

Circadian gene *mPer2* overexpression induces cancer cell apoptosis

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The *Period2* gene, an indispensable component of the circadian clock, not only modulates circadian oscillations, but also regulates organic function. We examined whether overexpression of the mouse *Period2* gene (*mPer2*) in tumor cells influences cell growth and induces apoptosis. Overexpression of PERIOD2 in the mouse Lewis lung carcinoma cell line (LLC) and mammary carcinoma cell line (EMT6) results in reduced cellular proliferation and rapid apoptosis, but not in NIH 3T3 cells. Overexpressed mPER2 also altered the expression of apoptosis-related genes. The mRNA and protein levels of *c-Myc*, *Bcl-X_i* and *Bcl-2* were downregulated, whereas the expression of *p53* and *bax* was upregulated in mPER2-overexpressing LLC cells compared with control cells transferred with empty plasmid. Our results suggest that the circadian gene *mPeriod2* may play an important role in tumor suppression by inducing apoptotic cell death, which is attributable to enhanced pro-apoptosis signaling and attenuated anti-apoptosis processes. (*Cancer Sci* 2006; 97: 589–596)

Circadian rhythms, which are daily oscillations regulated by an endogenous clock,^(1–3) enable organisms to adapt to daily environmental changes such as light, temperature and social communication, and serve to synchronize multiple molecular, biochemical, physiological and behavioral processes.^(4–6) Recent studies suggest that the circadian system is not only required for proper growth control, but is also involved in the circadian regulation of cell proliferation and apoptosis.^(7–9) It has been reported that 2–10% of all mammalian genes are clock-controlled.^(4,5,10–12) Most of these show tissue- or organ-specific expression and are involved in organ function. Only a small set of clock-controlled genes are expressed in multiple organs. Among them are genes that encode key regulators of cell cycle progression.^(10,11) Deregulation of the circadian clock may disturb the expression of clock-controlled genes and can have a profound influence on organ function.

It is now documented that alterations in circadian rhythm can be associated with cancers in both animal and human tumors.^(13,14) Up to now, at least eight core circadian clock genes have been identified in mammals and humans,^(2,15) and the *Period2* gene is regarded as an indispensable component of the circadian clock.⁽¹⁶⁾ Gene targeting studies have demonstrated that the deletion of *mPer2* induces arrhythmicity at both the behavioral and molecular levels.⁽¹⁷⁾ Mice without mPER2 function have a transient rhythm with a shortened period length of 22 h. A majority of mutant mice lose the persistence of cir-

cadian rhythm when placed in free-running conditions.⁽¹⁸⁾ The mice deficient in the *mPer2* gene were cancer-prone. After γ radiation, these mice showed a marked increase in tumor development and reduced apoptosis in thymocytes. 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.⁽¹⁹⁾

The relationship between *mPer2* and cancer still lacks direct demonstration. In the present study, we transferred pcDNA3.1(+)-*mPer2* into LLC cells, EMT6 cells and NIH 3T3 cells, assessed the effect of mPER2 overexpression on these cells and explored the possible signaling pathways leading to apoptotic cell death. Results show that overexpression of mPER2 may induce tumor cell apoptosis by the p53-mediated mitochondrial signaling pathway.

Materials and Methods

Plasmid

The eukaryotic expression vector pcDNA3.1(+)-*mPer2* containing a cDNA copy of *mPer2* (GenBank number NM_011066) was used in this study. The *mPer2* was confirmed as being in frame with no mutations by DNA sequencing.

Cell culture and transfection

Lewis lung carcinoma is a cell line established from the lung of a C57BL mouse bearing a tumor resulting from an implantation of primary Lewis lung carcinoma. EMT6 was established from a transplantable murine mammary carcinoma that arose in a BALB/cCRGL mouse after implantation of a hyperplastic mammary alveolar nodule. LLC, EMT6 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with antibiotics (BioWhittaker, Walkersville, MD, USA) and 10% fetal calf serum (Hyclone) in an atmosphere of humidified 95% air and 5% CO₂ at 37°C. The cells were transfected with the plasmids indicated using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA). Cell lysates were prepared 48 h later for the examination of protein expression.

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Abbreviations: EMT6, mouse mammary carcinoma cell; LLC, Lewis lung carcinoma cell; *mPer2*, mouse *Period2* gene; mPER2, mouse PERIOD2 protein; ODN, antisense phosphorothioate oligonucleotide; RT-PCR, reverse transcription-polymerase chain reaction; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

Antisense phosphorothioate oligonucleotides

Antisense phosphorothioate oligonucleotides for mPER2 mRNA were used to block mPER2 expression. The efficacy of this method and sequences of ODN have been described previously.⁽²⁰⁾ The antisense sequence targeting the coding region of mPER2 was 5'-CACGTATCCATTCATGTGCG-3', and the randomized control was 5'-ACCATGTTACCTGACCTGT-3'. All ODN were purified and amplified using high-performance liquid chromatography and phosphorothioated to increase membrane permeability. Oligonucleotides were used at a final concentration of 10 μ M.

