Rhythmic Control of the ARF-MDM2 Pathway by ATF4 Underlies Circadian Accumulation of p53 in Malignant Cells

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Abstract
The sensitivity of cancer cells to chemotherapeutic agents varies according to circadian time. Most chemotherapeutic agents ultimately cause cell death through cell-intrinsic pathways as an indirect consequence of DNA damage. The p53 tumor suppressor gene (TRP53) configures the cell deaths induced by chemotherapeutic agents. In this study, we show that the transcription factor ATF4, a component of the mammalian circadian clock, functions in circadian accumulation of p53 protein in tumor cells. In murine fibroblast tumor cells, ATF4 induced the circadian expression of p19ARF (Cdkn2a). Oscillation of p19ARF interacted in a time-dependent manner with MDM2, a specific ubiquitin ligase of p53, resulting in a rhythmic prevention of its degradation by MDM2. Consequently, the half-life of p53 protein varied in a circadian time-dependent manner without variation in mRNA levels. The p53 protein accumulated during those times when the p19ARF–MDM2 interaction was facilitated. Notably, the ability of the p53 degradation inhibitor nutlin-3 to kill murine fibroblast tumor cells was enhanced when the drug was administered at those times of day during which p53 had accumulated. Taken together, these results suggested that ATF4-mediated regulation of the p19ARF–MDM2 pathway underlies the circadian accumulation of p53 protein in malignant cells. Furthermore, they suggest an explanation for how the sensitivity of cancer cells to chemotherapeutic agents is enhanced at those times of day when p53 protein has accumulated, as a result of circadian processes controlled by ATF4. Cancer Res; 73(8); 1–11. © 2013 AACR.

Introduction
Daily rhythmic variations in biological functions are thought to affect the efficacy and/or toxicity of drugs; the potency of a large number of drugs varies depending on the time of day when the drugs are administered (1). It has been suggested that administering drugs at appropriate times of day can improve the outcome of pharmacotherapy by maximizing potency and minimizing the toxicity of the drug, whereas administering them at inappropriate times of day can induce severe side effects (2). A chronopharmacological strategy can also improve tumor response, overall survival, and dose-limiting toxicity in cancer patients who are subjects in ongoing prospective randomized clinical trials.

Circadian oscillations in biological function are associated with time-dependent changes in the efficacy and/or toxicity of many drugs. Recent molecular studies of the circadian biological clock system have revealed that oscillations in the transcription of specific clock genes play a central role in the generation of circadian rhythms (3–5). In mammals, 24-hour rhythms in different tissues are coordinated by a master clock located in the suprachiasmatic nuclei of the anterior hypothalamus. The master circadian clock follows a daily light/dark cycle and, in turn, synchronizes subsidiary oscillators in other brain regions and many peripheral tissues through neural and/or hormonal signals (6–8). These subsidiary oscillators coordinate a variety of biological processes, producing circadian rhythms in physiology and behavior.

p53 protects cells from uncontrolled growth through activating genes that induce cell-cycle arrest, DNA repair, and apoptotic cell death (9, 10). Because of its role in conserving stability by preventing genome mutation, p53 is known as "the guardian of the genome." Under nonstress conditions, the protein levels of p53 are tightly regulated by the E3 ubiquitin ligase MDM2 at posttranscriptional level (11–13). When cells are exposed to genotoxic stress such as chemotherapeutic treatments, p53 proteins are rapidly accumulated via a decrease in their degradation (14). Although previous reports show that the protein levels of p53 exhibit a significant circadian oscillation in the human oral epithelium and mouse tissues (15, 16), it remains to be clarified whether the oscillation...
of p53 protein levels affects the susceptibility of tumor cells to chemotherapeutic agents.

