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Per2 Inhibits K562 Leukemia Cell Growth In Vitro and In Vivo Through Cell Cycle Arrest and Apoptosis Induction

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Abstract Per2 regulates other molecular and biochemical processes beyond their established role in the regulation of the mammalian circadian clock, herein we investigated the growth inhibiting potential of Per2 in human K562 leukemia cells and the underlying mechanisms .The results showed that over-expression of Per2 induced not only cell cycle arrest at G2/M phase but also an increase in apoptosis, which was confirmed by characteristic morphological changes, FCM and evident DNA fragmentation. Further experiments confirmed both up-regulation of P53 and down-regulation of CylinB1and C-myc. On the other hand, while P53 was found to be down-regulated. CylinB1 and C-myc were up-regulated. after Per2 knockdown. In leukemia mice, Per2 transfection was shown to suppress cellular proliferation and accelerate apoptosis of K562 cells. Moreover, fewer leukemia cells were found to have infiltrated into the livers and spleens of the mice from the Per2 transfected group as compared with those from the control group. In summary, Per2 displayed a significant anti-tumor effect through cell cycle arrest and apoptosis induction in K562 cells. These data further support the emerging role of the circadian clock in critical aspects of cancer development and thorough research is underway on the mechanism of Per2 in the leukemia.

Keywords Circadian clock · Per2 · Apoptosis · Cell cycle arrest · K562 cell

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Introduction

A circadian clock controls various physiological and behavioral rhythms. In mammals, a master circadian clock exists in the suprachiasmatic nucleus of the hypothalamus, and slave oscillators can be found in most tissues [1, 2]. The rhythms of the circadian clock are controlled by the interaction between positive and negative feedback loops consisting of key clock regulators (including Clock and Bmal1), two cryptochromes (Cry1 and Cry2), and three Period (mPer1, mPer2, and mPer3) [3, 4]. Gene knockout studies in mice revealed differential roles for the three mPer proteins in the mammalian circadian rhythm [5, 6]. The Period (Per) genes are key circadian rhythm regulators in mammals, furthermore, Period2 (Per2) is an essential component of the mammalian clock mechanism and robust circadian expression of Per2 is essential for the maintenance of circadian rhythms. Recent studies have demonstrated that Per2 regulates other molecular and biochemical processes beyond their established role in the mammalian circadian clock. More importantly, Per2 appears to act as a tumor suppressor in mice [7, 8]. The deregulated expression of the Per2 gene can be found in breast cancers [9]. The increased proliferation and decreased apoptosis that are associated with clock disruption in tumors can be subverted through Per2 overexpression in malignant cells [10–12]. Moreover, Per2 was found to be downregulated in chronic

myeloid leukemia (CML) [13] though its function and the underlying mechanism of Per2 in CML were not known.

The circadian clock and cell cycle are two global regulatory systems that interact at the level of genes, proteins, and biochemical signals in most eukaryotic organisms [14]. The expression of cell cycle genes such as P53, Cyclins, and c-Myc are under circadian control and could be directly regulated by the circadian transcriptional complex [15]. Cell cycle checkpoints are important growth arrest mechanisms that ensure the orderly progression of cell cycle events and prevent aberrant mitosis in response to DNA damage. Investigations on roles of Per2 in tumorigenesis suggest a complex interaction between Per2 and many known regulators involved in various aspects of cell growth and apoptosis. The disruption or the reinforcement of the tumor circadian timing system, respectively, accelerates or slows down cancer growth through modifications of the clock-controlled genes.

In both the in vitro and in vivo studies, we focused on the growth inhibiting effect of Per2 in CML cells and its underlying mechanisms. We showed that the circadian gene Per2 played an important role in the regulation of both growth and apoptosis of human CML leukemia cells. Further studies exploring into its underlying mechanisms suggested that core clock factor Per2 may exert its tumor suppression effect through transcriptional regulation of cell cycle-related genes including P53, CylinB1 and C-MYC. These data further support the emerging role of the circadian clock in critical aspects of cancer development.

Materials and Methods

Cell Line and Plasmids

The human K562 leukemia cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, Utah), 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2.

The mammalian Per2-expression plasmid (pcDNA3.1-Per2) was a gift from Professor Steven Reppert (University of Massachusetts Medical School, USA), pcDNA3.1 empty vector was from our own laboratory. Per2 was confirmed as being in frame with no mutations by DNA sequencing. The cells were transfected with the plasmids indicated using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA). Stable cell lines were generated by transfecting K562 cells with pcDNA3.1-Per2 as well as control pcDNA3.1 empty vector, followed by G418 (800 mg/ml) selection.