We treated the cells with 100 nM TPA to induce endogenous mPER2 expression in LLC cells.⁽²¹⁾ Antisense oligonucleotides were used to block TPA-induced mPER2 expression. To enhance cellular uptake, antisense and control oligonucleotides were added to the cultures using Lipofectamine 2000 reagent (Invitrogen). Following the addition of TPA and oligonucleotides for 24 h, cells were grown in serum-free medium. At 48 h later, cells were collected, fixed, and processed for flow cytometric analysis. We analyzed serum starvation-induced apoptosis in three groups: (i) TPA alone; (ii) TPA and randomized oligonucleotides; and (iii) TPA and antisense oligonucleotides targeting *mPer2*.

Cell growth and proliferation

Cell growth and proliferation was assessed using the cell colony-forming assay, cell growth curves and the MTT assay according to the standard protocol. Each assay was repeated three times.

Flow cytometry

The cells were harvested, washed with phosphate-buffered saline and fixed in 70% ethanol for 30 min at 4°C, then treated with 50 μ g/mL of RNase A (Sigma, St Louis, MO, USA), stained with 50 μ g/mL propidium iodide for 20 min at 4°C without light, and analyzed by flow cytometry for DNA synthesis and cell cycle status (Beckman Coulter Elite, Miami Lakes, FL, USA).

To analyze the expression of apoptosis-related proteins, the cells were harvested, 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 a dilution of 1 : 100 for 30 min and incubated with fluorescein-isothiocyanate-conjugated secondary antibodies (Chemicon, Temecula, CA, USA) at a dilution of 1:100 for 30 min. The cells were then analyzed by flow cytometry.

Antibodies

Mouse antibodies against mPER2, P53, c-MYC, BCL-2, BAX, BCL-X_L and actin were purchased from Sigma. Rabbit anti-goat IgG and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunohistochemistry

Immunohistochemistry was carried out using the avidin-biotin complex method. The numbers of PER2-positive and PER2-negative tumor cells were determined in four random fields ($\times 100$ magnification), and the percentage of PER2-positive cells was then calculated.

Morphological study

The cells were harvested and prepared for electron microscopy in the conventional manner. They were stained with uranyl acetate and lead citrate, and observed with an H-600 IV transmission electron microscope (Hitachi, Tokyo, Japan).

DNA isolation and fragmentation assay (laddering)

DNA from each group was extracted using phenol/chloroform followed by ethanol precipitation. The DNA was dissolved in distilled water, and the concentration was measured spectrophotometrically. DNA (5 μ g) from each sample was analyzed by electrophoresis on a 1.8% agarose gel containing ethidium bromide.

RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen). RT-PCR for mouse *Per2*, *p53*, *c-Myc*, *Bcl-2*, *Bax*, *Bcl-X_L* and *GAPDH* mRNA was carried out. Details of the primers and the GenBank accession numbers are given in Table 1.

Western blot analysis

Cells were lysed with cold RIPA lysis buffer containing protease inhibitors, and proteins were collected by centrifugation. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and transferred electro-phoretically to polyvinylidene difluoride (PVDF) membrane (Pierce). Detection was carried out using an enhanced chemiluminescence reagent (Pierce).

Statistical analysis

Data were presented as the mean \pm SD, and the Student's *t*-test was used for comparisons among different groups. *P*-values of less than 0.05 were considered statistically significant.

Table 1. Gene nomenclature, GenBank accession code, primer sequences, and predicted sizes of the amplified products for the different genes studied

| Gene | Accession number | Forward primer | Reverse primer | Size (bp) |
|--------------------------|------------------|----------------------|---------------------|-----------|
| <i>Per2</i> | NM_011066 | GCAACGAGCCCTCAGACA | GGACCCACGGATGAACCT | 495 |
| <i>p53</i> | AF161020 | ATGGAGGATTCACAGTCGGA | TCAGTCTGAGTCAGGCCCA | 323 |
| <i>c-Myc</i> | X01023 | CCCAAGGGAAGACGATG | GAGCGGGTAGGGAAAGA | 225 |
| <i>Bcl-2</i> | AH001858 | GCTACCGTCGTGACTTCGC | TCCCAGCCTCCGTTATCC | 268 |
| <i>Bax</i> | L22472 | GATGCGTCCACCAAGAA | AGTAGAAGAGGGCAACCAC | 565 |
| <i>Bcl-X_L</i> | L35049 | AGGCAGGCGATGAGTTT | CCTTGCTACGCTTTCCAC | 412 |
| <i>GAPDH</i> | M88354 | TCACTGCCACCCAGAAGA | AAGTCGCAGGAGACAACC | 318 |

Results

Effects of mPER2 on cell growth and proliferation

Parental LLC, EMT6 and NIH 3T3 cells normally produce very low and barely detectable levels of mPER2. We established mPER2-overexpressing cells by transfecting mPER2 cDNA in a sense orientation into LLC, EMT6 and NIH 3T3 cells. The cells transfected with empty vector served as controls. Successful transfer of the *mPer2* gene using Lipofectamine 2000 was confirmed by western blotting (Fig. 1A). It was also evident by immunohistochemistry that the mPER2 protein was expressed in approximately 80% of pcDNA3.1(+)-*mPer2*-transfected LLC cells, but not in empty vector-transfected LLC cells (Fig. 1B), confirming the high transfection efficiency. The transfection efficiency in NIH3T3 and EMT6 cells was similar to that in LLC.

