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# Modulation of Circadian Rhythm of DNA Synthesis in Tumor Cells by Inhibiting Platelet-Derived Growth Factor Signaling

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**Abstract.** Circadian synchronization of cell proliferation is observed not only in normal healthy tissues but also in malignant solid tumors. However, the proliferation rhythm of tumor cells is often different from that of normal cells. We reported here that the peculiar rhythm of tumor cell proliferation was modulated by inhibition of platelet-derived growth factor (PDGF) signaling. DNA synthesis in tumor cells implanted in mice showed a 24-h oscillation apparently differing from that of normal bone marrow cells. Continuous administration of AG1295 (10 $\mu$g/h, s.c.), a PDGF receptor tyrosine kinase inhibitor, substantially suppressed DNA synthesis in the implanted tumor cells but not in the healthy bone marrow cells. During the administration of this drug, the rhythm of DNA synthesis in the tumor cells was synchronized with that in bone marrow cells. The present results suggest that the circadian rhythm of DNA synthesis in tumor cells is modulated by PDGF receptor signaling, which is activated following tumor progression. Because the rhythmic patterns of clock gene expression in tumor cells did not differ significantly from those in other healthy tissues, the enhanced signal transduction of PDGF receptor may cause an alteration in the rhythmicity of tumor cell proliferation without changing in the intracellular molecular clockwork.

***Keywords***: platelet-derived growth factor (PDGF), tumor progression, DNA synthesis, circadian rhythm, clock gene

## Introduction

The proliferation of cells in many mammalian tissues frequently occurs during a certain time of day. Synchronous progression through each stage of the cell cycle is implicated as the cause of the circadian variation in cell proliferation (1). Recent studies have suggested that intracellular molecular clockwork determines the daily time windows during which cells can traverse certain stages of the cell cycle (2). The cyclically expressed clock gene products control the timing of cell cycle progression by regulating the expression of cell cycle regulatory genes (2, 3). The circadian synchronization of cell cycle progression is observed not only in healthy normal tissues but also in malignant tumors. However, the proliferation rhythm of tumor cells is often different from that of non-tumor cells (4 – 6). Several possible mechanisms have been proposed to account for this phenomenon. It has been suggested that some aspect of tumor progression alters the intracellular molecular clock function (7, 8). Alternatively, potent mitogenic stimuli may enhance the growth of tumor cells, thus modulating the timing of their proliferation.

In solid tumors, platelet-derived growth factor (PDGF) signaling participates in various processes of tumor progression, including autocrine stimulation of tumor cell growth, recruitment of tumor stroma fibroblast, and induction of tumor angiogenesis (9). We

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reported previously that tyrosine kinase activity of the PDGF receptors on sarcoma tumor cells is fluctuated rhythmically with a period length of about 24 h (10). In this study, we investigated the possibility that PDGF signaling affects the circadian rhythm of tumor cell proliferation.

## Materials and Methods

*Materials*

AG1295 a PDGF-receptor tyrosine kinase inhibitor, was purchased from Calbiochem (San Diego, CA, USA). The compounds were dissolved in a sterilized saline and continuously administered to the tumor-bearing mice. 5-Bromo-2'-deoxyuridine (BrdU) was purchased from Wako Chemicals Inc., Ltd. (Osaka). Each compound was dissolved in sterile saline.

*Animals and cells*

Male ICR mice (5-week-old) were purchased from Charles River Japan, Inc. (Kanagawa). They were housed under a standardized light/dark cycle. Under the light/dark cycle, zeitgeber time (ZT) 0 was designated as lights on and ZT12 as lights off. Sarcoma 180 tumor cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai). The tumor cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. A 50-$\mu$l volume of $1.5 \times 10^6$ viable tumor cells was inoculated into the right hind footpads of each mouse. After the tumor size reached approximately 200 $mm^3$, the tumor-bearing mice were used for each experiment. An osmotic minipump (model 2001; ALZET, Palo Alto, CA, USA) was implanted under the dorsal hypodermic region of tumor-bearing mice and used for continuous administration of AG1295 (10 $\mu$g/h, s.c.) or saline. The dosage was selected based on a preliminary study. The animals were treated in accordance with the guidelines stipulated by the animal care and use committee of Kyushu University.

