my laboratory indicate that several GRE sites in HMG-CoA reductase gene are important for glucocorticoid induction in liver cells (Kohata, K., Shirai, H., Oishi, K., Ishida, N., in preparation). Fig. 2 summarizes the pathways of HMG-CoA reductase gene expression mentioned above. The expression of HMG-CoA reductase gene are regulated by peripheral clock as well as central clock.

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1.8. Circadian clock and torpor-change of lipid metabolism at hibernation

The tumor suppressor gene product, patched1, has a sterol-sensing domain like HMG-CoA reductase (Borjigin et al., 1999). Patched1 is the mammalian homologue of the *Drosophila hedgehog* receptor and its transcription is strictly regulated in a circadian manner in rat pineal gland. Sterol metabolism in the pineal clock might be an interesting field because this neuroendocrine gland plays an important role in the regulation of seasonal clocks that might correlate with annual lipid cycles in hibernators (Dark, 2005).

A recent breakthrough with torpor and hibernation field by Dr. C.C. Lee's group is that constant darkness like hibernation condition induced 5′-AMP and increased fat catabolic enzyme procolipase in murine blood but not increased in light–dark (LD) cycle condition. Under constant dark for mice, 5′-AMP induced procolipase and torpor. These data suggests that procolipase may have a role for the maintenance of circadian state of brain for hibernation (Zhang et al., 2006). As procolipase induction is disrupted in per1/per2 double mutant mice, this is the first report to link between molecular clock and torpor at substantial level in mammals. The change of mammalian energy source from sugars to lipids during torpor by enterostatin is an old system as well as circadian system. Further study should clarify a role of clock genes for the hibernation and lipid catabolism.

Uncited reference

Miyazaki et al. (2004).

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Photocurrent of nanoassembled Si film in contact with electrolyte

S. Mamykin [a,*], A. Kasuya [a], A. Dmytruk [a], N. Ohuchi [b]

[a] *Center for Interdisciplinary Research, Tohoku University, Sendai 980-8578, Japan*
[b] *Graduate School of Medicine, Tohoku University, Sendai 980-8579, Japan*

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Abstract

Nanoassembled Si thin films on conducting glass have been prepared. Films are photoactive and demonstrate "n-type" behavior in contact with electrolyte. Good correlation has been found between band gap obtained from luminescence and photocurrent. Up to such a small particle sizes the Si is still indirect optical material. Si nanoparticles demonstrate significantly reduced electron affinity 2.4 eV with much higher position of the bottom of conducting band compared to bulk Si while position of valence band did not change.

Keywords: Thin films; Electrode materials; Electronic band structure; Luminescence; Photocurrent

1. Introduction

Porous Si is a material of interest because of fundamental physical properties of quantum sized objects and possible promising applications in Si-based industry. Nanosized crystallites (<5 nm) have electrical and optical properties which are different from the bulk Si. Large specific surface area, increased band gap, luminescence in the visible region, changed electronic structure are basic properties used to design different devices such as solar cells with antireflecting coating [1], gas sensors, cold cathodes, flat panels, super capacitors, etc. [2,3]. It has also been proposed to use porous Si to improve hydrogen generation by p-Si in photoelectrolyses [4].

The usual way is to study porous Si on the Si wafer used for preparation. Unfortunately, in many cases, it is difficult to separate the properties of highly resistive, some times very thin layer of nanosized Si from the bulk material. The purpose of this report is to show a simple way to examine optical and photoelectrochemical properties of nanosized Si without influence of bulk Si. Prepared porous Si was separated from Si wafer and deposited as film on different substrate (conducting glass).

2. Experimental

Si nanoparticles were made by anodic etching of 0.12 Ω cm (1 0 0) p-type Si wafer with following dispersion of particles in toluen:ethanol (2:1) mixture by ultrasonic bath. The piece of Si wafer of rectangular form with 2 cm width was immersed into electrolyte on depth of 5 mm. Anodic current was about 40 mA/cm^2 and processing time was 15 min. HF (46%):H_2O_2 (30%):methanol (97%) 3:3:1 mixture was used as electrolyte. After anodization and before ultrasonic bath, the porous Si was carefully rinsed by methanol. Films were prepared by electrophoretic deposition [6] of Si nanoparticles on conducting glass from suspension. Deposited films were calcinated at 150 °C in N_2 atmosphere. As prepared films have yellow color and demonstrate interference maxima and minima during transmittance measurements suggesting films uniformity. The thickness measured by SEM was about 1 μm (Fig. 1). Spectra of luminescence have been measured by Jusco FP-750 spectrofluorometer. Photoelectrochemical measurements were carried out in 0.1 M H_2SO_4 electrolyte. CHI440 potentiostat and three-electrode cell was used to record photocurrent. All potentials are versus Ag/$AgCl_2$ reference electrode. Thousand Watts Xenon lamp with monochromator was used as source of white and monochromatic light. Actual white light intensity was 60 mW/cm^2.