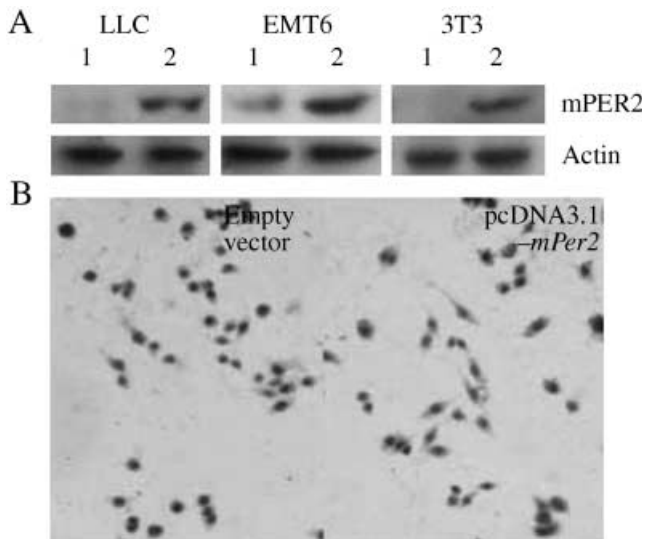
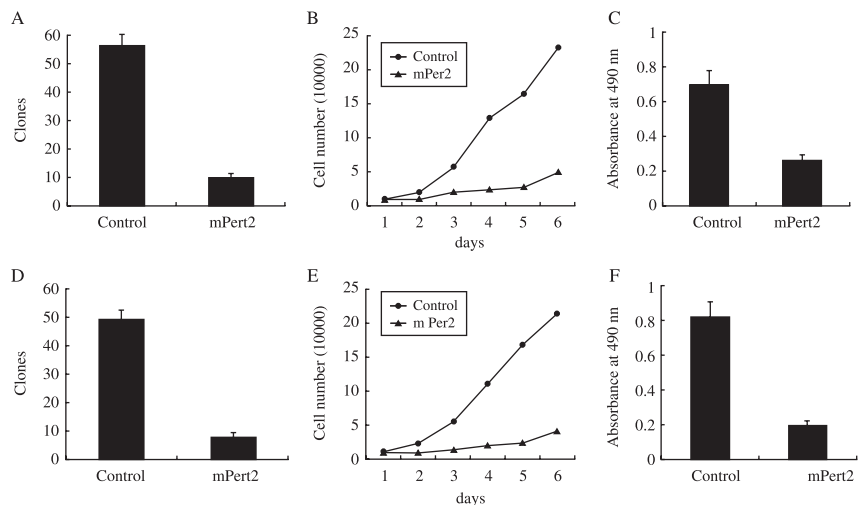


Fig. 1. Detection of mouse *Period2* (*mPer2*) expression by western blotting and immunohistochemistry. (A) Western blot analysis of mPER2 expression. (B) Immunohistochemistry of the pcDNA3.1(+)-*mPer2*-transfected Lewis lung carcinoma (LLC; right panel) cells and control cells (left panel). The right panel shows positive staining for mPER2.

Fig. 2. Analysis of the growth-inhibitory effect of mouse PERIOD2 (*mPER2*) overexpression on (A–C) Lewis lung carcinoma cells and (D–F) EMT6 cells. (A,D) Colony formation after gene transfection. mPER2 suppressed colony formation dramatically. The colony-forming efficiency of mPER2-overproducing cells was significantly lower than control cells in three independent experiments ($P < 0.01$). (B,E) Cell proliferation assay to quantify the number of viable cells. The numbers under the horizontal axis indicate the days for cell culture. The results indicate a significant difference in cell growth speed between the two groups ($P < 0.01$). (C,F) Growth-inhibitory effect of mPER2 on tumor cells detected by MTT assay. Absorbance at 490 nm showed a significant decrease in the growth of mPER2-overproducing cells compared with vector control cells ($P < 0.01$).



To test the effect of *mPer2* on cell growth, we carried out colony formation assays, cell growth curves and the MTT assay. *mPer2* dramatically suppressed cell growth in LLC and EMT6 cells, but not in NIH 3T3 cells. The colony-forming efficiency of mPER2 overexpressing cells (mean 10/dish for LLC cells, 8/dish for EMT6 cells) was significantly lower than in control cells (mean 56/dish in LLC cells, 49/dish in EMT6 cells) ($P < 0.01$) (Fig. 2A,D). Cell growth curves indicated a significant difference in cell growth speed between the two groups for both LLC and EMT6 cells ($P < 0.01$; Fig. 2B,E). *mPer2* also exhibited a statistically significant growth-inhibitory effect on LLC and EMT6 cells when evaluated by the MTT assay. Absorbance at 490 nm showed that mPER2 overexpression in tumor cells resulted in a significant decrease in cell growth, compared with control cells ($P < 0.01$) (Fig. 2C,F). In NIH 3T3 cells, there was no significant difference between the mPER2-overexpressing cells and the control cells.