Activating transcription factor (ATF)/cAMP response element (CRE)-binding (CREB) proteins induce the CRE-mediated gene transcription depending on the cAMP stimulation. Extensive activation of cAMP-dependent signaling is often observed in malignant tumor cells, which probably contributes to abnormal cell proliferation, cAMP-dependent signaling oscillates in a circadian time-dependent manner, which in turn also sustains core oscillation machinery of the circadian clock (17). Among the ATF/CREB family proteins, ATF4 constitutes a molecular link connecting cAMP-dependent signaling to the circadian clock (18). In this study, we showed that ATF4 caused the circadian accumulation of p53 protein in tumor cells. ATF4 repressed the expression of p19ARF in a circadian time-dependent manner; the oscillation of p19ARF resulted in periodic prevention of MDM2-mediated degradation of p53 protein. We thus investigated how the circadian accumulation of p53 protein affects the susceptibility of tumor cells to chemotherapeutic agents.

Materials and Methods

Animals and cells

Male Balb/c Nu/Nu mice were purchased from Charles River. Heterozygous Atf4-null (Atf4+/−) mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). The animals were housed in a temperature-controlled (24 ± 1°C) room under a 12-hour light:12-hour dark cycle. Under the light/dark cycle, zeitgeber time (ZT) 0 was designated as lights on and ZT12 as lights off. The animals were cared in accordance with the guidelines established by the Animal Care and Use Committee of Kyushu University (Fukuoka, Japan).

Mouse fibroblast-like tumor cell lines UV.BAL-5.4G (RCB2025) were provided by RIKEN Bio Resource Center (Tsukuba, Japan). The cells are established by UV irradiation, and were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented 10% FBS at 37°C in a humidified 5% CO2 atmosphere. The tumor cell lines expressed wild-type p53 protein (Supplementary Fig. S1). To assess the circadian oscillation of p53 expression in cultured cells, semi-confluent cultured UV.BAL-5.4G cells were stimulated with 10 nmol/L dexamethasone (DEX) for 2 hours; the medium was then replaced with DMEM supplemented with 2% FBS. Cells were harvested for extraction of protein or RNA at the indicated times after DEX stimulation. Mouse embryonic fibroblasts (MEF) were prepared by standard techniques from embryos of wild-type or Atf4-null (Atf4+/−) mice, and cells were maintained in DMEM supplemented with 10% FBS, 20 μmol/L β-mercaptoethanol, and 1 × nonessential amino acid mix. For oncogenic transformation, MEFs were infected with retroviral vectors expressing H-rasV12 and SV40LT (19).

Balb/c Nu/Nu mice were inoculated with UV.BAL-5.4G (2 × 10⁵ cells/mouse) or oncogenic-transformed MEFs (2.5 × 10⁶ cells/mouse). Tumor volume was measured as described previously (19), and tumor-bearing mice were used for each experiment after the tumor volume reached at 200 mm³.

Determination of p53 protein stability

To determine the stability of p53 protein in cultured UV. BAL-5.4G cells, 50 μg/mL of cycloheximide (CHX) was added to media and cell lysates were prepared at selected time intervals. Cells were lysed in 25 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1% Triton X-100 supplemented with protease inhibitor mixtures and proteasome inhibitor (100 nmol/L; MG132). To investigate the time-dependent difference in the stability of p53 protein in vivo, tumor masses formed by oncogenic wild-type or Atf4+/− cells were removed from tumor-bearing mice at ZT2 and ZT14, and single-cell suspension was prepared by standard methods (19). Cells were incubated in the presence of 50 μg/mL of CHX. After incubation, nuclear fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology), and they were immunoprecipitated with anti-p53 antibodies (PAb246; Calbiochem). The precipitants were analyzed by Western blotting using a polyclonal anti-p53 antibody (sc 6243; Santa Cruz Biotechnology Co., Ltd.).

Western blotting

Cell lysates were prepared from tumor masses or cultured cells were extracted as described earlier. The protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology). The lysate samples were separated on 5%, 10%, or 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were reacted with antibodies against p53 (sc-6243), ATF4 (sc-200), p19ARF (sc-22784), MDM2 (sc-812), or ACTIN (sc-1616). These antibodies were purchased from Santa Cruz Biotechnology. Anti-glutathion S-transferase (GST) antibodies (Wako Co., Ltd.) were used to detect recombinant GST-fused p53 (rgt-p53) protein. The immunocomplexes were further reacted with horseradish peroxidase–conjugated secondary antibodies. The membranes were photographed and the density of each band was analyzed by using LAS4000 mini (Fiji film).