3. Results and discussion

Freshly prepared (dispersed in toluene:ethanol) Si nanoparticles show luminescence with peak at 2.28 eV (545 nm, Fig. 2). From luminescence maximum, the particle size can be estimated to be around ~2 nm [5]. Excitation spectrum (dashed curve in Fig. 2.) is proportional to absorption and can be used to estimate optical band gap which is about 2.5 eV.

Photoelectrochemical properties of films have been studied in 0.1 M H_2SO_4 electrolyte using three-electrode cell. Working electrode demonstrates different potentials in darkness and under white light (Fig. 3) at the open circuit conditions. This photovoltage is about 0.5 V and shows "n-type" behavior. It suggests more than 0.5 V band bending at the equilibrium. The polarity of the voltage says that photogenerated electrons go to substrate

* Corresponding author. Tel.: +81 22 795 57 58; fax: +81 22 795 57 50.
E-mail address: mamykin@cir.tohoku.ac.jp (S. Mamykin).

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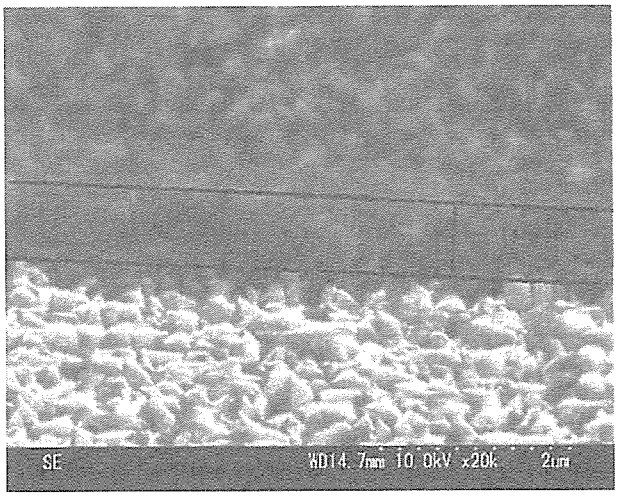

Fig. 1. SEM image of scratched and tilted sample. Si film thickness is about 1 μm.

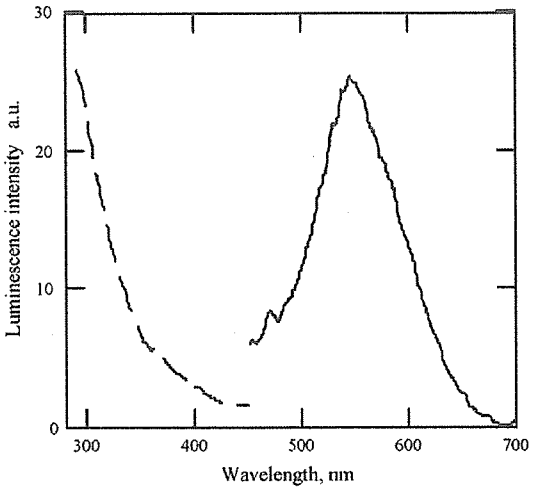

Fig. 2. Emission spectra (solid curve) and excitation (dashed curve) of luminescence of freshly prepared Si nanoparticles in toluene/ethanol mixture.

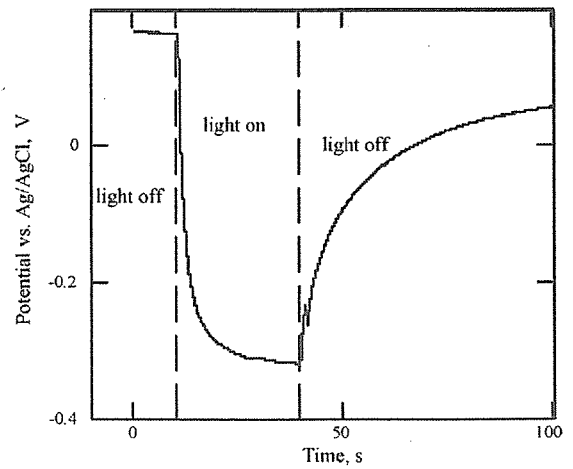

Fig. 3. Photopotential of sample vs. $Ag/AgCl_2$ reference electrode measured in 0.1 M H_2SO_4 electrolyte.