The plasmid pGenesil-3-SiPer2 and pGenesil-3-HK negative control was constructed by Wuhan Genesil Biotechnology Co., Ltd. Cells were plated in 6-well plate the day before to reach 30% of confluency by the time of transfection. About 100 pmol of siRNA oligo was transfected by Lipofectamine2000 according to the protocol provided by the manufacturer. The knocking down effect was examined 48–72 h later. The sequences of RNA oligos used were as following:

pGenesil-3-HK negative control 5′-GATCCGACTT CATAAGGCGCATGCTTCAAGACGGCATG CGCCTTATGAAGTCTTTTTTGTCGACA -3′ 5-AGCTTGTCGACAAAAAAGACTTCATAAGGC GCATGCCGTCTTGAAGCATGCGCCTTATGAAG TCG-3′

pGenesil-3- SiPer2 5'-GATCC GAACACAACCCATCTACAATTCAA GACGTTGTAGATGGGTTGTGTTCTTTTTTGTC GACA -3′ 5-AGCTTGTCGACAAAAAAGAACACAACC CATCTACAACGTCTTGAATTGTAGATGGGT TGTGTTCG -3′

Animal Experiments

These studies were conducted in accordance with the animal care guidelines instituted by the Animal Studies Committee of Chongqing Medical University.

Twenty-eight 4- to 6-week-old Balc/C nude mice (purchased from National Laboratory Animal Center Beijing, China) were maintained at the Animal Facility of Chongqing Medical University. Animals were kept in filter-top cages on sterile bedding, provided with sterile food and acidic water ad libitum and housed under a standardized 12 h:12 h light/ dark cycle at room temperature of 24±°C and a humidity of 60±10%. The animals were adapted to the 12 h:12 h light/dark cycle for 2 weeks before the experiments. Under the 12 h:12 h light/dark cycle, times was referred to as light onset (hours after light onset), so-called Zeitgeber time (ZT). ZT 0 was designated as lights on and ZT 12 as lights off. The animals were randomized into two groups: subcutaneous tumor group and leukemia mice group. Tumors were induced by subcutaneous injection of 1×10^7 leukemia cells into the right supra scapular area of each mouse. Leukemia mice were induced by injection of 2×10^7 leukemia cells into caudal vein. Three kinds of cells including pcDNA3.1-Per2 K562 cells, pcDNA3.1 K562 cells and the control K562 cells were injected into each group.

Flow Cytometric Analysis of Cell Cycle

After 48 h transient transfection cells were harvested, washed in cold PBS, fixed in 70% ethanol and stored at

4°C. Cells were treated with RNase-A solution (500 U/ml) at 37°C for 15 min and stained by propidium iodide (50 μg/ml) in 1.12% sodium citrate at room temperature before analysis. Flow cytometric determination of DNA content was analyzed by COULTER EPICS XL Flow Cytometer (Coulter Corp., Miami, FL, USA). The fractions of the cells in G0/G1, S and G2/M phase were analyzed using cell cycle analysis software, Multicycle (Phoenix Flow System, San Diego, CA, USA).

Flow Cytometric Analysis of Apoptosis

After 48 h transient transfection, cells were collected, washed with cold PBS for three times, and cell apoptotic death was quantified using FITC labeled Annexin-V (Responsif, Erlangen, Germany) and PI double staining assay. Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed with CellQuest software (BD Biosciences).

DNA Gel Electrophoresis for the Analysis of DNA Fragmentation

After 48 h transient transfection ,DNA laddering was detected by isolating fragmented DNA using the SDS/proteinase K/RNase A extraction method, which allows the isolation of only fragmented DNA without the contamination from genomic DNA. Briefly, approximately 5×10^6 K562 cells were treated as indicated, harvested, and pellets were suspended in lysis buffer (0.1 M NaCl, 50 mM Tris-HC1, pH 7.5, 10 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 20 g/mL protease K). After a 30-minute incubation on ice and centrifugation at 14,000 g for 30 min, cellular DNA was extracted and treated for 1 h at 37°C with RNase A. Finally, DNA preparations were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining via the Gel Imaging System.

Transmission Electron Microscopy

K562 cells from different groups were cultured for 48 hours, collected and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3, containing 2% sucrose and 1 mmol/L calcium chloride overnight at 4°C, washed several times in cold cacodylate buffer containing 5% sucrose, and post-fixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer containing 2% sucrose for 3 hours at 4°C. After washing with distilled water, the cells were stained en bloc with uranyl acetate, dehydrated through graded ethanols and propylene oxide, infiltrated, embedded, and polymerized in Polybed resin. Cells were then infiltrated for 6 hours with 100% Polybed resin, embedded in fresh resin, and polymerized overnight at 70°C. Thin sections were cut, stained with uranyl acetate and lead citrate, viewed and photographed with an H-600 IV transmission electron microscope (Hitachi, Tokyo, Japan).

