BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH

www.bjournal.com.br

ISSN 0100-879X
Volume 42 (10) 870-992 October 2009

BIOMEDICAL SCIENCES AND CLINICAL INVESTIGATION

Braz J Med Biol Res, October 2009, Volume 42(10) 882-891

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The Brazilian Journal of Medical and Biological Research is partially financed by

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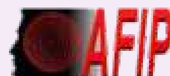

GE Healthcare

Brazilian Journal of Medical and Biological Research (2009) 42: 882-891
ISSN 0100-879X

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Abstract

Period2 is a core circadian gene, which not only maintains the circadian rhythm of cells but also regulates some organic functions. We investigated the effects of *mPeriod2* (*mPer2*) expression on radiosensitivity in normal mouse cells exposed to ^{60}Co-γ-rays. NIH 3T3 cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce endogenous *mPer2* expression or transfected with pcDNA3.1(+)-*mPer2* and irradiated with ^{60}Co-γ-rays, and then analyzed by several methods such as flow cytometry, colony formation assay, RT-PCR, and immunohistochemistry. Flow cytometry and colony formation assay revealed that irradiated NIH 3T3 cells expressing high levels of *mPer2* showed a lower death rate (TPA: 24 h 4.3% *vs* 12 h 6.8% and control 9.4%; transfection: pcDNA3.1-*mPer2* 3.7% *vs* pcDNA3.1 11.3% and control 8.2%), more proliferation and clonogenic survival (TPA: 121.7 ± 6.51 *vs* 66.0 ± 3.51 and 67.7 ± 7.37; transfection: 121.7 ± 6.50 *vs* 65.3 ± 3.51 and 69.0 ± 4.58) both when treated with TPA and transfected with *mPer2*. RT-PCR analysis showed an increased expression of *bax*, *bcl-2*, *p53*, *c-myc*, *mre11*, and *nbs1*, and an increased proportionality of *bcl-2*/*bax* in the irradiated cells at peak *mPer2* expression compared with cells at trough *mPer2* expression and control cells. However, no significant difference in *rad50* expression was observed among the three groups of cells. Immunohistochemistry also showed increased protein levels of P53, BAX and proliferating cell nuclear antigen in irradiated cells with peak *mPer2* levels. Thus, high expression of the circadian gene *mPer2* may reduce the radiosensitivity of NIH 3T3 cells. For this effect, *mPer2* may directly or indirectly regulate the expressions of cell proliferation- and apoptosis-related genes and DNA repair-related genes.

Key words: Circadian; *mPer2*; Radiation; Cell death; Proliferation; DNA repair

Introduction

Radiobiology studies have shown that ionizing radiation is a DNA-damaging agent inducing cell death, gene mutations and chromosome aberrations even at low doses (1). Most of these reactions are induced by hydroxyl radicals (indirect effects) and by one-electron oxidation (direct effects) resulting from exposure to ionizing radiation (2). Cell DNA molecules are important targets of radiation injury (3,4). Activation of the damage checkpoint occurs in response to many types of genomic lesions, including double-strand DNA breaks, single-strand DNA breaks and chemical modification of DNA by UV and γ irradiation (5,6). The cell cycle potentially stalls at several phases to provide ample time for the cell to repair DNA lesions before the S-phase (G1 arrest) and/or mitosis (G2 arrest). When cells recognize DNA injuries, especially double-strand DNA breaks, they activate the DNA damage checkpoint and repair the damage. When cells fail to fully repair the disordered DNA they activate the apoptotic cell death pathways. This fine tuning of the balance between DNA repair and apoptosis may be mediated by the DNA-binding properties of the related proteins and by their transactivation of gene transcription such as the P53 protein (7-9).

Circadian genes constitute the physiological basis of circadian clocks, which control the daily oscillations of biochemical, physiological, and behavioral processes of most organisms and enable these processes to occur at appropriate times of day (10-12). Recently, it has been shown that circadian genes not only play an important

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Research supported by the China National Natural Science Foundation (Nos. 30871357 and 30700393) and by CMB (No. 88-486).
Received January 23, 2009. Accepted July 22, 2009. Available online September 4, 2009.

role in controlling circadian rhythms, but also participate in other physiological and pathological activities, such as drug dependence, tumor development and radiation response (13-15).

The *Period2* (*Per2*) gene, an indispensable component of the circadian clock, not only modulates circadian oscillations, but also regulates other organic functions. *mPeriod2* (*mPer2*) gene-deficient mice are cancer prone. After γ radiation, these mice showed a marked increase in tumor development and reduced thymocyte apoptosis. Temporal expression of the genes involved in cell cycle regulation and tumor suppression, such as *Cyclin D1*, *Cyclin A*, *Mdm-2*, and *Gadd45*, were reportedly altered in *mPer2* mutant mice (16). The *mPer2* gene may play an important role in tumor suppression by regulating DNA damage-response pathways.

It previous studies from our laboratory, Zhang et al. (17) observed that high expression of the circadian gene *mPer2* might reduce the radiosensitivity of irradiated mouse tumor cells such as LLC and EMT6 cells. In the present study, we focus on the effects of the circadian gene *mPer2* on irradiated normal cells such as NIH 3T3 cells. We treated NIH 3T3 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce endogenous circadian *mPer2* expression, or established *mPer2*-overexpressing cells by transfecting pcDNA3.1(+)-*mPer2* into NIH 3T3 cells. We then exposed the cells to ^{60}Co-γ-rays, assessed the effects of *mPer2* on cell death, proliferation and clonogenic survival after radiation, and explored the possible mechanism involved. We trust that the present study will offer a new theory for radiochronotherapy and provide a new way to protect normal cells against radiation injury.