Effects of mPER2 on cell death and apoptosis

Cell proliferation and apoptotic damage of DNA were determined by flow cytometry. The LLC and EMT6 cells overexpressing mPER2 had much higher apoptotic peaks than the vector control cells (Fig. 3). DNA from cells after gene transfection was isolated and analyzed for fragmentation into nucleosomes. DNA laddering was much more evident in mPER2-overproducing tumor cells than in empty vector-transfected cells (Fig. 4). There were different morphological characteristics in the cells overexpressing mPER2 compared with the control cells when studied by transmission electron microscopy. Control cells had intact membranes, organelles and normal nuclear morphology. Apoptotic cells in the pcDNA3.1(+)-*mPer2* group showed homogeneous chromatin condensation within the nucleus (Fig. 5).

Effects of mPER2 on the expression of apoptosis-related genes

We choose LLC cells to study the mechanism underlying the tumor suppressive effects of *mPer2*. Details of the primers and the GenBank accession numbers are given in Table 1. RT-PCR analysis showed that the expression of c-Myc, Bcl-X_L

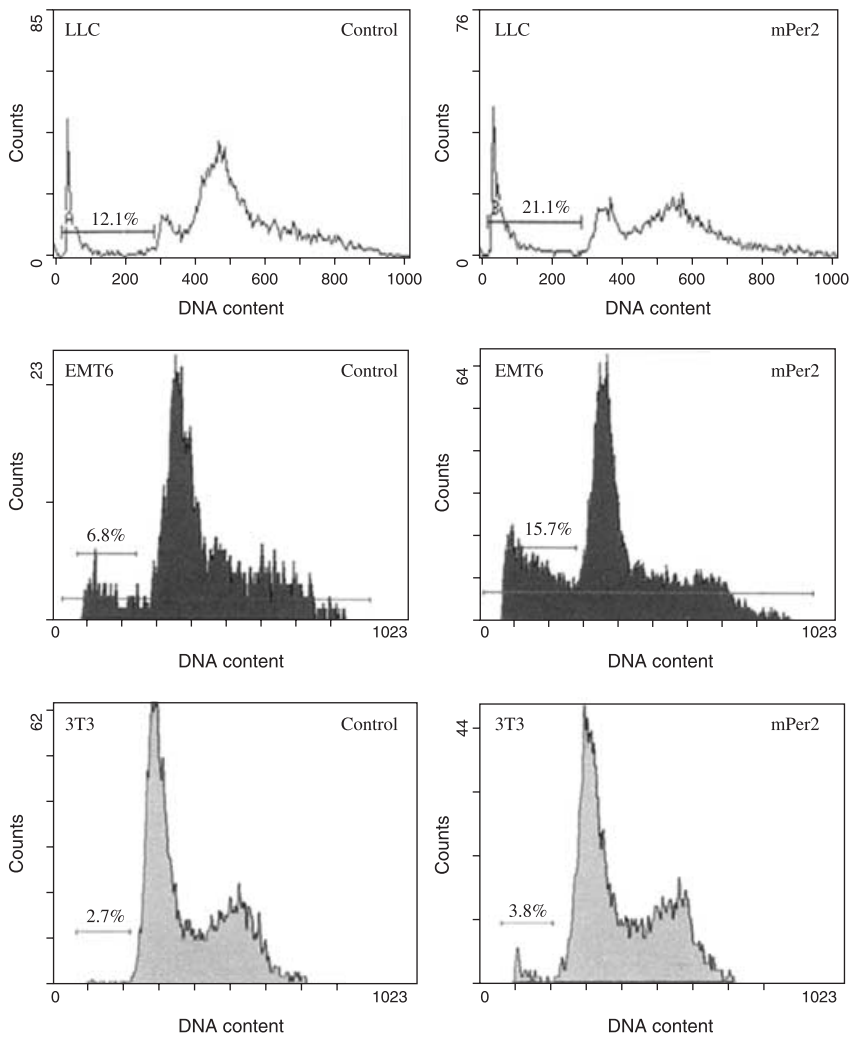


Fig. 3. Flow cytometric analysis of apoptosis in Lewis lung carcinoma (LLC), EMT6 and NIH 3T3 cells. Horizontal and vertical axes represent DNA content and cell number, respectively. The percentage of sub-G₁ cells undergoing apoptosis is indicated (bar). The LLC and EMT6 cells overexpressing mouse PERIOD2 (mPER2) had a much higher apoptotic peak than the vector control cells.