Anti-tumor Nude Mice Experiment

Balb/c AnN-Foxn1 nude mice (4–6 weeks old) were purchased from the National Laboratory Animal Center (Beijing, China) and kept in an animal facility for 1–2 weeks before use. All animal experimental procedures were conducted and approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. 15 nude mice were randomized into 3 groups, namely the pcDNA3.1-Per2 K562 group, pcDNA3.1 K562 group and the untreated K562 group. Mice from each group were injected subcutaneously between the scapulae with 1×10^7 chronic myeloid leukemia cells as indicated above. At experimental end (30 days later), mice were sacrificed by cervical dislocation, and tumor specimens were taken, photographed, and tumor weights and tumor diameters were subsequently determined.

Anti-leukemia Nude Mice Experiment

K562 cells from different groups were propagated in liquid culture as described above. Cells were washed twice in PBS, counted, and resuspended at a density of 10^7 cells per 0.2 mL PBS. Recipient nude mice received 10^7 cells intravenously by tail vein injection. Injected mice were subsequently evaluated on a daily basis for weight loss, failure to thrive, splenomegaly, or hind-limb paralysis. Mice that appeared premorbid were killed. Part of the leukemia-bearing mice were sacrificed after 4 weeks of transplantation and evaluated for the diffusion of leukemia cells into bone marrow, liver, spleen and lung, and the other remaining animals were scored for their survival durations.

Liver and spleen organs were removed and fixed in neutrally buffered 10% formalin at room temperature for 16 hours before they were embedded in paraffin and sectioned. All tissues were stained with hematoxylin and eosin for light microscopy. Photomicrographs were obtained using an Olympus BH2 microscope (Olympus, Melville, NY) with 100×Plan Achromat oil objective, and were acquired with an Olympus Qcolor5 digital camera.

TUNEL Assay

To detect apoptotic cells in tumors, sections were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) (Roche Molecular Biochemicals) according to the manufacturer's instruc-

tions. After deparaffinization, the sections were treated with 20 g/ml proteinase K for 10 min, with 3% H_2O_2 in methanol for 10 min and 0.1% Triton X-100 in 0.1 % sodium citrate for 10 min at 37°C. Then the sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37°C in the humidified atmosphere in the dark. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with Mayers hematoxylin.

Reverse Transcription (RT)-PCR Analysis

Total RNA was isolated with Trizol reagent (Invitrogen). RT-PCR for Per2, p53, c-myc, cyclinB1 and β-actin mRNA was carried out. Details of the primers and the GenBank accession numbers are given in Table 1. PCR was performed using 1 μl of cDNA in 40 μl PCR reaction buffer containing 15 pmol of each primer, 0.1 mM dNTP, and 0.3 units Taq polymerase (Promega). Conditions of these reactions were as follows: prehealing at 94°C for 5 mins; 30 cycles at 94°C,30 s, 56°C, 30 s, 72°C, 30 s followed by elongation at 72°C for 10 mins.

Western-Blot

Cells were lysed with lysis buffer (50 mM Tris [Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% nonidet P-40 [NP-40]); subsequently cell lysates were resolved on 4% to 15% gradient sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs) and transferred to PVDF membranes (Sigma, St Louis, MO). Immunoblots were incubated with the following antibodies: Per2, P53, c-MYC, CyclinB1 and GADPH, which were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) Blots were developed using Supersignal WestPico chemiluminescent substrate (Pierce), imaged and analyzed for its photodensitometry by the Bio-Rad Gel Imaging System.

Statistical Analysis

Data were expressed as means±standard error of the mean (S.E.M.). Differences between two groups were evaluated by unpaired Student's *t*-test. $P \leq 0{\cdot}05$ denoted the presence of a statistically significant difference.

Results

Expression of Per2 led to Cell Cycle Arrest and Apoptosis Induction in K562 Cells

After 48 h transient transfection, the cells from different groups were collected and their cell cycle distribution was analyzed. We demonstrated that expression of Per2 led to cell cycle arrest in K562 cells by showing that the pcDNA3.1- Per2 -K562 cells had a significantly increased number of cells (**36.04**%) in the G2/M phase compared with those of the pcDNA3.1-K562 cells (**12.48**%) and the K562 cells from the control group(**9.71** %) ($P < 0.01$, Fig. 1a, b). On the other hand, as is shown in Fig. 1c, d, e, f, expression of Per2 induced nuclear condensation, cell shrinkage and membrane blebbing, and the apoptotic bodies were found under the transmission electron microscope (Fig. 1e). Moreover, it was found that Per2 expression induced an accumulation of fragmented DNA (Fig. 1f), a hallmark of apoptotic cell death. Flow cytometric analysis further confirmed the massive apoptosis induced by the expression of Per2 (Fig. 1c and d). These results suggest that after cell cycle arrest, Per2-transfected cells may undergo apoptosis.