Quantitative RT-PCR analysis

Total RNA was extracted using RNAiso reagent (Takara Co., Ltd.). The complementary DNA (cDNA) was synthesized by reverse transcribing 0.4 μg of RNA using a ReverTra Ace qPCR RT kit (Toyobo). The cDNA equivalent of 12 ng of RNA was amplified by PCR in a real-time PCR system (Applied Biosystems, Life Technologies). Sequences for PCR primers are described in Supplementary Table S1.

Vector construction

Specific silencing of the endogenous Atf4 in tumor cells was achieved by using a shRNA-expressing retrovirus vector. Nucleotides 1114–1132 of the mouse Atf4 (NM_009716) coding sequence were chosen as the target for shRNA. The Atf4-shRNA–encoding oligonucleotides were created as indicated below, each containing the 19-nucleotide target sequence of Atf4, followed by a short spacer and an antisense sequence of the target: 5′-GAGCATTCTTTAGTTAGAGCTCACCCTAAACTAAGGAATGCTC3′. The Atf4-shRNA–encoding sequence was cloned into the BamHI and BglII sites of the pDON-AI2 vector (Takara) and transfected into GST-hi...
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Small interfering RNA

Oncogenic transformed Atf4−/− cells were transfected with siRNA against p19arf (Santa Cruz Biotechnology). This siRNA is designed to prevent the expression of p19ARF, and the downregulation efficacy was confirmed in previous studies (19). On day 7 after oncogene introduction, cells were transfected with siRNA (20 nmol/L) and used for experiments at 48 hours after transfection.

Determination of the binding capacity of MDM2 to p53 protein

Tumor masses formed by oncogenic wild-type or Atf4−/− cells were removed from tumor-bearing mice at ZT2 and ZT14. The masses were homogenized in a lysis buffer as described earlier. Equal amounts of cell lysates (1 mg protein) were precleared with 30 μL of protein G-Sepharose beads. After preclearing, 1 μg anti-p19ARF antibodies (sc-32748; Santa Cruz Biotechnology) and 30 μL protein G-Sepharose beads were added into lysates, and the mixtures were incubated for 12 hours at 4°C. The beads and supernatants were separated by centrifugation. The beads were washed 3 times with washing buffer and subjected to Western blot analysis. After addition of rGST-p53 protein into the supernatants (1 μg/mL), the mixtures were further immunoprecipitated with anti-MDM2 antibodies (sc-965; Santa Cruz Biotechnology) as described earlier. The cell lysates, supernatants, and immunoprecipitations were analyzed by 5%, 10%, or 15% SDS-PAGE followed by immunoblotting with antibodies against p53 (sc-6243), p19ARF (sc-22784), or MDM2 (sc-812).

Determination of cytotoxicity

Tumor masses formed by UV.BAL-5.4G or oncogenic-transformed wild-type and Atf4−/− cells were removed from tumor-bearing mice at ZT2 or ZT14. Single-cell suspensions were prepared by standard methods (19). Cell suspensions were mixed with 100 μmol/L nutlin-3, 500 μmol/L bleomycin, or 1 mmol/L dacarbazin, and thereafter seeded on 24-well plate. At 2 hours after seeding, we confirmed attachment of cells on the well bottom. The culture medium was replaced to drug-free medium, and cells were incubated for indicated times. The dosage of drugs was selected based on previous reports (20–23). A significant antitumor effect of nutlin-3 is observed in mice when they were administered orally with the drug at the dosage of 200 mg/kg (20). Oral administration of the same dosage of nutlin-3 into mice results in peak plasma concentration at 100 μmol/L (21). Similar reason was also applicable in both cases of bleomycin and dacarbazin (22, 23). The viable cells were detected by staining with 5 μmol/L calcein-AM. Cell viability was determined by using a microscope to count viable cells. The number of viable cells just after treatment with drugs (0 hour) was set at 100.