*Immunoprecipitation and immunoblot analysis*

Tumor masses were removed at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22 on day 5 after the initiation of treatment with AG1295 or saline. Tumor masses were homogenized with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 2 mM PMSF, and 1 mM $Na_3VO_4$). After centrifugation, the supernatants were treated with protein A-Sepharose beads to remove nonspecific binding proteins and then incubated at 4°C for 12 h with antibodies against either $\alpha$-PDGF receptor or $\beta$-PDGF receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) plus protein A-Sepharose beads. The immune complex-bound beads were washed with PBS three times, mixed with sample buffer, boiled, and centrifugated. The immune complexes in the supernatants were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The transferred proteins were reacted with antibodies against phospho-Tyrosine (p-Try, Santa Cruz Biotechnology) or against PDGF receptors. Specific antigen/antibody complexes were made visible by using horseradish peroxidase–conjugated secondary antibodies and Super Signal Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA).

*Immunohistochemical analysis*

To determine DNA synthesis, tumor-bearing mice received a single i.p. injection of BrdU (100 mg/kg) at six different time point as outlined above. Tumor masses were removed at 1 h after BrdU injection and fixed for 4 h with 4% paraformaldehyde in 0.1 M phosphate buffer. The fixed tumor masses were frozen and sectioned at 30-$\mu$m thickness. Suspensions of bone marrow cells were also prepared from femurs of the tumor-bearing mice. The cells were smeared onto microscope slides and fixed for 20 min with 70% ethanol. Detection of BrdU-positive cells was performed by immunofluorescence using FITC-conjugated anti-BrdU antibodies (Oxford Biotechnology Ltd., Oxford, UK). The total number of cells in tumor masses and bone marrow were counted in the bright field after haematoxylin staining. The percentage of BrdU-positive cells was assessed by counting the numbers of positive cells among at least 300 cells from each sample.

*Quantitative RT-PCR analysis*

Total RNA was extracted from tumor masses and bone marrow by using TRIzol reagent (Invitrogen, Carsbad, CA, USA). The cDNA of cell cycle regulatory genes [Cyclin E1, Cyclin A2, and cyclin-dependent kinase (CDK) 2], circadian clock genes (*Clock*, *Bmal1*, *Per*, *Cry*), and glyceraldehydes-3-phosphate dehydrogenase *(GAPDH)* were synthesized and amplified by using a superscript one-step RT-PCR system (Invitrogen). This RT-PCR reaction was performed using 100 pmol each of the forward and reverse primers (Table 1). To evaluate the quantitative reliability of this method, kinetic analysis of amplified products was performed to ensure that signals were derived only from the exponential phase of amplifications as described previously (11). The ratios of the amplified target to the amplified internal control (calculated by dividing the value of each cell cycle regulatory gene or clock gene by that of GAPDH) were compared among groups.

**Table 1.** Primers used for detection of the genes

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *Cyclin E1* | 5'-TGAGCT TGA ATA CCCTAGGA-3' | 5'-TCTAGAAGCTCTGCGATCTG-3' |
| *Cyclin A2* | 5'-GTCCTTCATGGAAAGCAGTC-3' | 5'-CTAGCAGCATAGCAGCCGT-3' |
| *CDK2* | 5'-AGGTGGAGAAGATTGGAGAG-3' | 5'-TCCTTCTCAGTAATGACTGA-3' |
| *Clock* | 5'-AAGATTCTGGGTCTGACAAT-3' | 5'-TTGCAGCTTGAGACATCGCT-3' |
| *Bmal1* | 5'-TATCACACTACGAAGTCGAT-3' | 5'-ATCTATCATATCGATGCCTA-3' |
| *Per1* | 5'-CCAGGCCCGGAGAACCTTTTT-3' | 5'-CGAAGTTTGAGCTCCCGAAGTG-3' |
| *Per2* | 5'-ACACCACCCCTTACAAGCTTC-3' | 5'-CGCTGGATGATGTCTGGCTC-3' |
| *Cry1* | 5'-TGATACAGATGGCCTGTCCT-3' | 5'-GTCATGATGGCGTCAATCCA-3' |
| *Cry2* | 5'-GTTGCCAACTATGAGAGACCT-3' | 5'-CGTTCAATGTTGAGCCGACTA-3' |
| *GAPDH* | 5'-GACCTCAACTACATGGTCTACA-3' | 5'-ACTCCACGACATACTCAGCAC-3' |

*Statistical analysis*

The significance of the 24-h variation in each parameter was tested by analysis of variance (ANOVA). The statistical significance of differences among groups was analyzed by ANOVA. A 5% level of probability was considered to be significant.