Material and Methods

Cell culture

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with antibiotics (BioWhitaker, USA) and 10% fetal calf serum (Hyclone) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Induction of rhythm by TPA treatment

TPA, the classic protein kinase C activator, was purchased from Promega (USA). We treated NIH 3T3 cells with 100 nM TPA to induce endogenous circadian *mPer2* expression. At time zero, TPA was added to the medium, which was replaced with serum-free DMEM after 2 h. TPA treatment without serum can trigger the induction of the circadian oscillation of expression of some genes, including *mPer2*, with an approximate period of 24 h in NIH 3T3 cells. And the trough level of *mPer2* mRNA occurs 12 h after TPA treatment and peaks 24 h after TPA treatment (18). Cells were then divided into three radiotreatment groups: a) control group (cells irradiated without TPA treatment), b) TPA 12-h group (cells irradiated at 12 h after TPA treatment with trough *mPer2* level), and c) TPA 24-h group (cells irradiated at 24 h after TPA treatment with peak *mPer2* level).

Cell transfection

The eukaryotic expression vector pcDNA3.1(+)-*mPer2* containing a cDNA copy of *mPer2* (GenBank No. NM_011066) was used in this study. The *mPer2* gene was confirmed as being in frame with no mutations by DNA sequencing. The cells were transfected with the indicated plasmids using the lipofectamine 2000 transfection reagent (Invitrogen, USA). Cell lysates were prepared 48 h later for the examination of protein expression and radiotreatment. The cells were divided into three groups: a) control group (cells without transfection), b) pcDNA3.1 group (cells transfected with the empty vector pcDNA3.1), and c) pcDNA3.1-*mPer2* group (cells transfected with pcDNA3.1-*mPer2*).

Antibodies

Mouse antibodies against mPER2, P53, BAX and proliferating cell nuclear antigen (PCNA) were purchased from Sigma (USA). Rabbit anti-goat IgG and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA).

Western blot analysis

At 48 h after transfection, cells were lysed with cold RIPA lysis buffer (Sigma-Aldrich, USA) containing protease inhibitors, and proteins were collected by centrifugation. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, USA) and transferred electro-phoretically to a polyvinylidene difluoride membrane (Pierce). Detection was carried out using an enhanced chemiluminescence reagent (Pierce).

Radiotreatment

All cell groups were irradiated with γ-rays, with a total absorbed dose of 4 Gy, using a ^{60}Co teletherapy machine (Phoenix, Japan). Cells were exposed at a dose rate of 115.38 cGy/min in an exposure field of 25 x 25 cm, with spacing of 80 cm. Cells were then processed for flow cytometry and colony formation assay to test cell death, proliferation and clonogenic survival.

Flow cytometry

To determine the expression of mPER2 protein, the cells were harvested at 48 h after transfection, fixed in 70% ethanol for 30 min at 4°C, and incubated with 0.1% saponin for 20 min. They were then incubated with primary antibodies (Sigma) at 1:200 dilution for 30 min and with fluorescein-isothiocyanate-conjugated secondary antibodies (Chemicon, USA) at 1:150 dilution for 30 min, and analyzed by flow cytometry (Beckman Coulter Elite ESP, USA).

Six hours after radiotreatment, all cells were digested with trypsin, harvested, washed with phosphate-buffered

saline (PBS), fixed in 70% ethanol for 30 min at 4°C, treated with 50 µg/mL RNase A (Sigma), stained with 50 µg/mL propidium iodide for 20 min at 4°C without light, and analyzed by flow cytometry using an instrument equipped with a 488-nm argon laser for the determination of DNA synthesis and cell cycle status. Data were collected in linear mode and analyzed with the Multicycle Software (Beckman Coulter, USA). Apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in so-called "sub-G1" peaks on DNA histograms. Four distinct phases were recognized by flow cytometry in a proliferating cell population, including the G0/G1, S- (DNA synthesis phase), G2 and M-phases (mitosis).

Colony formation assay

Six hours after radiotreatment, all cells were digested with trypsin, statically cultured in DMEM in the cell culture incubator (SANYO, Japan) for 14 days, washed with PBS, fixed with methanol, stained for 15 min and washed again. The preparation was then photographed with the Omegapic formatter and analyzed.

RT-PCR

Total RNA of cells treated by TPA was isolated with Trisol reagent (Invitrogen) 30 min after irradiation. RT-PCR for mouse *bax*, *bcl-2*, *p53*, *c-myc*, *rad50*, *mre11, nbs1*, and *GAPDH* mRNA was carried out. The primer sequences were 5'-GATGCGTCCACCAAGAA-3' and 5'-AGTAGAAG AGGGCAACCAC-3' for *bax*, 5'-CCCAAGGGAAGACG ATG-3' and 5'-GAGCGGGTAGGGAAAGA-3' for *bcl-2*, 5'-CCCAAGGGAAGACGATG-3' and 5'-GAGCGGGTAG GGAAAGA-3' for *c-myc*, 5'-GCAACGAGCCCTCAACA-3' and 5'-GGACCCACGGATGAACCT-3' for *p53*, 5'-TTTGG CGGAGTACCTATC-3' and 5'-CACCACTCGGTAGTTGT AAT-3' for *rad50*, 5'-GGCGAAGCAGTTCAAGAG-3' and 5'-GGCTGTTGTCGGGTAGAT-3' for *mre11*, 5'-GGAAGCC GACACCTCATC-3' and 5'-CACAATCATTTACGCACAG-3' for *nbs1*, and 5'-TCACTGCCACCCAGAAGA-3' and 5'-AAGTCGCAGGAGACAACC-3' for *GAPDH*. RT-PCR products were detected by 1% agarose electrophoresis and analyzed according to the integral optical density method with a Gel-Pro analyzer. Lane-to-lane variation in the amount of loaded mRNA was controlled internally by normalizing the level of each gene to that of *GAPDH*.