Immunohistochemical analysis

The viable cells were stained with 5 μmol/L calcein-AM. After staining with calcein-AM, cells were fixed with 4% (w/v) paraformaldehyde in phosphate buffer, pH 6.9, and thereafter treated with 10% normal sheep serum for 1 hour. Cells were incubated with the rabbit anti-p53 antibodies (sc-6243; Santa Cruz Biotechnology) at 4°C for 24 hours. After washing with cold PBS, cells were incubated with anti-rabbit IgG Cy3-conjugated antibodies (Sigma) for 12 hours. The viable and p53 positive cells were detected under a 20× objective lens.

Statistical analysis

The statistical significance of differences among groups was determined by ANOVA and post hoc Bonferroni comparisons. A 5% probability was considered significant.

Results

ATF4 regulates the stability of p53 protein through the ARF–MDM2 pathway

We showed previously that the protein levels of p53 were significantly higher in oncogenic Atf4−/− cells than in normal cells (19). To investigate the role of ATF4 in the expression of p53 in malignant tumor cells, the levels of p53 protein and its mRNA were assessed in ATF4-downregulated tumor cells. In this experiment, UV.BAL-5.4G cells were used because of excessive ATF4 expression (data not shown). Infection of UV.BAL-5.4G with retrovirus vector-expressing shRNA targeting Atf4 resulted in a dose-dependent inhibition of ATF4 protein expression (Fig. 1A, top). Downregulation of ATF4 significantly increased the protein levels of p53, but had little effect on its mRNA levels (Fig. 1A, bottom), suggesting that ATF4 regulates protein levels of p53 in tumor cells without induction of its mRNA expression.

To investigate the possibility that ATF4 regulates the expression of p53 protein at posttranscriptional level, we explored the stability of p53 protein in ATF4-downregulated tumor cells. UV.BAL-5.4G cells were infected with Atf4 shRNA- or control shRNA-expressing retrovirus vectors. Then 50 μg/mL of CHX was added to the media, and cell lysates were prepared at selected time intervals. In control shRNA-infected cells, the half-life of p53 protein was approximately 0.65 hours (Fig. 1B). However, the half-life in Atf4 shRNA-infected cells was prolonged about 9-fold (5.9 hours). A deficiency in ATF4 in malignant tumor cells seems to induce the stabilization of p53 protein.

The stability of p53 protein is mainly regulated by its phosphorylation and interaction with specific ubiquitin ligases MDM2, which promote p53 protein ubiquitination and its proteasomal degradation (11–13). Although p19ARF (also known as p14ARF in humans) also stabilizes p53 by sequestering MDM2 (11), the levels of both p19ARF mRNA and its protein were significantly increased in ATF4-downregulated
UV.BAL-5.4G cells \( (P < 0.01; \text{Fig. 1C}) \). These results suggest that p19ARF is involved in the regulation of p53 protein stability by ATF4.

To further evaluate the role of p19ARF in the regulation of the stability of p53 protein by ATF4, we assessed the protein levels of p53 and p19ARF in oncogenic \( \text{Atf4}^{-/-} \) cells. MEFs were prepared from embryos of wild-type or \( \text{Atf4}^{-/-} \) mice, and infected with retroviral vectors expressing H-ras \( V12 \) and SV40LT for oncogenic transformation (19). As compared to wild-type oncogenic cells, the protein levels of both p53 and p19ARF were obviously abundant in oncogenic \( \text{Atf4}^{-/-} \) cells (Fig. 1D). The elevated levels of both p53 and p19ARF proteins in \( \text{Atf4}^{-/-} \) oncogenic cells were decreased by overexpression of ATF4 (Fig. 1E). Furthermore, the levels of p53 protein in \( \text{Atf4}^{-/-} \) oncogenic cells were also decreased by downregulation of p19ARF (Fig. 1F). Taken together, these results suggest that ATF4 affects the stability of p53 protein by repressing the expression of p19ARF.