## Results

*Influence of continuous administration of AG1295 on the phosphorylation of PDGF receptors on tumor cells*

In the saline-treated mice (control), the amount of tyrosine phosphorylation in $\alpha$- and $\beta$-PDGF receptors on tumor cells, which had been implanted in mice, increased during the light phase and decreased during the dark phase (Fig. 1). The fluctuation in the phosphorylation of PDGF receptors was consistent with previous observations (10). Continuous administration of the tyrosine kinase inhibitor AG1295 (10 $\mu$g/h, s.c.) not only suppressed the phosphorylation of PDGF receptors in the implanted tumor cells, but also damped the fluctuation in their phosphorylation (Fig. 1).

*Influence of continuous administration of AG1295 on the 24-h variation in DNA synthesis in tumor cells*

To explore the role of PDGF receptor signaling in the circadian control of tumor cell proliferation, we investigated the temporal profile of DNA synthesis in the implanted tumor cells during the administration of AG1295. Because the rhythmic phase of DNA synthesis in tumor cells is often different from that of cells in healthy normal tissues such as bone marrow (4, 5), we compared the temporal profile of DNA synthesis in tumor cells with those in bone marrow cells. Both tumor and bone marrow cells from saline-treated mice showed significant 24-h variations in DNA synthesis ($P$<0.05, respectively; Fig. 2, left panel). However, the DNA synthesis rhythm in tumor cells was different from that in bone marrow cells. The number of BrdU-positive bone marrow cells increased from late dark phase to early light phase, whereas the number of BrdU-positive tumor cells peaked around the early dark phase. This

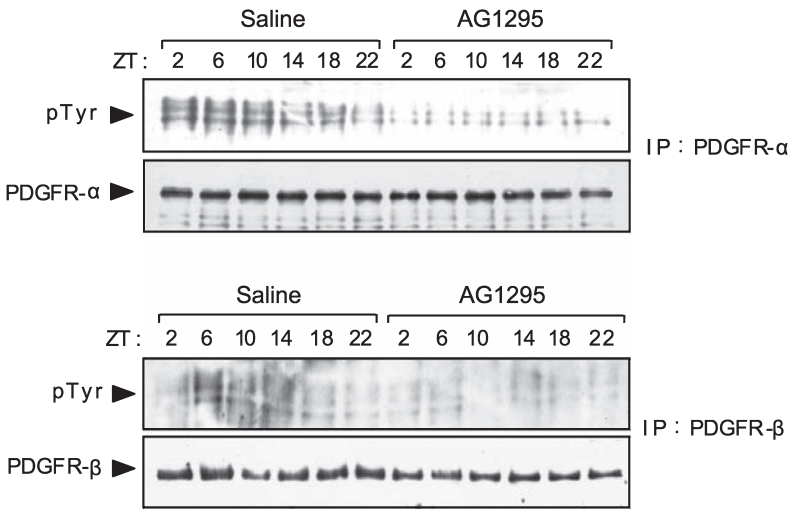


**Fig. 1.** Continuous administration of AG1295 (10 $\mu$g/h, s.c.) inhibits the circadian oscillations in the phosphorylation of PDGF receptors ($\alpha$-PDGF-R and $\beta$-PDGF-R) on sarcoma 180 tumor cells. Whole cell lysates were immunoprecipitated (IP) with antibodies against $\alpha$-PDGF-R and $\beta$-PDGF-R, and the antigens were separated by SDS-polyacrylamide gel electrophoresis. The transferred proteins were reacted with antibodies against phosphorylated tyrosine (pTyr).