Immunohistochemistry

Six hours after irradiation, the cells treated with TPA were used for immunohistochemistry. The test was carried out using the avidin-biotin complex method. The numbers of P53-, BAX- and PCNA-positive and -negative NIH 3T3 cells were determined in four random fields at 100X and 400X magnification, and the percentages of positive cells were calculated.

Statistical analysis

Data are reported as means ± SD. One-way ANOVA was used to compare difference among groups and P values of less than 0.05 were considered to be statistically significant.

Results

The effects of radiation on NIH 3T3 cells treated with TPA

Parental NIH 3T3 cells normally produce very low and barely detectable levels of *mPer2*. In the present study, NIH 3T3 cells, TPA treatment was used to induce endogenous *mPer2* expression and to trigger the induction of the circadian oscillation of expression of various clock and clock-related genes, including *mPer2*. The expression level of *mPer2* mRNA oscillated within an approximate period of 24 h, with the trough occurring after 12 h and the peak after 24 h of TPA treatment.

The death and proliferation of cells irradiated after TPA treatment were determined by flow cytometry. The cells irradiated at peak *mPer2* expression had much lower apoptotic peaks than cells irradiated at trough *mPer2* expression and than control cells irradiated without TPA treatment ($P < 0.01$; Figure 1).

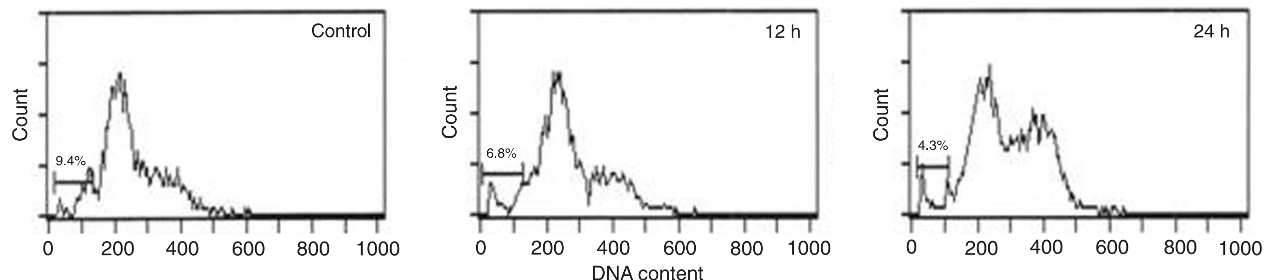


Figure 1. Apoptosis in NIH 3T3 cells irradiated after 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. Control: cells irradiated without TPA treatment; 12 h: cells irradiated 12 h after TPA treatment; 24 h: cells irradiated 24 h after TPA treatment. The percentage of sub-G1 cells undergoing apoptosis is indicated by the bar. The cells of the TPA 24-h group had a significantly lower apoptotic peak than the cells of the TPA 12-h group and of control cells ($P < 0.01$, one-way ANOVA).

The G0/G1, S-, G2, and M-phases of the cell cycle were also analyzed by flow cytometry, with the following cell percentages being observed in each phase: control group, G0/G1 (59.3 ± 1.07%), S (29.2 ± 0.30%), and G2/M (11.7 ± 0.21%); TPA 12-h group, G0/G1 (58.8 ± 0.91%), S (32.2 ± 0.66%) and G2/M (9.4 ± 0.47%); TPA 24-h group, G0/G1 (53.2 ± 0.73%), S (42.7 ± 0.43%), and G2/M (4.1 ± 0.34%). The S-phase fraction was greater in TPA 24-h group cells than in TPA 12-h group cells and control cells (P < 0.01; Figure 2). Clonogenic survival was determined by the colony formation assay. The colony-forming efficiency was significantly higher in TPA 24-h group cells than in TPA 12-h group cells and control cells (P < 0.01; Figure 3). Irradiated NIH 3T3 cells expressing high levels of *mPer2* showed less cell death and more cell proliferation and clonogenic survival. Three independent experiments demonstrated similar results.

Role of radiation in NIH 3T3 cells with up-regulated *mPer2*

We established *mPer2*-overexpressing cells by transfecting pcDNA3.1(+)-*mPer2* into NIH 3T3 cells. Successful transfection of the *mPer2* gene using the lipofectamine 2000 reagent was confirmed by Western blotting (Figure 4A). It was also evident by flow cytometry that the fluorescence intensity of mPER2 protein expression in pcDNA3.1(+)-*mPer2*-transfected cells was