**ATF4 is required for circadian accumulation of p53 protein in tumor cells**

The expression of p53 protein in the thymuses of mice exhibits circadian oscillation (16). In this study, we also found a significant circadian accumulation of p53 protein in UV.BAL-5.4G tumor cells implanted in mice \( (P < 0.05; \text{Fig. 2A, top}) \). The circadian accumulation of p53 protein was occurred without rhythmic change in its mRNA expression (Fig. 2A, bottom). A similar time-dependent accumulation of p53 protein was also detected in cultured UV.BAL-5.4G cells whose circadian clocks were synchronized by DEX treatment (Fig. 2B), suggesting that the oscillation in the expression of p53 protein is cell autonomous.

ATF4 acts not only as a regulator of p53 protein levels, but also as a component of the mammalian circadian clock (18). To investigate whether ATF4 regulates the circadian accumulation of p53 protein, we assessed the temporal expression profiles of p53 in tumor masses formed by oncogenic wild-
type or Atf4\textsuperscript{-/-} cells. As compared with wild-type tumor cells, the p53 protein levels in Atf4\textsuperscript{-/-} tumor cells increased at both ZT2 and ZT14, but the protein levels did not show a significant time-dependent variation (Fig. 3A). Because there was no significant time-dependent oscillation of p53 mRNA levels in both wild-type and Atf4\textsuperscript{-/-} tumor cells (Supplementary Fig. S2), ATF4 seems to regulate the circadian accumulation of p53 protein at posttranscriptional level.

To test this possibility, we explored whether ATF4 induces a circadian change in the stability of p53 protein in tumor cells. As shown in Fig. 3A, the levels of p53 protein in wild-type tumor cells prepared at ZT2 were low. We thus conducted immunoprecipitation by using 2 types of anti-p53 antibodies to detect low levels of p53 protein. In the wild-type tumor cells, the half-life of p53 proteins varied depending on the time of their preparation. The half-life of p53 protein in wild-type tumor cells prepared at ZT2 was much shorter than that of p53 protein in cells prepared at ZT14 (Fig. 3B). In contrast, the half-life of p53 protein in Atf4\textsuperscript{-/-} tumor cells was considerably longer than that from wild-type cells, but the time of preparation did not cause significant differences in the half-life of p53 protein in Atf4\textsuperscript{-/-} tumor cells (Fig. 3B). These results suggest that circadian accumulation of p53 protein in wild-type tumor cells is associated with the time-dependent change in its stability; the circadian change in that stability is caused by ATF4.

**ATF4 regulates the circadian change in the p53 protein stability through the p19ARF–MDM2 pathway**

MDM2 protein in UV.BAL-5.4G tumor masses was constantly expressed throughout the day, whereas the levels of both ATF4 and p19ARF proteins in the tumor masses exhibited obvious circadian oscillations (Fig. 4A, top). The accumulation of p19ARF varied almost inversely with ATF4 protein expression. The levels of p19ARF mRNA in UV.BAL-5.4G tumor masses showed a significant circadian oscillation, with higher levels from the late light phase to the early dark phase ($P < 0.05$; Fig. 4A, bottom). Similar significant time-dependent expression of p19ARF was also detected in wild-type tumors ($P < 0.05$; Fig. 4B). However, neither p19ARF mRNA nor protein showed significant time-dependent oscillation in the Atf4\textsuperscript{-/-} cell tumor masses. The protein levels of p19ARF was elevated at both time points, suggesting that constant elevation of p19ARF in Atf4\textsuperscript{-/-} cells may prevent the circadian change in the MDM2 binding to p53 protein.