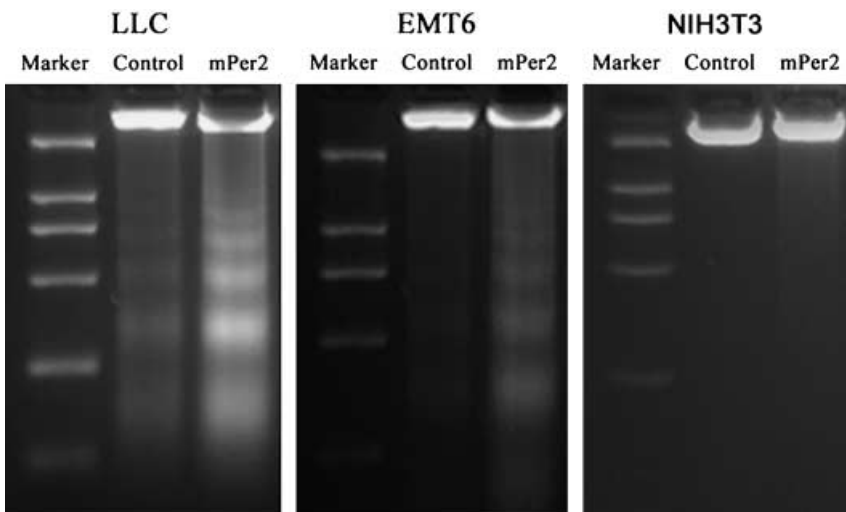


Fig. 4. DNA was isolated and analyzed by 1.8% agarose gel electrophoresis. DNA fragmentation (laddering) was much more evident in DNA samples from mouse PERIOD2 (mPER2)-overproducing Lewis lung carcinoma and EMT6 cells than in vector control cells. There was no difference in DNA fragmentation (laddering) between mPER2-overproducing NIH 3T3 cells and controls.

and Bcl-2 mRNA were downregulated, whereas the expressions of p53 and Bax mRNA were upregulated in mPER2-overexpressing LLC cells compared with vector control cells (Fig. 6A). The housekeeping gene *GAPDH*

was used to ensure the integrity of the RNA and equality of loading.

After protein normalization to actin levels, western blot analysis also demonstrated that the level of P53 in mPER2-

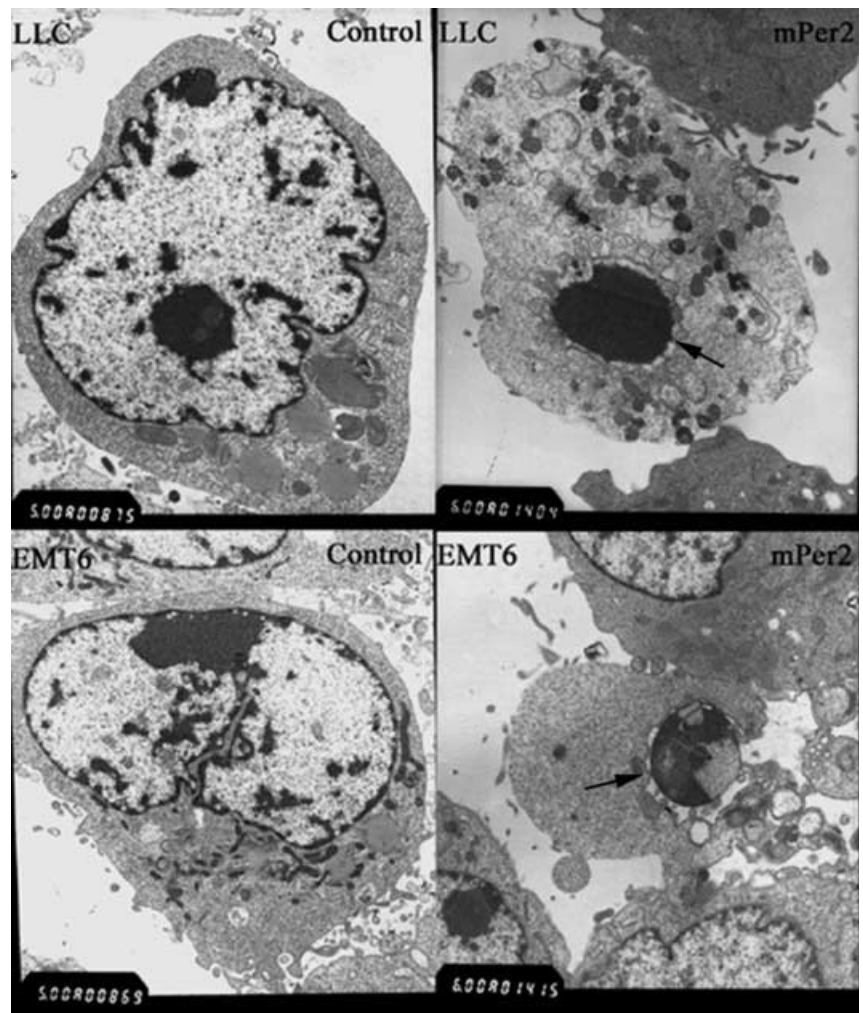


Fig. 5. Electron microscopic evaluation of Lewis lung carcinoma and EMT6 cells. Vector control cells had intact membranes and organelles, and normal nuclear morphology. Apoptotic cells in mouse PERIOD2 (mPER2)-overexpressing cells showed homogeneous chromatin condensation within the nucleus. Arrows indicate chromatin condensation within the nucleus. Magnification: $\times 6000$.

overexpressing cells was much higher than in vector control cells (Fig. 6B). In contrast, there was an apparent decrease in the levels of BCL-2 and c-MYC protein in mPER2-overproducing cells, compared with control cells. As shown in Fig. 6, the amount of BAX protein in mPER2-overexpressing cells tended to be higher, and the amount of BCL-X_L protein lower, than those in vector control cells. The densitometric results were used to calculate a ratio of BAX to BCL-X_L. The results revealed that the ratio was higher in mPER2-overexpressing cells than in vector control cells.