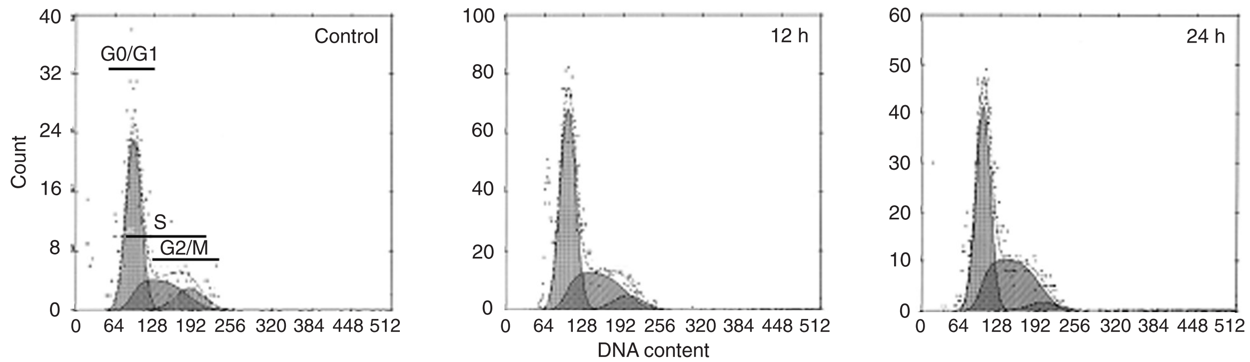
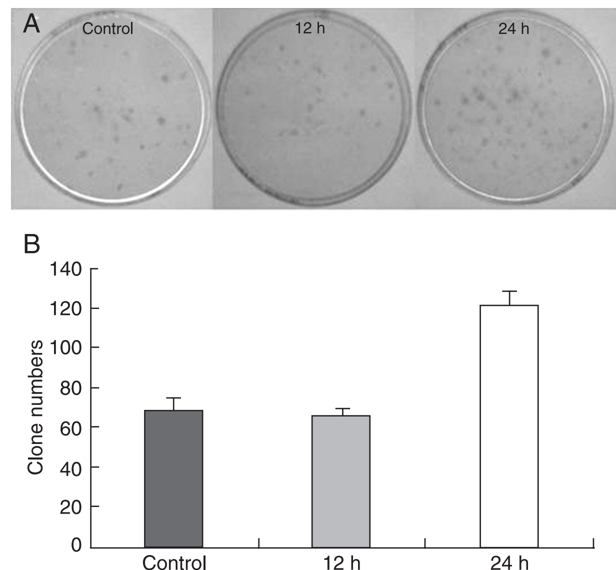


Figure 2. The cell cycle in NIH 3T3 cells irradiated after 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. Control: cells irradiated without TPA treatment; 12 h: cells irradiated 12 h after TPA treatment; 24 h: cells irradiated 24 h after TPA treatment. G0/G1, S and G2/M phases are indicated. The S-phase fraction of the TPA 24-h group was significantly higher than that of the TPA 12-h group and of control cells (P < 0.01, one-way ANOVA).

Figure 3. Colony formation of 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated NIH 3T3 cells after irradiation. *A*, Control: cells irradiated without TPA treatment; 12 h: cells irradiated 12 h after TPA treatment; 24 h: cells irradiated 24 h after TPA treatment. *B*, High expression of mPER2 dramatically increased the clonogenic survival of irradiated NIH 3T3 cells. The colony-forming efficiency of TPA 24-h group cells was significantly higher than that of TPA 12-h group cells and of control cells (P < 0.01, one-way ANOVA).



higher than in pcDNA3.1-transfected cells and in control cells without transfection (Figure 4B).

The effect of *mPer2* on the death, proliferation and clonogenic survival of cells irradiated after transfection was determined by flow cytometry and the colony formation assay. The results revealed that the apoptotic peak was lower in *mPer2* overexpressing cells than in empty vector cells and control cells (P < 0.01; Figure 5). Analysis of cell cycle distribution showed: control group, G0/G1 (46.3 ± 1.21%), S (40.9 ± 1.67%), and G2/M (12.8 ± 0.78%); pcDNA3.1 group, G0/G1 (55.6 ± 0.84%), S (39.0 ± 0.56%), and G2/M (5.4 ± 0.41%); pcDNA3.1-*mPer2* group, G0/G1 (45.9 ± 1.04%), S (46.5 ± 0.72%), and G2/M (7.6 ± 0.54%). The S-phase fraction of *mPer2* overexpressing cells was higher than that of empty vector cells and control cells (P < 0.01; Figure 6). mPER2 dramatically increased the clonogenic survival of irradiated NIH 3T3 cells. The colony-forming efficiency of *mPer2* overexpressing cells was also higher than that of other cells (P < 0.01; Figure 7). Three independent experiments demonstrated that irradiated NIH 3T3 cells overexpressing *mPer2* also showed less cell death and more cell proliferation and clonogenic survival.

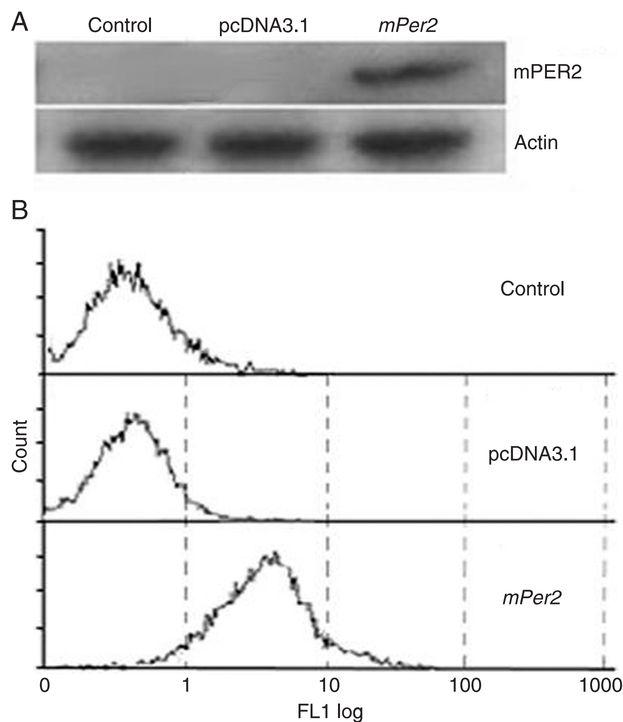


Figure 4. Detection of mPER2 expression by Western blot analysis and flow cytometry. Control: cells without transfection; pcDNA3.1: cells transfected with pcDNA3.1; *mPer2*: cells transfected with pcDNA3.1-*mPer2*. *A*, Western blot analysis of mPER2 expression. The mPER2 expression of *mPer2* group cells was significantly higher than that of pcDNA3.1 group cells and of control cells. *B*, Flow cytometric analysis of mPER2 expression. The fluorescence intensity of mPER2 expression in *mPer2* group cells was also significantly higher than that of pcDNA3.1 group cells and of control cells (P < 0.01, one-way ANOVA).