MDM2 promotes the degradation of p53 protein by functioning as an E3 ubiquitin ligase. The ubiquitination occurs through binding of MDM2 to the transactivation domain of the p53 protein (11–13). To investigate the circadian time dependency of the binding ability of MDM2 to p53 protein, we conducted immunoprecipitation experiment as shown in Fig. 4C. The protein levels of p53 in wild-type tumor cells prepared at ZT14 were approximately 4-fold higher than those in wild-type cells prepared at ZT2 (Fig. 4C). As a result, the half-life of p53 protein in wild-type tumor cells prepared at ZT2 was much shorter than that at ZT14 (Fig. 4C). In contrast, the half-life of p53 protein in Atf4\textsuperscript{-/-} tumor cells was considerably longer than that from wild-type cells, but the time of preparation did not cause significant differences in the half-life of p53 protein in Atf4\textsuperscript{-/-} tumor cells (Fig. 4C). These results suggest that circadian accumulation of p53 protein in wild-type tumor cells is associated with the time-dependent change in its stability; the circadian change in that stability is caused by ATF4.
at ZT2 (Fig. 3A). Such large difference in p53 contents could hinder accurate comparison of the binding capacity of MDM2 to p53 between the 2 times. We thus added constant amount of rGST-p53 protein in tumor cell lysates and evaluated the binding capacity of MDM2 to exogenous p53 protein. The results of immunoprecipitation analysis revealed that MDM2 protein in wild-type tumor cells precipitated together with p19ARF in a circadian time-dependent manner (Fig. 4C, IP: p19ARF). The amount of MDM2 associated with p19ARF was greater at ZT14 compared with that at ZT2. Consequently, much amount of p19ARF-unbound MDM2 remained in supernatants prepared from ZT2 of wild-type tumor cell lysates (Fig. 4C, Supernatant A). The p19ARF-unbound MDM2 in wild-type tumor cells precipitated together with rGST-p53 in a circadian time-dependent manner (Fig. 4C, IP: MDM2). The amount of rGST-p53 associated with MDM2 was greater at ZT2 compared to that at ZT14.

In Atf4−/− tumor cells, most of p19ARF in Atf4−/− tumor cell lysates precipitated with anti-p19ARF antibody, but substantial amount of p19ARF-unbound MDM2 remained in the supernatants (Fig. 4C, Supernatant A). The p19ARF-unbound MDM2 in the supernatants prepared from Atf4−/− cells could be associated with rGST-p53, but the association between MDM2 and rGST-p53 did not exhibit the time-dependent variation (Fig. 4C, IP: MDM2). These results suggest that the time-dependent interaction between p19ARF and MDM2 underlies circadian change in the binding capacity of MDM2 to p53 protein. Constant interaction between p19ARF and MDM2 in Atf4−/− tumor cells may prevent the circadian oscillation of MDM2 binding to p53 protein.

ATF4 forms heterodimers with C/EBPa that act to repress p19Arf gene transcription through the C/EBP-ATF response element (CARE; ref. 19). The CARE sequence is located between −6263 and −6255 with respect to the transcriptional start site of the p19Arf gene. Chromatin immunoprecipitation analysis revealed that the amount of ATF4 binding to CARE within the p19Arf 5′-flanking region varied time dependently (Supplementary Fig. S3). These results suggest that ATF4 periodically suppresses the expression of p19Arf mRNA, thereby causing the circadian oscillation in its protein levels.

Dosing time-dependent change in the cytotoxicity of chemotherapeutic agents

Because the p53 protein levels in UV.BAL-5.4G and oncogenic wild-type tumor cells oscillated in a circadian time-dependent manner, we investigated whether that oscillation affects the cytotoxic effect of chemotherapeutic agents. Several studies have showed that the dosing time-dependent change in the cytotoxicity of chemotherapeutic agents is associated not only with circadian oscillation in the sensitivity of tumor cells to the drugs but also with their pharmacokinetics (24–25). To exclude the influence of circadian change in the distribution of drugs into the tumor cells, single-cell suspensions were prepared from UV.BAL-5.4G tumor masses at ZT2 and ZT14 (Fig. 5A). Cells were treated with chemotherapeutic agents, and p53 protein levels and cell viability were determined at indicated times.