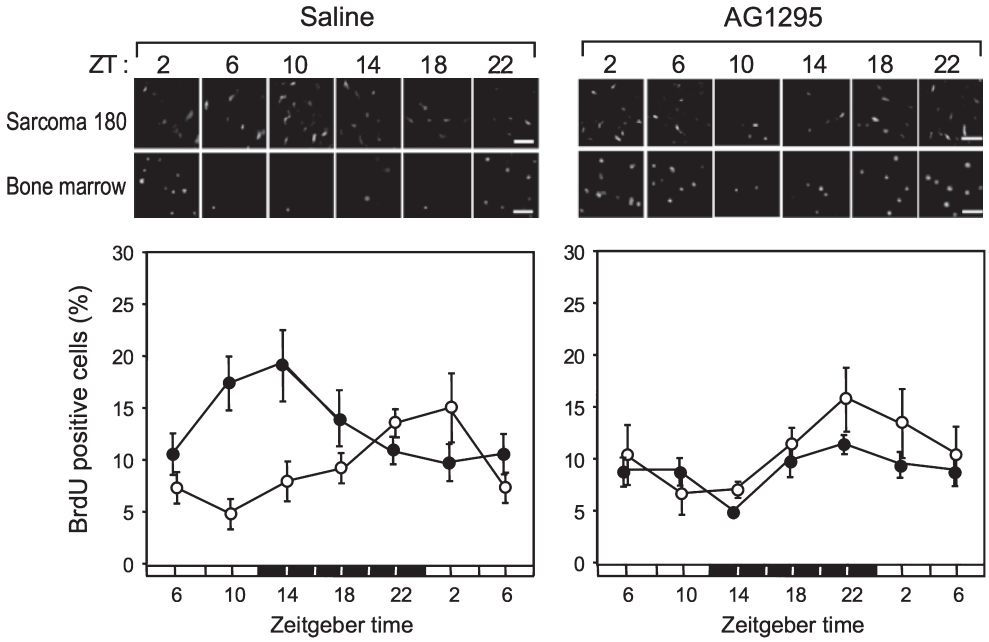


**Fig. 2.** Influence of continuous administration of AG1295 (10 $\mu$g/h, s.c.) on the daily variations in DNA synthesis in implanted sarcoma 180 tumor cells (closed circle) and in healthy bone marrow cells (open circle). Tumor-bearing mice were continuously administered AG1295 (10 $\mu$g/h, s.c.) or saline by means of osmotic minipumps for 5 days. DNA synthesis of cells in tumor and bone marrow were assessed by BrdU labeling. Each point represents the mean ± S.E.M (n = 4 – 6). Upper panels show the representative micrographs of BrdU-positive cells from sarcoma 180 tumor masses and bone marrow. Scale bars represent 30 $\mu$m.

result confirms previous findings that the proliferation rhythm of tumor cells shows different phase from that of non-tumor cells (4 – 6).

Although continuous administration of AG1295 had little effect on the rhythmicity of DNA synthesis in bone marrow cells, the treatment substantially decreased DNA synthetic activity in the implanted tumor cells (Fig. 2, right panel). During the administration of this drug, the DNA synthesis in tumor cells still exhibited significant 24-h variation ($P$<0.05), synchronizing itself with the rhythm of DNA synthesis in bone marrow cells. These results indicate that PDGF-receptor signaling influences the proliferation rhythm of tumor cells, but not that of bone marrow cells.

*Modulation of tumor expression of cell cycle regulatory genes by AG1295*

Because progression through each stage of the cell cycle is precisely coordinated and controlled by a family of CDK, which are activated by binding to cyclins (12), we investigated whether inhibition of PDGF receptor signaling by AG1295 influenced the expression of the cell cycle regulators in the implanted tumor cells. During the cell cycle progression, cyclin E/CDK2 and cyclin A/CDK2 complexes lead to the full inactivation of pRB. This, in turn, results in release and depression of E2F transcription factors and causes cell entry into the S-phase (13). In the saline-treated mice, time-dependent variations in mRNA levels of E1 and A2 cyclins were observed in the implanted tumor cells (Fig. 3A). The mRNAs accumulated from the light phase to the early dark phase, which corresponded to the peak time of DNA synthesis in tumor cells (Fig. 2, left panel). Although continuous administration of AG1295 had a marked effect on the expression of cell cycle regulators in the implanted tumor cells, the mRNA levels in each type of cell cycle regulatory gene still exhibited time-dependent variation (Fig. 3A). The mRNA levels of both E1 and A2 cyclins accumulated from the mid dark phase to the late dark phase (Fig. 3A). During the administration of this drug, the rhythmic pattern of the expression of E1 and A2 cyclins in the implanted tumor cells resembled those observed in bone marrow cells (Fig. 3B).