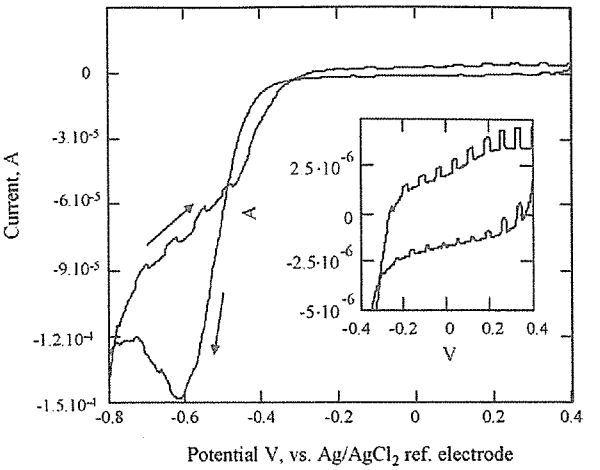

Fig. 4. Cyclic voltammetry under chopped white light of 60 mW/cm^2 intensity. The inset shows enlarged region of small current.

and holes go to contact with electrolyte. So, originally p-type Si was converted to n-type Si nanoparticles. This is in the agreement with high internal resistance of porous Si [2].

Cyclic voltammetry (Fig. 4) measured under chopped light allows to measure photo and dark current simultaneously. Significant dark current at negative potentials was present caused by reaction between substrate (fluorine doped SnO_2) and electrolyte. After dark current subtraction, the photocurrent (Fig. 5) shows three significant regions. The first is from 0.4 to −0.2 V. It increases from negative to positive potential with increasing of electric field in nanoparticles helping to separate photogenerated charge. This photocurrent is relatively small due to lack of hole scavenging at contact with electrolyte. Probably, the main holes consumer is oxidation reaction of Si. Second region is from −0.6 to −0.2 V. The photocurrent significantly increases due to increasing of reaction probability between generated holes and hydrogen. Third region is from −0.6 to negative side of potential. The photocurrent decreases with decreasing of electric field inside of Si particle at more negative potentials and riches zero at

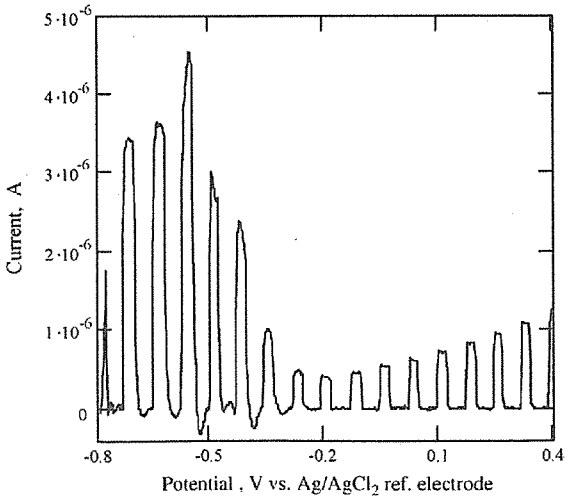

Fig. 5. Photocurrent of Si nanoparticles vs. applied potential.

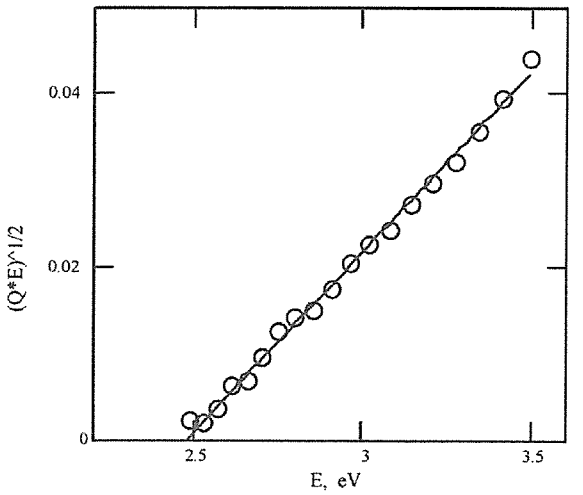

Fig. 6. Spectral dependence of quantum yield (Q) vs. photon energy. Special scale was applied to show indirect optical transitions. Measured optical band gap is 2.5 eV.