Per2 Overexpression Upregulated the Expression of P53 and Downregulated Those of c-Myc and CyclinB1

The K562 cell line was stably transfected by the Per2 expression vector (pcDNA3.1-Per2) or the empty vector (pcDNA3.1). Clones were selected on the basis of G418 resistance, and Per2 expression was demonstrated by RT-PCR and Western blot (Fig. 2a and b).

RT-PCR analysis showed that both expressions of c-Myc and cyclinB1 mRNA were downregulated, whereas the expression of p53 mRNA was upregulated in Per2-overexpressing K562 cells as compared with those of the empty vector-transfected K562 cells or the non-transfected control cells (Fig. 2c). The housekeeping gene β-actin was

Table 1 DNA sequences for all the primers

Gene	Primer	Length (bp)
β-actin(BC001301)	5′- CTGACTGACTACCTCATGAAGATC -3′5′- GTGAGAAGGTCGGAAGGAAGG-3'	258
P53(NM_000546.3)	5′-AGAATCTCCGCAAGAAAGG-3′5′-GCAAGCAAGGGTTCAAAGAC-3′	417
C-MYC(NM_002467.)	5′- CAACCCTTGCCGCATCCAC -3′5′- CCTCCTCGTCGCAGTAGAA -3′	318
cyclinB1(NM_031966.2)	5′- TTTCTGCTGGGTGTAGGTC-3′5′- AGGTTCTGGCTCAGGTTCT-3′	407
Per2 (NM_022817)	5′- TGTATTTTTAGTAGAGACAGGGT -3′5′- CATAACAGCAGAGTAAGATTTTG-3′	444