Fluorescence signals of apoptosis-related proteins were detected by flow cytometry in the LLC cells transfected with pcDNA3.1-*mPer2* and in control cells (Fig. 6C). The results also demonstrated increased amounts of P53 and BAX in mPER2-overexpressing cells than in vector control cells. In contrast, there was an apparent decrease in BCL-2, BCL-X_L and c-MYC levels in mPER2-overproducing cells, compared with control cells.

Effects of antisense oligonucleotides on mPER2 expression and serum starvation-induced cell apoptosis

Reverse transcription-polymerase chain reaction analysis was carried out to detect expression of mPER2 mRNA. As

shown in Fig. 7A, treatment with antisense oligonucleotides resulted in an obvious reduction in the expression of mPER2 mRNA in comparison with the controls. As demonstrated by flow cytometric analysis (Fig. 7B), the inhibition of endogenous *mPer2* expression by antisense oligonucleotides prevented cells from undergoing serum starvation-induced cell death, compared with the controls ($P < 0.01$).

Discussion

In the present study, the overexpression of mPER2 in mouse LLC and EMT6 cells was first described. As a core circadian gene, *mPer2* has the function of not only maintaining the circadian rhythm of cells, but also sustaining the normal cell cycle. *mPer2* exhibited a significant growth-inhibitory effect on LLC and EMT6 cells, as evaluated by MTT assay, cell growth curves and cell colony-forming assay. Several lines of evidence, including flow cytometry, DNA laddering and ultra-microstructure, consistently documented cell apoptosis in LLC and EMT6 cells. However, *mPer2* overexpression neither inhibited cell growth nor induced apoptosis in NIH 3T3 cells. The inhibition of endogenous *mPer2* expression by antisense