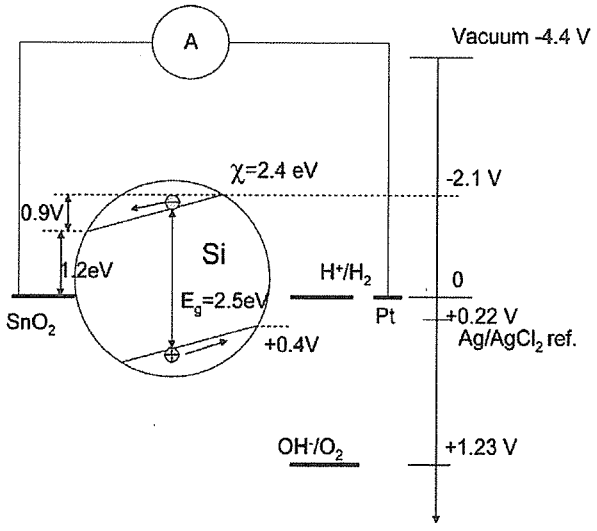

Fig. 7. Bands positions for Si nanoparticles in contact with electrolyte.

flat bands. Estimated flat band potential is about −0.9 V versus $Ag/AgCl_2$ reference electrode.

Spectral dependence of photocurrent was recorded at fixed applied bias (+0.2 V) where short circuit condition is performed. Behavior of the photocurrent quantum yield (Q) versus photon energy (Fig. 6) demonstrates indirect band gap 2.48 eV. This is much higher than band gap of bulk Si (1.12 eV) due to quantum size effect.

On the basis of obtained band gap (2.48 eV) and flat band potential (−0.9 V) it is possible now to estimate the electron affinity χ and band positions of nanosized Si (Fig. 7). Taking into account the low concentrations of free carriers due to trap by surface states [2], the Fermi level is fixed at the middle of band gap. Summarizing, the flat band potential (−0.9 V) and distance between Fermi level and bottom of conduction band (−1.25 V), the position of conduction band against $Ag/AgCl_2$ is found to be −2.15 V. Valence band position can be found by subtracting the value of band gap (2.5 eV) and it is +0.35 V. Electron affinity as distance between bottom of conduction band and vacuum level can be found using potential of $Ag/AgCl_2$ in vacuum scale (+4.6 eV) and it is ~2.4 eV. This is significantly lower then for bulk Si (3.8 eV). It is interesting to mention that valence band position of bulk Si (+0.4 versus $Ag/AgCl_2$) is almost the same as in the case of nanosized Si.

4. Conclusions

Nanoassembled Si films on conducting glass are photoactive and demonstrate "n-type" behavior. Good correlation has been found between band gap obtained from luminescence and photocurrent. Up to such a small particle sizes, the Si is still an indirect optical material. Si nanoparticles demonstrate significantly reduced electron affinity 2.4 eV with much higher position of the bottom of conducting band compared to bulk Si while position of valence band did not change.