mPer2 up-regulated the expressions of cell proliferation- and apoptosis-related genes and DNA repair-related genes and proteins

We chose RT-PCR analysis and immunohistochemistry to study the mechanism of the effects of *mPer2* gene expression on the response of NIH 3T3 cells to radiation. RT-PCR analysis showed that the mRNA levels of *bax*, *bcl-2*, *p53*, *c-myc*, *mre11*, and *nbs1* were up-regulated in the irradiated cells at peak *mPer2* expression compared with cells at trough *mPer2* expression and control cells, with the expression of *c-myc* and *bcl-2* being particularly up-regulated. However, there was no significant difference in *rad50* level among the three cell groups (Figure 8). Immunohistochemistry also showed increased protein levels of P53, BAX and PCNA in irradiated NIH 3T3 cells with high mPER2 expression (Figure 9). Percentages of positive cells were: P53, control (34.1 ± 2.7%), 12 h (40.2 ± 3.9%), and 24 h (61.3 ± 3.6%); BAX, control (20.7 ± 4.1%), 12 h (22.8 ± 4.3%), and 24 h (37.1 ± 6.4%); PCNA, control (29.4 ± 3.2%), 12 h (33.7 ± 5.6%), and 24 h (87.3 ± 4.7%). Three independent experiments demonstrated similar results.

Discussion

Circadian clocks are molecular time-keeping mechanisms that reside in a diverse range of cell types in a variety of organisms. The primary role of these cell-autonomous clocks is to maintain their own approximately 24-h molecular rhythms and to drive the rhythmic expression of genes involved in physiology, metabolism and behavior (19,20). The circadian clock is composed of multiple, single-cell circadian oscillators, which, when synchronized, generate coordinated circadian outputs that regulate overt rhythms. Eight clock genes involved in the interacting transcriptional-/translational-feedback loops that compose the molecular clockwork have been cloned (21).

Parental NIH 3T3 fibroblasts normally produce very low and barely detectable levels of circadian genes. TPA treatment is as effective as serum shock in triggering the induction of circadian gene expression in cultured cells, including *mPer2*. As a core circadian gene, *mPer2* not only maintains the circadian rhythm of cells, but also sustains the normal cell cycle. After TPA treatment, parental NIH 3T3 cells present circadian oscillation of *mPer2* expression within an approximate period of 24 h, with the trough at zeitgeber time 12 (ZT12)and the peak at ZT24 (18,22,23).

In the present study, we treated NIH 3T3 cells with TPA to induce endogenous *mPer2* expression, and cells were irradiated at ZT12 with trough *mPer2* level and at ZT24 with peak *mPer2* level. The death, proliferation and clonogenic survival of irradiated cells were determined by flow cytom-

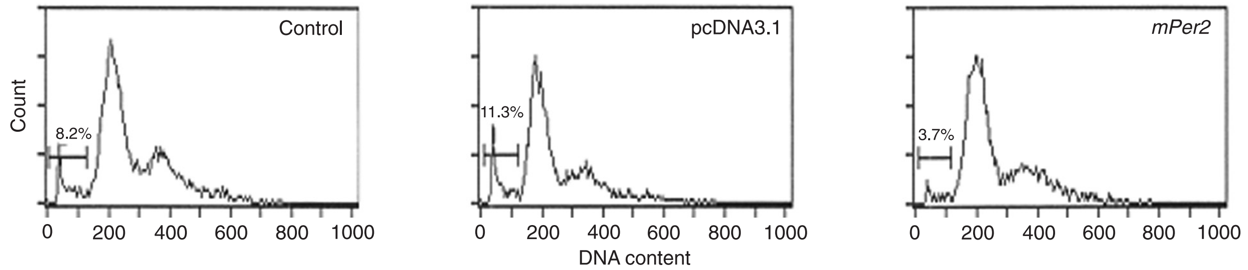


Figure 5. Apoptosis in NIH 3T3 cells irradiated after transfection. Control: cells without transfection; pcDNA3.1: cells transfected with pcDNA3.1; *mPer2*: cells transfected with pcDNA3.1-*mPer2*. The percentage of sub-G1 cells undergoing apoptosis is indicated by the bar. The apoptotic peak of *mPer2* overexpressing cells was lower than that of empty vector cells and of control cells (P < 0.01, one-way ANOVA).