At 8 hours after treatment with nutlin-3, a potent inhibitor of p53 degradation, the number of p53-positive cells and their signal intensity were obviously increased, but the...
accumulation of p53 protein was greater when cells were treated with nutlin-3 at ZT14 (Fig. 5B). Consistent with the dosing time dependency of p53 accumulation, induction in the expression of p53 target genes, Bax, p21, Noxa, and Puma was also significantly enhanced in cells treated with nutlin-3 at ZT14 (\(P < 0.05\), respectively; Fig. 5C). These findings suggest that the growth-suppressive properties of p53 are changed according to the dosing time of nutlin-3. In fact, the cytotoxic effect of nutlin-3 on UV BAL-5.4G tumor cells also varied significantly depending on the time when the cells were treated (Fig. 5D, left). Similar dosing time dependency was also found in tumor cells treated with genotoxic drugs, bleomycin and dacarbazine (Fig. 5D, middle and right panels). The cytotoxic effect of these drugs was enhanced at the time of day when p53 protein is abundant.

In the final set of experiment, we investigated whether ATF4 was involved in the dosing time-dependent change in the cytotoxic effect of nutlin-3. To this end, cell viability was assessed in nutlin-3–treated wild-type and Atf4/−/− cells implanted in mice. Significant dosing time-dependent difference in the cytotoxic effect of nutrient-3 was found when the drug was applied to cells prepared from wild-type tumor masses (\(P < 0.01\); Fig. 6). Conversely, there was no significant dosing time-dependent difference in the
The cytotoxic effect of nutlin-3 on oncogenic Atf4−/− cells. Furthermore, the viability of nutlin-3–treated Atf4−/− cells was more rapidly decreased as compared with wild-type cells. Because the number of p53-positive cells and their signal intensity in nutlin-3–treated Atf4−/− cells were greater than those in wild-type cells (Fig. 6), a potent accumulation of p53 protein may induce the severe cytotoxic effect of nutlin-3 on Atf4−/− tumor cells at both time points.
Regulation of Circadian Degradation of p53 Protein by ATF4

Figure 6. ATF4 is required for circadian change in the sensitivity of tumor cells to nutlin-3. Single tumor cell suspensions were prepared at ZT2 or ZT14 from oncogenic wild-type or Atf4−/− cells implanted in mice. Cells were treated with 100 μmol/L nutlin-3 as shown in Fig. 5A. For plots of cell survival rate, the mean basal value (0 hour) of each group is set at 100. Each value represents the mean ± SEM (n = 3). **P < 0.01; *P < 0.05; comparison is between groups. The microscopic photographs show the temporal changes in the nutlin-3–induced accumulation of p53 protein in oncogenic wild-type (top) and Atf4−/− cells (bottom). Double-labeled cells (either yellow or red nucleus surrounded by green cytoplasm) indicate the p53-positive cells.

Discussion

The molecular circadian clock operates at a cellular level and governs a wide variety of physiologic processes. This study suggests that this clockwork governs the time-dependent accumulation of p53 protein in tumor cells. In mammals, circadian rhythmicity in various biological processes is controlled by a molecular pacemaker composed of clock gene products (26–28). These gene products constitute an oscillating mechanism based on self-sustained transcriptional/translational feedback loops. The Clock gene encodes the CLOCK transcription factor, which dimerizes with BMAL1 to activate the transcription of Period (Per) and Cryptochrome (Cry) genes via E-box or E-box-like enhancer sequences (4, 29). Once PER and CRY proteins have reached critical concentrations, they attenuate CLOCK/BMAL1 transactivation, thereby generating circadian oscillation in their own transcription. In addition, CLOCK/BMAL1, PER, and CRY proteins regulate the expression of ATF4, which in turn sustains the transcriptional oscillation of Bmal1, Per2, and Cry1 gene (18). This mechanism interconnects the positive and negative limbs of circadian clockwork circuitry and also regulates 24-hour variation in output physiology through the periodic activation/repression of clock-controlled output genes (30). ATF4 also acts as an output component of the circadian clock and regulates the rhythmic expression of its target genes through the CRE (31).