*Circadian expression of clock genes in tumor cells*

In the final set of experiments, we investigated whether inhibition of PDGF-receptor signaling by AG1295 affected the rhythms in the expression of clock genes in the implanted tumor cells. In the saline-treated mice, the mRNA levels of the clock genes in the implanted tumor cells showed obvious 24-h variations (Fig. 4A). For *Per1*, *Per2,* and *Cry2*, the mRNA levels peaked around the early dark phase, whereas *Cry1* mRNA levels peaked around the late dark phase. *Bmal1* and *Clock* mRNA oscillations were antiphase to those of the *Per* and *Cry2* mRNA rhythms, with peak levels from the late dark phase to the early light phase. These patterns of clock gene expression were similar to those in bone marrow cells of saline-treated mice (Fig. 4B). Continuous administration of AG1295 had little effect on the rhythmicity of the expression of clock genes in both tumor and bone marrow cells (Fig. 4A). Taken

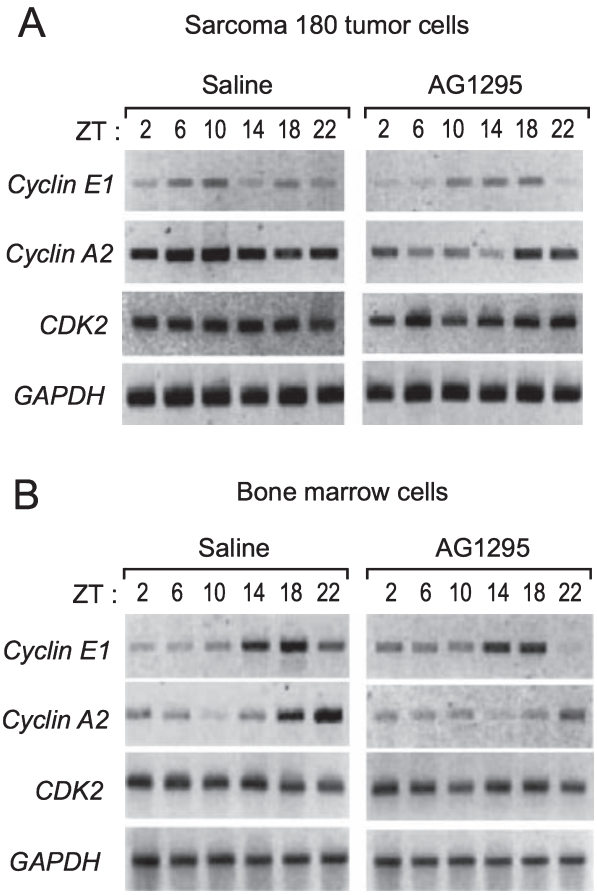


**Fig. 3.** Influence of continuous administration of AG1295 (10 $\mu$g/h, s.c.) on the rhythmic expression of cyclin E1, cyclin A2, and CDK2 in sarcoma 180 tumor cells (A) and bone marrow cells (B). Total RNA was extracted and analyzed for each cell cycle regulatory gene by RT-PCR. As an internal control, GAPDH mRNA was used because its transcripts were constant throughout the day. Data shown were confirmed in two independent saline-treated groups and three AG1295-treated groups.