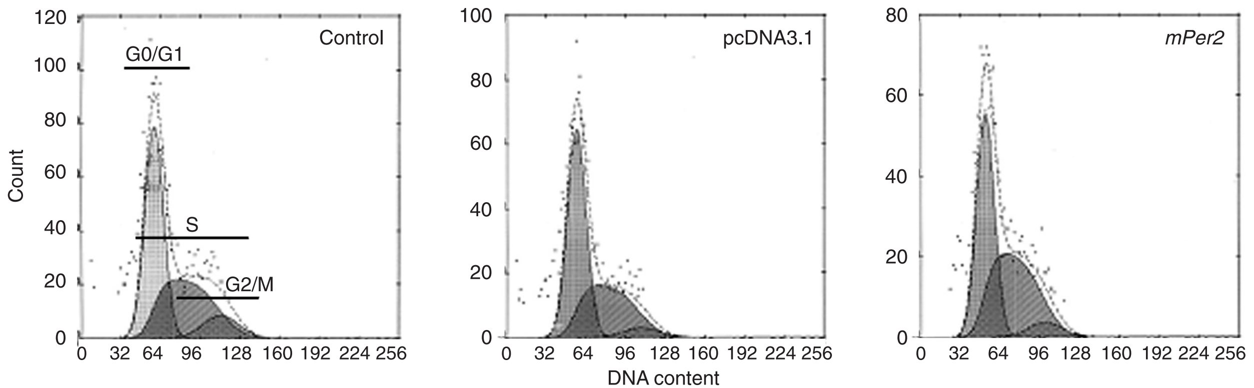
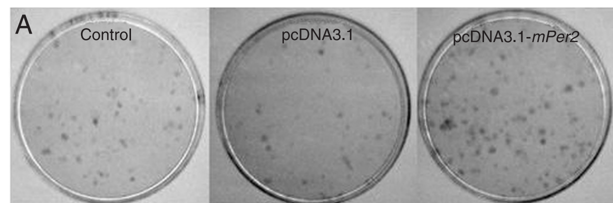
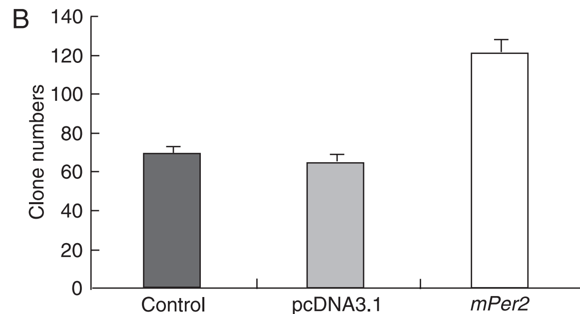


Figure 6. The cell cycle in NIH 3T3 cells irradiated after transfection. Control: cells without transfection; pcDNA3.1: cells transfected with pcDNA3.1; *mPer2*: cells transfected with pcDNA3.1-*mPer2*. G0/G1, S and G2/M phases are indicated. The S-phase fraction of *mPer2* overexpressing cells was higher than that of empty vector cells and of control cells (P < 0.01, one-way ANOVA).

Figure 7. Colony formation of transfected NIH 3T3 cells after irradiation. *A*, Control: cells without transfection; pcDNA3.1: cells transfected with pcDNA3.1; *mPer2*: cells transfected with pcDNA3.1-*mPer2*. *B*, mPER2 dramatically increased the clonogenic survival of irradiated NIH 3T3 cells. The colony-forming efficiency of *mPer2* overexpressing cells was significantly higher than that of empty vector cells and of control cells (P < 0.01, one-way ANOVA).