In murine fibroblast tumor cells, ATF4 functioned as a circadian regulator of p19ARF expression, causing a time-dependent interaction between p19ARF and MDM2, which resulted in periodic prevention of MDM2–mediated p53 degradation. Consequently, the half-life of p53 protein varied in a circadian time-dependent manner; the proteins accumulated during those times of day when p19ARF–MDM2 interaction was facilitated (Fig. 7). In contrast, the p53 mRNA levels in tumor cells failed to exhibit a significant circadian oscillation, suggesting that the molecular clock regulates the circadian expression of p53 protein at posttranscriptional level. In fact, the abundance of p53 protein in Atf4−/− tumor cells increased consistently throughout the day without a corresponding increase in mRNA levels.

In this study, we used retrovirus vectors expressing SV40LT as a transforming agent, together with H-rasV12. Because SV40LT inactivates p53 and prevents the phosphorylation of Rb by protein–protein interaction (32, 33), inactivation of the growth-suppressive properties of p53 has been shown to be essential for immortalization of MEFs by SV40LT. In fact, p53 protein in oncogene-introduced wild-type cells was precipitated together with SV40LT (Supplementary Fig. S4). However, substantial amount of SV40LT-unbound p53 protein still existed in wild-type cells, suggesting that extensive expression of p53 in oncogene-introduced cells overrides the binding capacity of SV40LT. Concomitant introduced H-rasV12 may also enhance the accumulation of p53 protein through DNA damage response. The SV40LT-unbound p53 protein in wild-type cells seemed to be involved in the circadian regulation of tumor cell sensitivity to chemotherapeutic agents.

Because cells deficient in p53 exhibit resistance to chemotherapeutic agents (34, 35), we explored whether the oscillation of p53 protein levels affects the susceptibility of tumor cells to chemotherapeutic agents. Nutlin is a cis-imidazoline analog that mimics 3 residues (Phe19, Trp23, and Leu26) in the helical transactivation domain, residues that are conserved across species and that are critical for binding to...
of conventional chemotherapeutic agents, bleomycin and decarbazine, were also enhanced by applying the drugs at the times of day during which accumulation of p53 protein was facilitated in tumor cells. Because there was no significant dosing time-dependent difference in the cytotoxic effect of nutlin-3 on oncogenic Atf4–/– cells, Atf4-mediated regulation of the ARF–MDM2 pathway could cause a circadian accumulation of p53 protein, thereby causing the time-dependent change in the susceptibility to chemotherapeutic agents.

The amount of p53 protein in oncogene-introduced Atf4–/– cells not only failed to show circadian variation, but also was greater than that in wild-type cells. This may be because of a decrease in the degradation of p53 through MDM2-mediated proteasomal pathway. Although the protein levels of p19ARF were elevated in Atf4–/– cells, MDM2 was not completely entrapped by p19ARF. The p19ARF-unbound MDM2 in Atf4–/– cells still had a capacity to bind to p53 protein. Nutlin-3 seemed to prevent the interaction between p19ARF-unbound MDM2 and p53 protein, resulting in severe cytotoxic effect on Atf4–/– cells.

The effectiveness and toxicity of chemotherapeutic agents vary with the dosing time. However, many drugs are still administered without regard to the time of day. Identification of the mechanism underlying the dosing time dependency of chemotherapeutic agents should help achieve better chronopharmacotherapy for treatment of cancers.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: M. Horiguchi, S. Koyanagi, S. Ohdo

Development of methodology: M. Horiguchi, S. Koyanagi, N. Matsunaga

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Horiguchi, S. Koyanagi, K. Kakimoto, N. Matsunaga

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Writing, review, and/or revision of the manuscript: M. Horiguchi, S. Koyanagi, S. Ohdo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. M. Hamdan

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### References


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Rhythmic Control of the ARF-MDM2 Pathway by ATF4 Underlies Circadian Accumulation of p53 in Malignant Cells


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