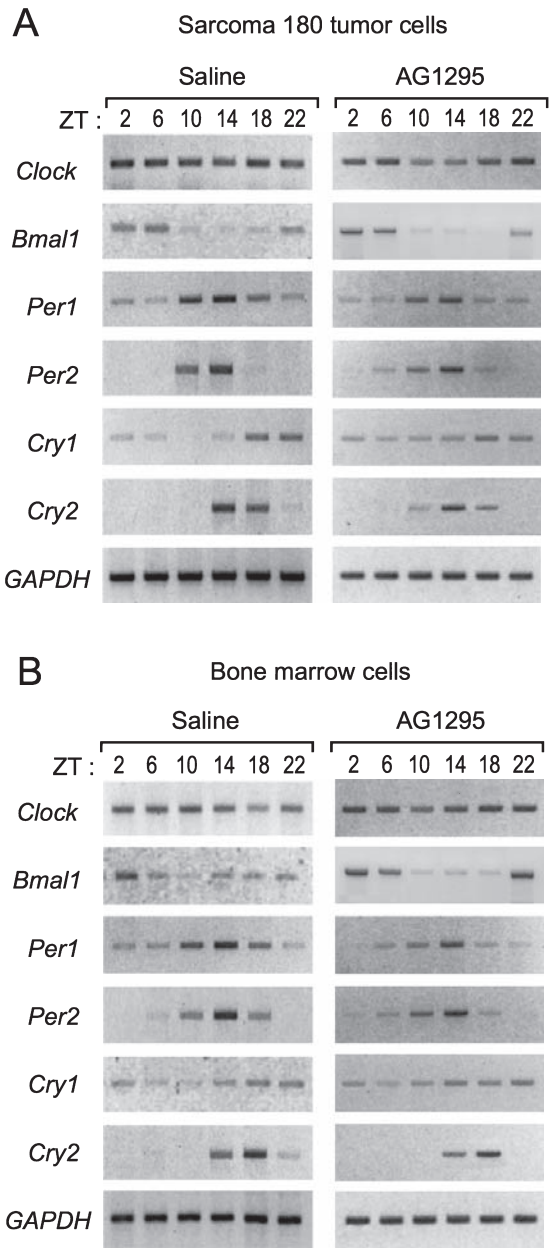


**Fig. 4.** Influence of continuous administration of AG1295 (10 $\mu$g/h, s.c.) on the rhythmic expression of clock genes in sarcoma 180 tumor cells (A) and bone marrow cells (B). Total RNA was extracted and analyzed for each clock gene by RT-PCR. As an internal control, GAPDH mRNA was used because its transcripts were constant throughout the day. Data shown were confirmed in two independent saline-treated groups and three AG1295-treated groups.

together, these findings suggest that inhibition of PDGF receptor signaling in tumor cells modulates the DNA synthesis rhythm without affecting clock gene expression.

## Discussion

PDGFs are a family of dimeric disulfide-bonded growth factors exerting their biological activities through activation of two structurally related tyrosine kinase receptors, the $\alpha$- or $\beta$-PDGF receptors (9). We reported previously that phosphorylation of PDGF receptors on the implanted sarcoma tumor cells exhibit significant 24-h variations (10), indicating that the tyrosine kinase activity of PDGF receptors varies according to the time of day. One possible mechanism accounting for the 24-h variation in the tyrosine kinase activity of PDGF receptors is that there is a time-dependent change in the amounts of ligand binding to PDGF receptors. Our previous study has demonstrated that the expression of vascular endothelial growth factor (VEGF), a member of the PDGF family of mitogens, in sarcoma 180 tumor cells implanted in mice shows a significant 24-h oscillation. The oscillation in the

expression of VEGF gene is governed by the core components of the circadian clock (11). It is therefore possible that the molecular components of the circadian clock affect the expression of PDGF genes, thereby causing the oscillation in its protein production. However, to our knowledge, no previous study has reported the daily oscillation in PDGF production from the tumor cells. Further study will be required to clarify the mechanism of 24-h variation in PDGF-receptor activity in tumor masses.

The present study demonstrated that continuous administration of AG1295 damped the fluctuation of PDGF receptor's phosphorylation, accompanied by modulation of the rhythmicity in DNA synthesis in the tumor cells. Circadian variation in cell proliferation is considered to be caused by synchronous progression of the cell cycle. During the cell cycle progression, cyclin E/CDK2 and cyclin A/CDK2 complexes lead to the full inactivation of pRB. This, in turn, results in release and depression of E2F transcription factors and causes cell entry into the S-phase (13). The activation of PDGF receptors signaling can induce the expression of various types of cell cycle regulators, leading to transition of quiescent cells into the proliferative stage of the cell cycle (14). Therefore, the mitogenic action of PDGF receptors signaling on tumor cells may lead to modulation of their proliferation rhythm by altering the expression of cell cycle regulators.