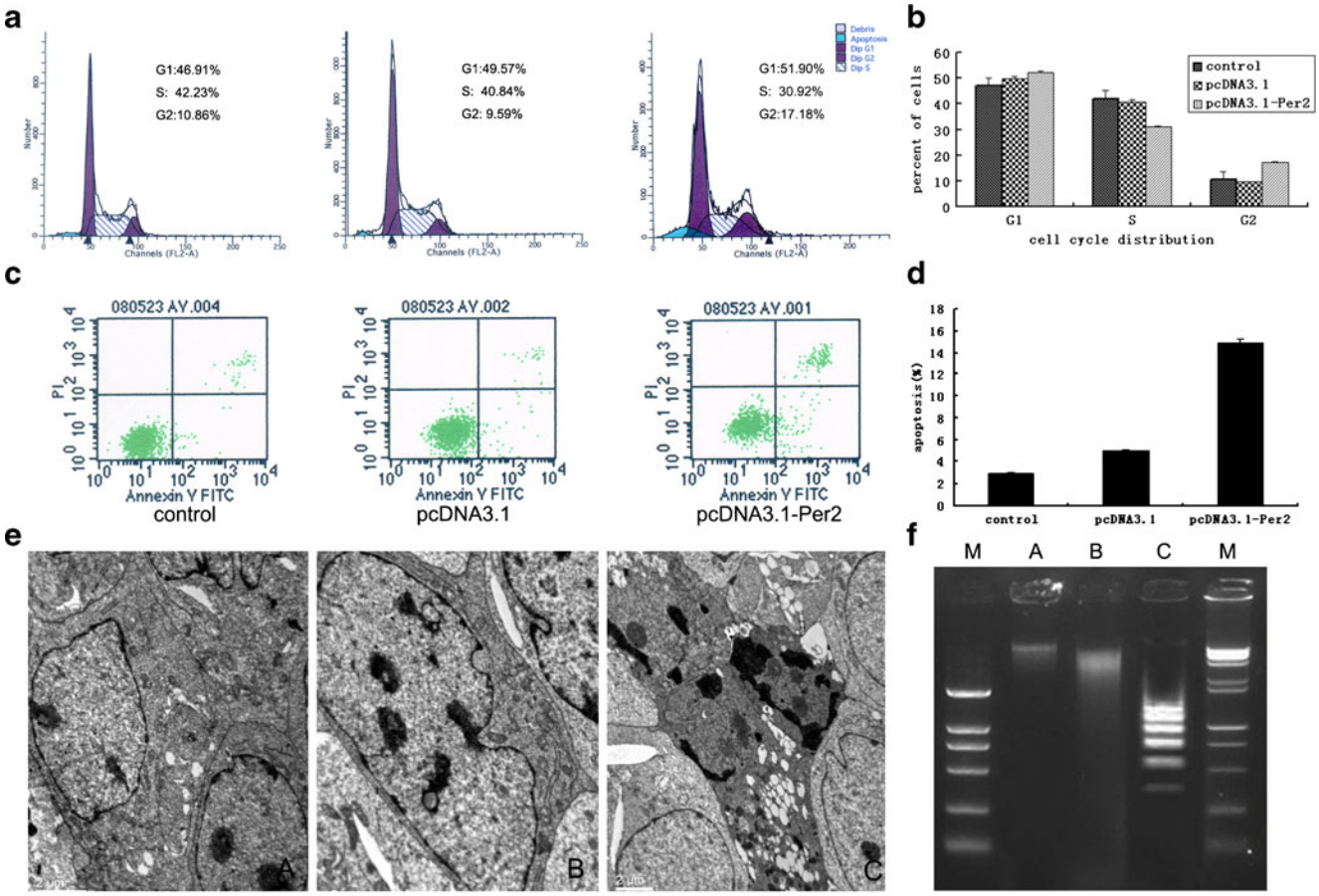

Fig. 1 Per2 overexpression induced cell cycle arrest and promoted apoptosis in K562 cells. **a**,**b** Cell cycle analysis. K562 cells were transiently transfected with either empty vector or a Per2 expression vector, and after 48 hours' transfection, they were collected and stained with propidium iodide (PI), and analyzed by flow cytometry for cell cycle analysis. **c**,**d** Apoptosis analysis. K562 cells were stained with annexin V-FITC and PI and analyzed by flow cytometry for apotosis. **e** Electron microscopic evaluation of the K562 cells. K562 control cells (*A*) and Empty Vector-tranfected cells (*B*) had intact membranes and organelles, and normal nuclear morphology, while the pcDNA3.1- Per2 -K562-cells (*C*) showed homogeneous chromatin condensation within the nucleus (Magnification:×6000). **f** DNA was isolated and analyzed by 1.8% agarose gel electrophoresis for the detection of DNA ladder. DNA fragmentation (laddering) was much more evident in DNA samples from pcDNA3.1- Per2 -K562-cells (*C*) than those from either the empty vector pcDNA3.1-K562 cells (*B*) or the control K562 cells(*A*)

included for the control of equal RNA loading. Results from western blot analysis further demonstrated that the level of P53 in Per2- overexpressing cells was much higher than that in vector control cells (Fig. 2d). In contrast, there was an apparent decrease in the levels of CyclinB1 and c-MYC protein in Per2- overproducing cells, compared with control cells.

Effects of SiPer2 on Cell Cycle-related Genes

K562 cells were transiently transfected either with an empty control SiRNA vector (**pGenesil-3-HK**) or Per2-SiRNA vector **(pGenesil-3 -SiPer2**). Two days post-transfection, RT-PCR and Western blot analysis were carried out to evaluate the expression level of Per2 mRNA and protein. As was shown in Fig. 3a and b , anti-sense oligonucleotides treatment resulted in an obvious reduction in the expression of Per2 mRNA and protein in comparison with that in the control group, and moreover, the expression of P53 mRNA and protein was downregulated and those of both c-MYC and CyclinB1 were upregulated after SiPer2 (Fig. 3c and d)

Effects of Per2 in the Chronic Myeloid Leukemia Nude Mice Model

High level of Per2 expression was observed in Per2-treated tumors, and Per2 mRNA in Per2-treated group was significantly higher than those in control groups treated with pcDNA 3.1 or k562 control (data not shown). The pcDNA3.1- Per2-k562 tumors were significantly smaller in size than tumors treated with pcDNA3.1 -K562 empty vector and control K562. Per2 overexpression resulted in