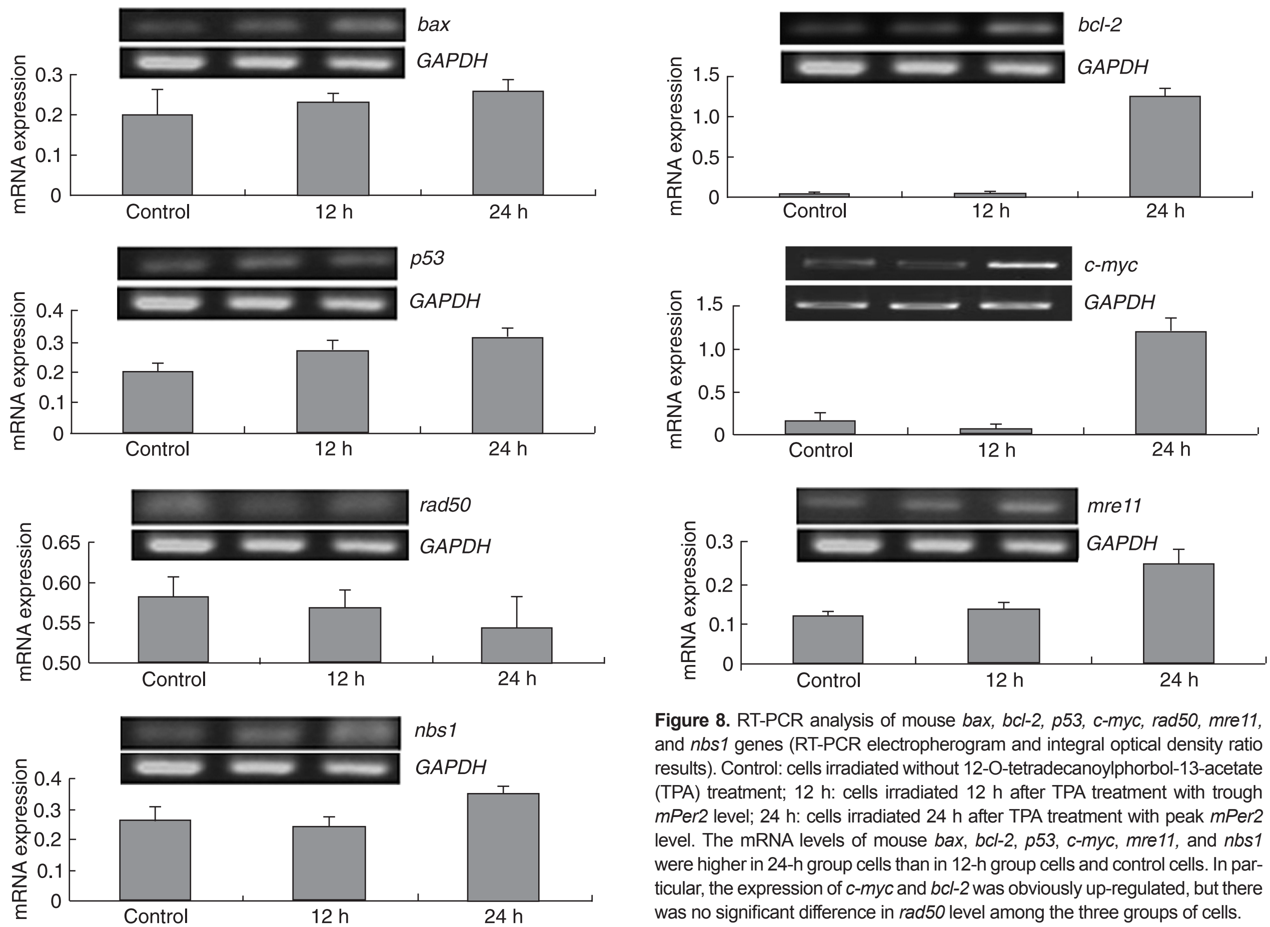


Figure 8. RT-PCR analysis of mouse *bax, bcl-2, p53, c-myc, rad50, mre11,* and *nbs1* genes (RT-PCR electropherogram and integral optical density ratio results). Control: cells irradiated without 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment; 12 h: cells irradiated 12 h after TPA treatment with trough *mPer2* level; 24 h: cells irradiated 24 h after TPA treatment with peak *mPer2* level. The mRNA levels of mouse *bax*, *bcl-2*, *p53*, *c-myc*, *mre11,* and *nbs1* were higher in 24-h group cells than in 12-h group cells and control cells. In particular, the expression of *c-myc* and *bcl-2* was obviously up-regulated, but there was no significant difference in *rad50* level among the three groups of cells.

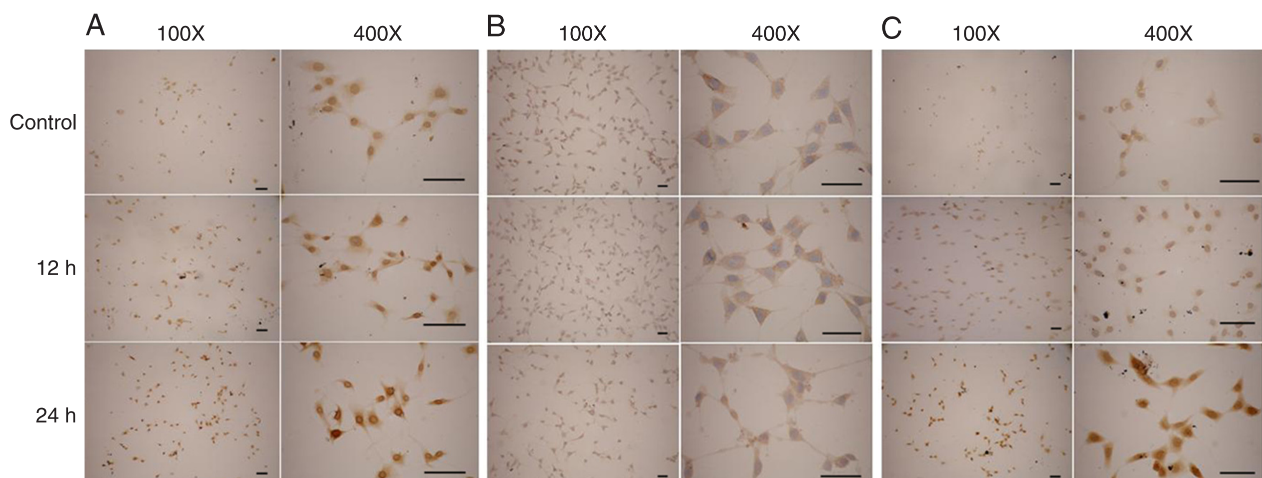


Figure 9. Expression of P53 (A), BAX (B) and proliferating cell number antigen (PCNA; C) proteins determined by immunocytochemistry (100X, 400X magnification). Control: cells irradiated without 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment; 12 h: cells irradiated 12 h after TPA treatment; 24 h: cells irradiated 24 h after TPA treatment. P53, BAX and PCNA levels were up-regulated in cells irradiated with peak *mPer2* level (24-h group) compared to cells irradiated with trough *mPer2* level (12-h group) and control cells. Magnification bar = 200 μm.

etry and colony formation assay. The results of the present study showed that high expression of *mPer2* might reduce cell death and enhance cell proliferation of irradiated NIH 3T3 cells. However, after TPA treatment, parental NIH 3T3 cells presented circadian expression not only of *mPer2*, but also of other clock and clock-related genes such as *mPer1* and *DBP* (18). Therefore, the radiobiological effects may result from *mPer2* or other clock and clock-related genes. In order to assess the effects of *mPer2* expression on the irradiated cells, we transfected pcDNA3.1(+)-*mPer2* into NIH 3T3 cells. The results showed that overexpression of mPER2 in NIH 3T3 cells resulted in reduced cell death and enhanced cell proliferation after radiation, suggesting that overexpressed *mPer2* may diminish the radiosensitivity of NIH 3T3 cells.

Fu et al. (16) reported that the loss of *mPer2* function resulted in increased tumor development and deficiencies in response to DNA damage in mice, suggesting that the *mPer2* gene functions in tumor suppression by regulating DNA damage-responsive pathways. Compared with wild-type mice, *mPer2* mutant mice showed a neoplastic growth phenotype and an increased sensitivity to γ radiation in thymocytes. Zhang et al. (17) also found that high expression of the circadian gene *mPer2* might diminish radiosensitivity of irradiated mouse tumor cells. LLC and EMT6 cells with high *mPer2* expression exposed to ^{60}Co-γ-rays presented reduced DNA damage, increased survival and clone-forming rate, which suggested that *mPer2* might protect cells against the radiation injury of γ-rays and increase the survival rate of tumor cells. The present study demonstrated that high expression of *mPer2* could diminish the radiosensitivity of NIH 3T3 cells, with reduced cell death, enhanced cell proliferation and increased clonogenic survival. Moreover, tumor cells and NIH 3T3 cells were irradiated at different times after TPA induction, i.e., at 12 h (trough time of *mPer2* expression) and at 24 h (peak time) after induction, whereas LLC cells were irradiated at 18 h (trough time) and 30 h (peak time) after induction. The present research combined with the previous research of Fu et al. (16) and Zhang et al. (17) would contribute to protecting normal cells against radiation injury in cancer radiotherapy. We propose that radiotherapy could be used against cancer at a certain time of *Per2* expression for the best ratio of tumor suppression and normal tissue protection, which can kill tumor cells and protect normal cells maximally. However, our hypothesis needs further study.