Although continuous administration of AG1295 substantially decreased DNA synthesis in the tumor cells and modulated their rhythmicity, the treatment had little effect on the rhythmicity of DNA synthesis in bone marrow cells. It is thus unlikely that DNA synthesis in bone marrow cells depends on the PDGF-receptor signaling. In fact, no significant levels of phosphorylated PDGF receptors were detectable in bone marrow cells, although PDGF-receptor proteins were detected (data not shown). Taken together, these findings suggest that low tyrosine kinase activity of PDGF receptors on bone marrow cells results in the inability of AG1295 to modulate their DNA synthesis rhythm. However, we were unable to rule out the possibility that the amount of AG1295 distributed in the bone marrow cells was insufficient to inhibit the tyrosine kinase activity of PDGF receptors. Further studies are required to clarify the distribution of AG1295 in bone marrow cells.

During the administration of AG1295, the DNA synthesis in tumor cells still exhibited significant 24-h variation, synchronizing itself with the rhythm of DNA synthesis in bone marrow cells. This result suggests that in the absence of PDGF signaling, circadian progression of the cell cycle in tumor cells is synchronized by the same mechanism functioning in bone marrow cells. Recent molecular dissections of the circadian biological clock system have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of circadian rhythms (15 – 17). Gene products from *Clock* and *Bmal1* form a heterodimer that activates the transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. Once the PER and CRY proteins have reached a critical concentration, they attenuate CLOCK/BMAL1 transactivation, thereby generating a circadian oscillation in their own transcription (18, 19). The CLOCK/BMAL1 heterodimers also activate the expression of clock-controlled output genes, allowing for a circadian control of cellular and physiological functions, which include cell cycle progression (2, 3). It has been suggested that some aspects of tumor progression influence the intracellular molecular clock function (6), thereby resulting in modulation of the rhythmicity in tumor cell proliferation. However, the rhythmic phase of clock gene expression in the implanted tumor cells was not affected by administration of AG1295. The rhythmicity of clock gene expression in the tumor cells was similar to those in bone marrow cells. It is thus unlikely that the peculiar rhythm of DNA synthesis in tumor cells is caused by altering the rhythmicity of clock gene expression. The expression of clock gene in implanted cells is subordinated to the dominance exerted by the central clock of the host animal (11, 20). The master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus follows a daily light/dark cycle and, in turn, synchronizes subsidiary oscillators in peripheral tissues through neural and/or humonal signals (21, 22). Although PDGF has the ability to induce the expression of *Per1* gene in cultured NIH3T3 cells (23), a more potent factor may transmit the circadian signaling from the SCN to the implanted tumor cells. Glucocorticoids are attractive candidates for such circadian signaling because they inhibit food-induced phase shifting of peripheral clock gene expression (24). In fact, we observed that 24-h rhythmicity in glucocorticoid secretion in sarcoma 180 tumor-bearing mice was similar to that in normal healthy mice (25). The normality of glucocorticoid secretion in tumor-bearing mice may maintain the rhythmicity of clock gene expression in the tumor cells. Therefore, transduction of PDGF receptor signaling may induce the expression of cell cycle–regulated genes without changing the intracellular circadian clockwork, resulting in alteration of the rhythmicity in tumor cell proliferation. Further studies are required to investigate whether this hypothesis is also applicable for other type of tumor cells.

The present findings in this animal model suggest that the difference in the rhythmic phase of cell proliferation

between tumor and bone marrow is caused by the activation of PDGF signaling. The cyclic activation of PDGF receptors on tumor cells appears to modulate the rhythm of tumor cell proliferation through inducing the expression of various types of cell cycle regulators. Because it is generally accepted that chemotherapy drugs exert a greater cytotoxic effect on proliferating cells, our present findings may provide an aid for better understanding of the circadian phase–dependent toxicity and anti-tumor effect of several chemotherapy drugs.

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