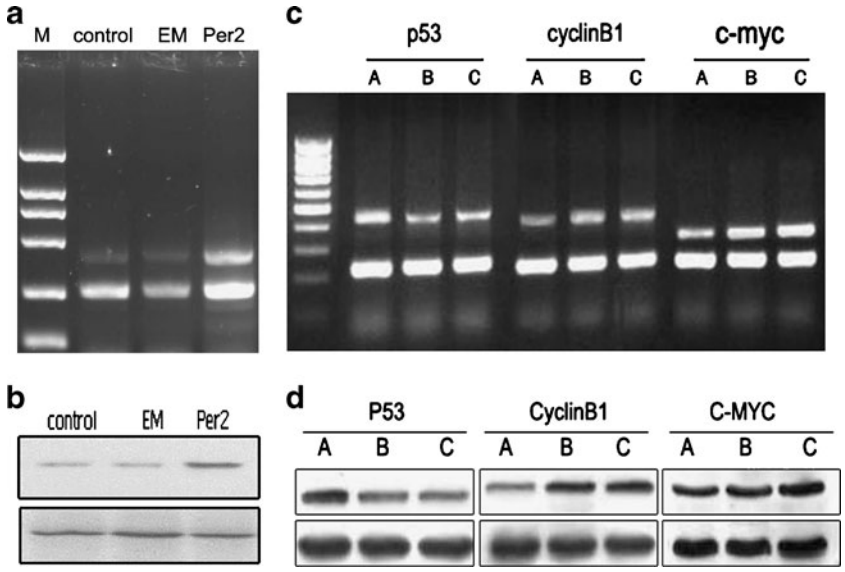

Fig. 2 Per2 regulates the expression of cell cycle-related gene. **a** RT-PCR showed the different mRNA expression level of the control K562 cells (control), pcDNA3.1- k562 (EM) and pcDNA3.1-Per2-k562 (Per2) cells. **b** Western blot analysis of Per2 expression in the pcDNA3.1-Per2-k562(Per2), control K562 cells(control) and. pcDNA3.1- k562 cells(EM).**c** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *p53*, *c-myc*, cyclinB1 in pcDNA3.1-Per2 -K562-cells (*A*), pcDNA3.1-K562 cells (*B*)and control K562 cells(*C*) .β-actin was used as loading control. **d** Western blot analysis of P53, C-MYC, CyclinB1. GAPDH was used as loading control

not only a marked reduction of mean tumor weight (control group, 5.63 g; pcDNA3.1 group , 4.86 g; Per2 group, 2.45 g; $P<0.01$; Fig. 4a) but also significant decrease in both tumor volume and growth rate ($P < 0.01$)(Fig. 4b). Furthermore, Per2 overexpression led to significant induction of apoptosis in tumors of the pcDNA3.1-Per2 group by as was demonstrated by TUNNEL assay(Fig. 4c and d) .

In leukemia nude mice, the pcDNA3.1- Per2-k562 cells in bone marrow were demonstrated to be less proliferative than both the pcDNA3.1 -k562 and the control K562 cells (Fig. 4e). There were less infiltration of the liver and spleen in the mice transplanted with pcDNA3.1- Per2-k562 cells than that in those injected with pcDNA3.1 -k562 or control K562 cells (Fig. 4f and g) and as was shown by the survival

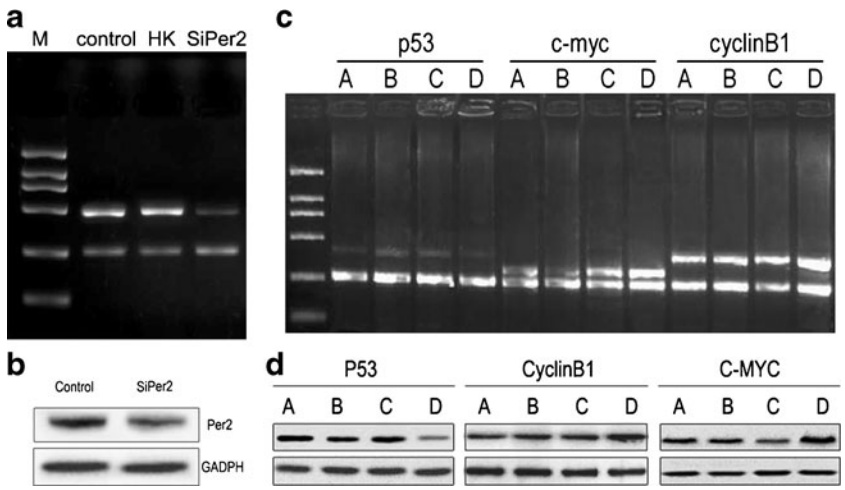

Fig. 3 The expression of cell cycle-related genes after SiPer2 treatment. **a** RT-PCR analysis of the Per2 mRNA expression in pGenesil-3 -SiPer2-k562 (SiPer2), pGenesil-3-HK -k562 negative control (HK) and K562 control (control). **b** Western blot analysis of Per2 protein expression in pGenesil-3 -SiPer2-k562 (SiPer2) and K562 control (control). **c** RT-PCR analysis of *p53*, *c-Myc* and CyclinB1 mRNA expression in control group (*A*), mock group (*B*), pGenesil-3-HK -k562 negative control group (*C*) and Genesil-3 -SiPer2-k562 group (*D*). β-actin was used as loading control. **d** Western blot analysis of P53, c-MYC and CyclinB1 protein expression. GAPDH was used as loading control