Without radiation, overexpression of mPER2 results in reduced proliferation and rapid apoptosis of tumor cells, but not of NIH 3T3 cells, suggesting that *mPer2* may play an important role in tumor suppression by inducing apoptotic cell death (24). However, irradiated NIH 3T3 cells with mPER2 overexpression showed reduced cell death, suggesting that *mPer2* may affect the radiation-induced cell death by other ways.

In response to ionizing radiation, cells immediately activate a series of biochemical pathways that promote cell survival while maintaining genetic integrity. The main cellular defense system against ionizing radiation exposure is composed of two distinct types of biochemical pathways, i.e., the DNA damage cell cycle checkpoint pathways and the DNA repair pathways (25). Arrest of replicative DNA synthesis after DNA damage is thought to occur to provide ample time for the cell to repair DNA lesions before the S-phase (G1 arrest) and/or mitosis (G2 arrest).

The RAD50-MRE11-NBS1 (MRN) complex plays an important role in the repair of DNA damage caused by radiation. The complex practically participates in all repair mechanisms, especially homologous recombination and non-homologous end-joining, which are the most important mechanisms in the repair of DNA double-strand breaks (26). In the present study, the levels of *rad50* expression of the various cell groups were not different, in agreement with reports showing that *rad50* expression is persistent and stable (27). As the core part of the MRN complex, MRE11 has not only nuclease activity, but also a connective effect on RAD50 and NBS1, and its expression changes rapidly after radiation (28). NBS1 is also an important part of the MRN complex (29). The irradiated NIH 3T3 cells expressing high levels of *mPer2* showed an increased expression of *mre1l* and *nbs1*, which suggested that *mPer2* could enhance the function of DNA damage repair MRN complex.

c-Myc, a proto-oncogene, plays an important role in both cell proliferation and apoptosis (30,31). Circadian regulators may target genes that are controlled by *c-myc*. *c-Myc* itself is also controlled by the circadian clock, and the level of *c-myc* mRNA oscillated in 24-h light/dark cycles in wild-type mouse livers, peaking at ZT14 (16). Gamma radiation may increase the expression of *c-myc*, whose overexpression could drive cells to progress through the cell cycle in the presence of genomic DNA damage in order to improve the efficiency of DNA repair (32). It has also been reported that *c-myc* directly regulates the transcription of the *nbs1* gene involved in DNA double-strand break repair (33). The present study showed that *c-myc* expression increased in irradiated NIH 3T3 cells with high expression of *mPer2*, thus suppressing cell death and enhancing cell repair.

BCL-2 and BAX are members of the BCL-2 family that is a key regulator of the mitochondrial response to apoptotic signals in the intrinsic pathway (34,35). *Bcl-2* is an antiapoptotic gene acting as a potent suppressor of apoptosis by blocking the release of cytochrome *c*, whereas *bax* is a proapoptotic gene with oppose functions acting as a promoter of cell death. The ratio of antiapoptotic-to-proapoptotic molecules determines the response to a death signal (36). In the present study, the high expression of *mPer2* increased the BCL-2/BAX ratio, thus suppressing cell death and enhancing cell growth.

Wild-type P53 protein levels rise dramatically after exposure to ionizing radiation. This rise results from as yet undefined changes in the post-transcriptional modifications

undergone by the P53 protein such as phosphorylation, binding to other proteins, or oligomerization. At subsequent end points of DNA-damage, a prolonged half-life was observed as well as increased DNA-binding activity of the P53 protein and enhanced transcriptional transactivation activity driven by this protein (37,38). P53 can keep genomic stability by mediating apoptosis and DNA repair (7-9,39) and can induce a transient arrest of the cell cycle at G1, so that the cells will have time to repair damaged DNA. Activated p53 can also eliminate cells through mechanisms involving prolonged arrest at G1 or apoptosis. Our study showed an increased expression of p53 in irradiated NIH 3T3 cells at peak *mPer2* expression, with decreased cell death. This indicated that a high expression of mPER2 up-regulated P53 expression, which could enhance DNA repair.

PCNA is a protein that acts as a processivity factor for DNA polymerase delta in eukaryotic cells. PCNA is an essential factor in cell proliferation and can be used as an index to evaluate cell proliferation (40). Since DNA polymerase delta is involved in resynthesis of excised damaged DNA strands during DNA repair, PCNA is important for both DNA synthesis and DNA repair. PCNA is also involved in the DNA damage tolerance pathway known as post-replication repair. In the present study, the irradiated cells with high expression of *mPer2* showed enhanced cell proliferation, and the increase in PCNA staining after DNA damage could be a result of DNA repair.

After radiation, the high expression of *mPer2* in NIH 3T3 cells results in reduced cell death and enhanced cell proliferation and clonogenic survival, which means that high expression of the circadian gene *mPer2* diminishes radiosensitivity of NIH 3T3 cells. A high expression of *mPer2* may up-regulate the expression of apoptosis-related genes, with an increased proportionality of *bcl-2*/*bax*, and may reduce cell death and enhance cell proliferation to diminish radiosensitivity. Moreover, *mPer2* may also up-regulate DNA repair-related genes to increase DNA-repair efficiency after radiation to diminish radiosensitivity. Future research should be focused on studying the detailed mechanisms by which the circadian clock controls genes related to radiosensitivity.

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