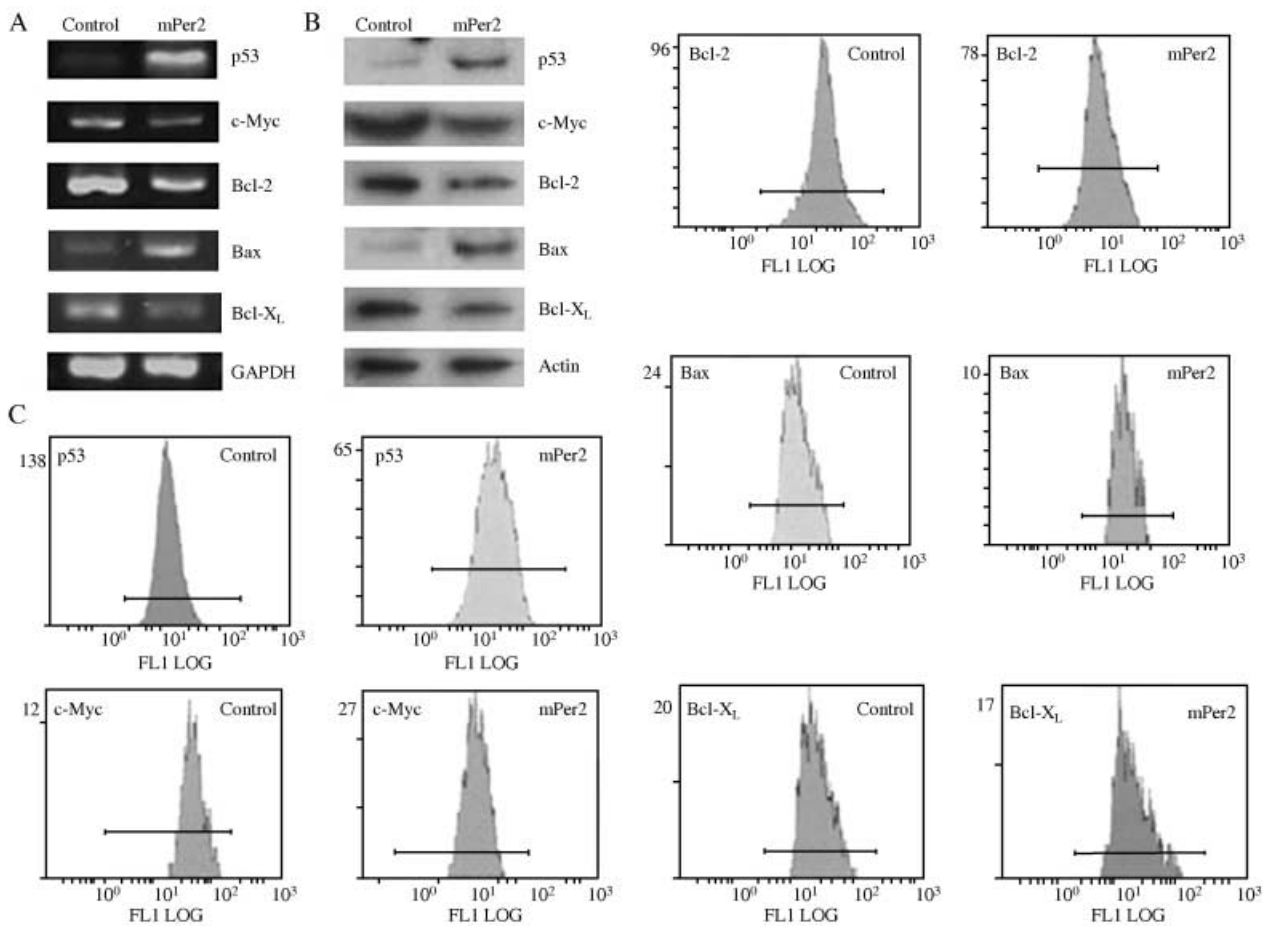


Fig. 6. Analysis of apoptosis-associated genes and proteins in Lewis lung carcinoma cells. (A) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of *p53*, *c-Myc*, *Bcl-2*, *Bcl-X_L*, and *Bax*. (B) Western blot analysis of P53, c-MYC, BCL-2, BAX and BCL-X_L. (C) Flow cytometric analysis of the expression of apoptosis-related proteins. RT-PCR, western blot analysis and flow cytometry all demonstrated increased amounts of P53 and BAX in mouse PERIOD2 (mPER2)-overexpressing cells than in vector control cells. In contrast, there was a decrease in BCL-2, BCL-X_L and c-MYC levels in mPER2-overproducing cells, compared with control cells.

oligonucleotides attenuated serum starvation-induced cell death.

The results of the present study suggest that overexpressed mPER2 may alter the expression of apoptosis-related genes. Recent studies have reported that approximately 7% of all clock-controlled genes identified in rodents regulate either cell proliferation or apoptosis. These clock-controlled genes involved in regulating the cell cycle and apoptosis include *c-Myc* and the tumor suppressor *p53*, as well as genes that encode caspases, cyclins and transcription factors.^(10,11,19,22) The rhythmic expression of several cyclins, as well as that of the tumor suppressor *p53*, are synchronized with the circadian oscillation patterns of *Per1* and *Bmal1* expression in human oral mucosa.^(23,24)

The tumor suppressor *p53* is a transcription factor that is activated in response to DNA damage or oncogenic transformation. Loss of *p53* in many cancers leads to impaired cell cycle regulation, genomic instability and inhibition of apoptosis.^(25–27) *p53* can induce a transient arrest in G₁ in cells, thus the cells have time to repair damaged DNA. Activated *p53* can also eliminate cells through mechanisms involving prolonged

arrest in G₁ or apoptosis. The elimination of damaged, stressed or abnormally proliferating cells by *p53* is considered to be the principal means by which *p53* mediates tumor suppression. The levels of *p53* mRNA and protein in mPER2-overexpressing LLC cells tended to be higher than in the vector control cells. This indicated that overexpressed mPER2 induced an increase in *p53* levels, which may contribute, at least in part, to apoptosis in mPER2-overexpressing cells.

The *c-MYC* oncoprotein is a transcription factor that promotes cell growth and proliferation, as well as apoptosis under certain conditions. The *c-Myc* P1 promoter is suppressed directly by the core circadian regulator *Bmal1*, indicating that *c-Myc* is a clock-controlled gene.⁽²⁴⁾ The expression of *c-Myc* mRNA shows a significant increase in all mouse tissues studied in *Per2* mutants. In the present study, there was an apparent decrease in *c-MYC* mRNA and protein levels in mPER2-overexpressing cells than in vector control cells. Downregulation of *c-Myc* is one possible mechanism by which overexpression of mPER2 could promote apoptosis. The overexpression of mPER2 induces *Bmal1* expression throughout 12:12 L : D cycles, and then increases intracellular levels of Bmal1–Npas2 or Bmal1–Clock