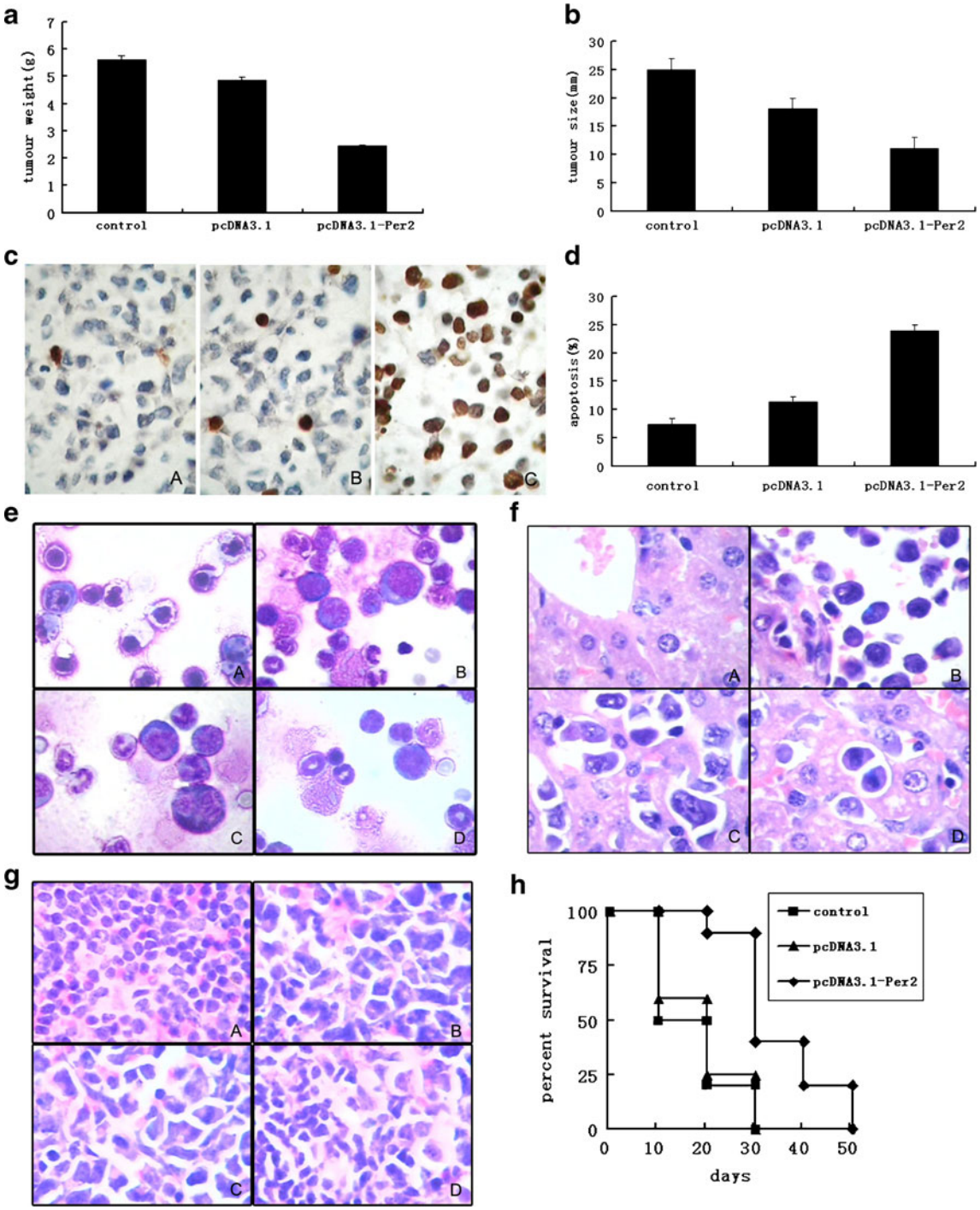

Fig. 4 Per2 inhibits the growth of both the subcutaneous tumor and leukemia in nude mice. **a** Per2 overexpression led to a marked reduction of tumor weight and the tumors in the pcDNA3.1-Per2-K562 transplanted mice were the smallest (P <0.01). **b** Per2 overexpression led to a marked reduction of tumor volume and the tumors in the pcDNA3.1-Per2-K562 transplanted mice showed the minimum volume (P <0.01). **c** Apoptotic cells in the tumors from different groups as detected by TUNEL assay with the tumors from the pcDNA3.1-Per2-K562 transplanted mice group (*C*) showed the most apoptotic cells than those from either the control K562 cell transplanted (*A*) or the pcDNA3.1- k562 cell transplanted ones (*B*). **d** The apoptosis rates of tumor cells in different groups. **e** Wright Giemsa staining of bone marrow cells from different groups. (*A*: normal group; *B*: the control K562 group; C: pcDNA3.1- k562 group; D: pcDNA3.1-Per2-K562 group). **f** Histology of the liver infiltrated by K562 leukemia cells **g** Histology of the spleen infiltrated by K562 leukemia cells. **h** survival curve of the mice from different cell transplanted groups

curve, the Per2-treated mice survived longer than those in the other two groups.