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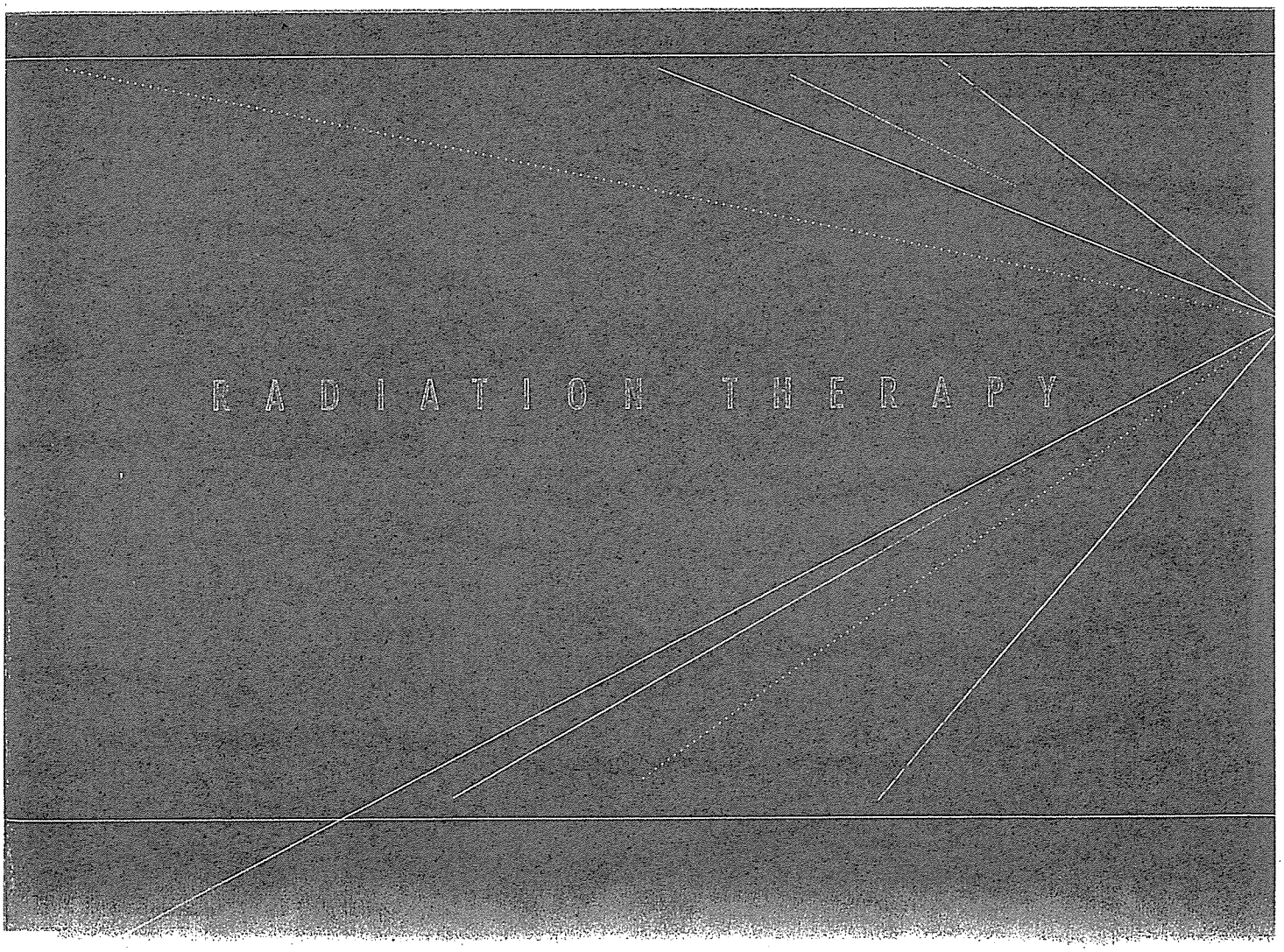
放射線治療

編集 山田章吾



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●執筆者

山田　章吾　東北大学大学院医学系研究科内科病態学講座放射線腫瘍学分野　教授

白土　博樹　北海道大学病院放射線科　助教授

藤田　勝久　北海道大学病院診療支援部放射線部門

石川　正純　北海道大学病院分子追跡放射線医療寄附研究部門　特任助教授

高井　良尋　東北大学医学部保健学科放射線治療技術学分野　教授

三津谷正俊　東北大学病院放射線部　副技師長

奈良崎覚太朗　東北大学病院放射線治療科

村上　昌雄　兵庫県立粒子線医療センター　医療部長

菱川　良夫　兵庫県立粒子線医療センター　院長

辻井　博彦　放射線医学総合研究所重粒子医科学センター　センター長

大内　憲明　東北大学大学院医学系研究科外科病態学講座腫瘍外科学分野　教授

武田　元博　東北大学病院乳腺内分泌外科　助教授

石田　孝宣　東北大学病院乳腺内分泌外科　講師

芝本　雄太　名古屋市立大学大学院医学研究科量子放射線医学分野　教授

林　　靖之　長崎大学医学部歯学部附属病院放射線科

中田　健生　市立札幌病院放射線科

晴山　雅人　札幌医科大学放射線医学　教授

坂田　耕一　札幌医科大学放射線医学　助教授

中村　和正　九州大学大学院医学研究院臨床放射線科学　講師

塩山　善之　九州大学大学院医学研究院臨床放射線科学

大賀　才路　九州大学大学院医学研究院臨床放射線科学

渋谷　　均　東京医科歯科大学大学院医歯学総合研究科腫瘍放射線医学　教授

大西　　洋　山梨大学大学院医学工学総合研究部放射線医学　助教授

淡河恵津世　久留米大学医学部放射線医学教室　講師

角藤　芳久　宮城県立がんセンター放射線科　医療部長

西村　恭昌　近畿大学医学部放射線医学教室放射線腫瘍学部門　教授

根本　建二　山形大学大学院医学系研究科放射線腫瘍学分野　教授

仲田　栄子　東北大学医学部保健学科診療放射線技術科学専攻

小野寺　浩　東北大学病院移植・再建・内視鏡外科