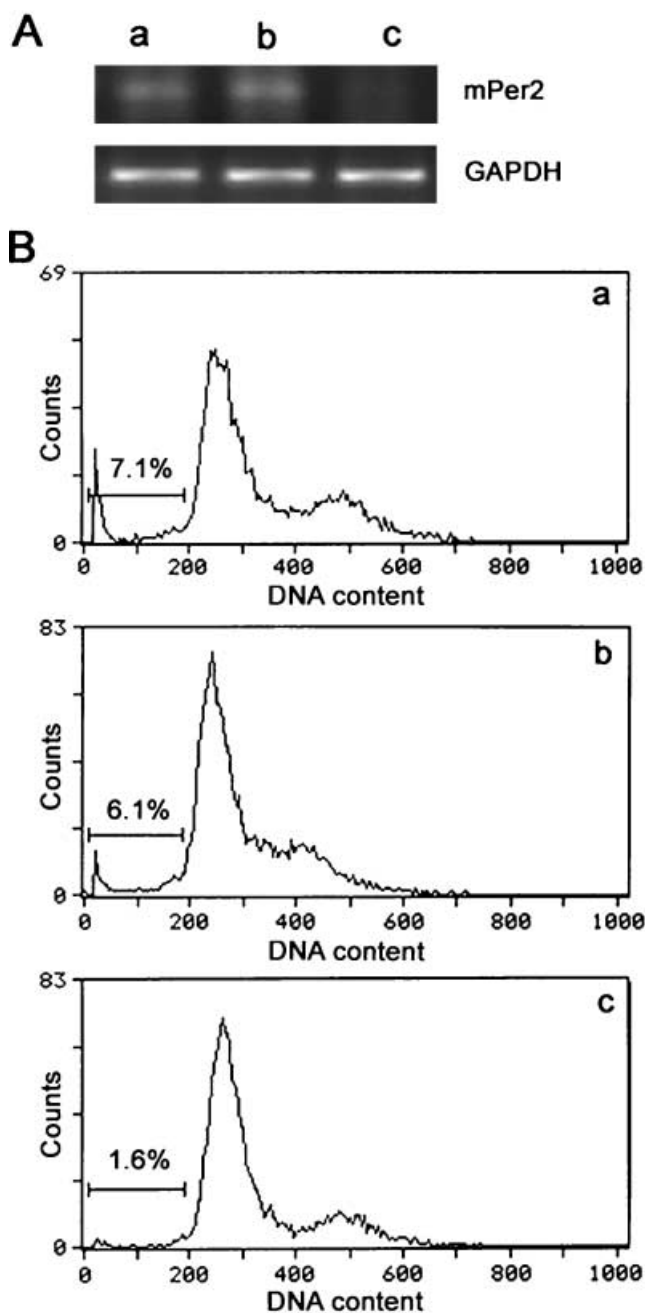


Fig. 7. Flow cytometric analysis of serum starvation-induced apoptosis in 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-treated Lewis lung carcinoma (LLC) cells. (A) Suppression of mouse *mPeriod2* (*mPer2*) expression by antisense oligonucleotides. Cells were treated with TPA to induce endogenous *mPer2* expression in LLC cells. Antisense oligonucleotides were used to block *mPer2* expression. *mPer2* expression was detected by reverse transcription-polymerase chain reaction analysis for cells treated with: (i) TPA alone; (ii) TPA and randomized oligonucleotides; or (iii) TPA and antisense oligonucleotides. (B) Flow cytometric analysis of serum starvation-induced apoptosis in LLC cells treated with: (i) TPA alone; (ii) TPA and randomized oligonucleotides; or (iii) TPA and antisense oligonucleotides. Horizontal and vertical axes represent DNA content and cell number, respectively. Cells in the sub-G₁ fraction (bar) were regarded as apoptotic, and their proportions are indicated. The apoptosis rate in groups i, ii and iii were 7.1, 6.1 and 1.6%, respectively.

proteins, and repression of *c-Myc*. *c-Myc* is demonstrated to be repressed by p53 in a number of mouse and human cell lines and mouse tissues. They provide evidence that p53 binds to the *c-Myc* promoter *in vivo* and represses the promoter through a mechanism that involves histone deacetylation.⁽²⁸⁾ Whether mPER2 downregulates *c-Myc* expression in a p53-dependent manner or not warrants further investigation.

Recent studies suggest that cytosolic p53 may interact directly with members of the Bcl-2 family of apoptosis regulators, thereby triggering mitochondrial outer membrane permeabilization and apoptosis. The Bcl-2 family of proteins is a key regulator of the mitochondrial response to apoptotic signals in the intrinsic pathway. The *Bcl-2* gene family comprises more than 20 different members that regulate apoptosis either positively or negatively.^(29,30) It includes antiapoptotic *Bcl-2* members, such as Bcl-2, Bcl-X_L, Bcl-W and Mcl-1, acting as potent suppressors of apoptosis by blocking the release of cytochrome *c*. Whereas proapoptotic members, such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk, act as promoters that have opposing functions and promote cell death, the pro-apoptotic proteins Bax, Bak and Bid translocate to the mitochondria with apoptotic stimuli. The ratio of antiapoptotic-to-proapoptotic molecules determines the response to a death signal.

In the present study, we checked the expression of BCL-2, BAX, BCL-X_L and the ratio of BAX to BCL-X_L. As shown in Fig. 6, the levels of BAX mRNA and protein were increased significantly in mPER2-overexpressing cells compared with vector control cells. In contrast, we showed an apparent decrease in the mRNA and protein levels of BCL-2 in mPER2-overexpressing cells compared with vector control cells. The mRNA and protein levels of BCL-X_L were decreased significantly in mPER2-overexpressing cells compared with vector control cells. The mean values for the Bax : Bcl-X_L mRNA ratios were higher in mPER2-overexpressing cells than in vector control cells. Western blot analysis also demonstrated an increased ratio of BAX to BCL-X_L in mPER2-overexpressing cells, compared with control cells. These may be involved in the effect of mPER2 on apoptosis.

In summary, overexpression of mPER2 results in reduced cellular proliferation and rapid apoptosis in tumor cells, but not in NIH 3T3 cells. Overexpressed mPER2 alters the expression of apoptosis-related genes and regulates the expression of proteins involved directly or indirectly in apoptosis. In addition to p53-dependent apoptosis, there may be other mechanisms by which overexpressed mPER2 induces apoptosis. The exact character of the tumor-associated death pathway activated by mPER2 still needs to be studied. An important clue to the nature of this apoptotic pathway may come from results showing that mPER2 preferentially kills tumorigenic cells but not non-tumorigenic NIH 3T3 cells, indicating that tumor-specific client proteins with which mPER2 interacts may be required for initiating the programmed cell death. Other circadian regulators may also play a similar role in tumor suppression. Cancer should no longer be treated as a local disorder, but a general disorder regulated by the circadian clock. Future research should be focused on studying the detailed mechanisms by which the circadian clock controls genes related to cell proliferation and apoptosis.

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