Discussion

In the present study, *Per2 was* overexpressed in K562 cells and its effects were evaluated. As a core circadian gene, *Per2* can not only maintain the circadian rhythm of cells, but also sustain the normal cell cycle by adjusting cell cycle related genes such as P53, c-Myc and cyclinB1. In our study, the over-expression of Per2 also exhibited a significant cell apoptosis inducing effect as was demonstrated by the results from flow cytometry, DNA laddering and ultramicrostructure observation. On the other hand, Per2 silencing in K562 cells showed the adverse control effect on these genes. Moreover, Per2 inhibited the growth of the K562 human leukemia cells in nude mice, and the life span of mice from the Per2-K562 cells-transplanted group was longer than that from the control group. Further experiments witnessed less leukemia cells-infiltrated liver and spleen of the mice from the Per2-K562 cells-transplanted group.

Progression through the cell cycle is monitored by cell cycle checkpoints that ensure proper replication and segregation of genetic material between daughter cells [16]. During mitosis, the spindle checkpoint monitors for proper attachment of the kinetochores on sister chromatids to spindle microtubules [17]. CyclinB1 was the key point of G2/M cell cycle stage, and investigations to date have demonstrated that CyclinB1 expression is likely to be correlated with the malignant potential of human tumors [18–20]. The high level of CylinB1 may accelerate the progress of G2/M stage. Without synthesis of CyclinB1 prior to the G2/M transition, the cell cannot enter mitosis and the cell cycle will arrest at G2 phase. Our results indicated that the disturbance of CyclinB1 by Per2 may lead to cell cycle arrest at G2/M which is in great concert with the results from earlier studies demonstrating that clock genes regulate the G2/M transition in regenerating murine livers [21] .Besides, Circadian gene regulates cell cycle and apoptosis proteins in mouse bone marrow and tumor [22]. It is conceivable that Per2 may control cell cycle progression by its transcriptional regulation on CyclinB1. These results together with our findings support a role for the circadian clock in orchestrating mitotic events.

p53 is a tumor suppressor gene whose activation can induce cell cycle arrest, DNA repair or apoptosis, or senescence, depending on the differential activation of *p53* target genes [23, 24]. The levels of both p53 mRNA and protein in Per2-overexpressing K562 cells tended to be higher than those in the vector control cells, indicating that mPer2 overexpression induced an increase in p53 levels, which may contribute, at least in part, to apoptosis in Per2-overexpressing cells. When mammalian cells contain damaged DNA, the p53 tumor suppressor and the Rb family of transcriptional repressors work together to down-regulate a large number of genes that encode proteins required for G2/M transition. Elimination of these essential cell cycle proteins helps to keep the cells arrested in G2 [25]. Meanwhile, P53 is a regulator of Cdc2-cyclin B1 and can transcriptionally suppress both genes. Moreover, previous studies indicated that circadian regulation of cyclin B1 appears to be indirect and to involve a p53-dependent mechanism, p53 helps to block entry into mitosis and strengthens the G2 arrest [26].

Conversely, c-*myc* is an oncogene that its activation has been linked with many human cancers [27–29].

The proto-oncogene c-myc has been shown to play a pivotal role in cell cycle regulation, metabolism, apoptosis, differentiation, cell adhesion, and tumorigenesis, and participates in regulating hematopoietic homeostasis. One key regulator of the cell division cycle and modulator of DNA damage control is c-myc [30], The identification of c-myc as a circadian regulated gene directly links circadian function to a key regulator of the cell cycles and Per2 can suppress c-myc expression indirectly through stimulating Bmal1 transcription [15]. Our studies revealed that c-myc is under circadian regulation of Per2. We assumed that Per2 suppressed the K562 cell proliferation through it transcriptional regulation of c-myc expression.

In the past decade, many of the molecular components of the circadian clock have been identified in several organisms. Therefore, the next steps to understand the circadian system as a whole will be to study tisssue-specific regulation of clock components and in particular Per2 function.

Our data established a strong link between the circadian clock and the cell cycle, which may explain how disruption of the clock may shift the cellular balance between proproliferative versus antiproliferative genes, increasing the risk for caner initiation and progression. It is likely that studies in this area over the next few years will yield yet more connections between the Per2 and signal transduction pathways and further increase our knowledge of how these factors interact and, just as importantly, how such interactions and subsequent cellular responses are exquisitely orchestrated.

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