FEBS Letters 584 (2010) 4731–4734

FEBS Letters

journal homepage: www.FEBSLetters.org

Per3, a circadian gene, is required for Chk2 activation in human cells

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ARTICLE INFO

Article history:
Received 21 July 2010
Revised 26 October 2010
Accepted 2 November 2010
Available online 9 November 2010

Edited by Angel Nebrada

Keywords:
Circadian clock
Per3
Cell cycle checkpoint
Chk2
DNA damage

ABSTRACT

***PER3* is a member of the *PERIOD* genes, but does not play essential roles in the circadian clock. Depletion of Per3 by siRNA almost completely abolished activation of checkpoint kinase 2 (Chk2) after inducing DNA damage in human cells. In addition, Per3 physically interacted with ATM and Chk2. Per3 overexpression induced Chk2 activation in the absence of exogenous DNA damage, and this activation depended on ATM. Per3 overexpression also led to the inhibition of cell proliferation and apoptotic cell death. These combined results suggest that Per3 is a checkpoint protein that plays important roles in checkpoint activation, cell proliferation and apoptosis.**

Structured summary:
MINT-8052850: *Chk2* (uniprotkb:O96017) *physically interacts* (MI:0915) with *Per3* (uniprotkb:P56645) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-8052875: *Per3* (uniprotkb:P56645) *physically interacts* (MI:0914) with *Chk2* (uniprotkb:O96017) and *ATM* (uniprotkb:Q13315) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

The circadian clock is responsible for multiple physiological and behavioral rhythms in many organisms [1,2]. In mammalian cells, the circadian clock is organized through a complex network of feedback loops that drive rhythmic expression patterns of core clock components. The members of the basic helix-loop-helix transcription factor, Bmal1 and Clock form heterodimers, and initiate expression of several target genes containing E-box enhancer element, including *PERIOD* (*PER*) and *CRYPTOCHROME* (*CRY1* and *CRY2*). Per and Cry proteins act as negative regulators of circadian target gene expression by directly interacting with and inhibiting transcriptional activity of Clock:Bmal1 [3].

In mammals, three *PER* genes, *PER1*, *PER2*, and *PER3*, have been identified [4,5]. Of these three genes, *PER1* and *PER2* have been shown to play essential roles in circadian clock. Although the clock continues to oscillate when a single *PER* gene is mutated [6–8], disruption of both *PER1* and *PER2* genes results in immediate loss of behavioral and molecular rhythmicity [7]. In contrast, the behavioral and molecular rhythms of *PER1/PER3* and *PER2/PER3* double mutant mice resembled the rhythms of mutant mice with disruption of *PER1* or *PER2* alone [9]. These results suggest that *PER3* is not involved in regulating normal circadian clock function.

Recently, a growing number of studies have suggested that the circadian clock components such as Clock:Bmal1, Tim, Per1, and Per2 are also involved in controlling the cell cycle and cellular response to genotoxic stresses [10,11]. Despite the similarity between Per3 and Per1 or Per2 proteins, the function of Per3 in cell cycle regulation or DNA damage response has not yet been reported.

In this study, we showed that Per3 plays an important role in the cellular responses to genotoxic stresses. The depletion of Per3 by siRNA treatment resulted in the abrogation of the ATM/Checkpoint kinase 2 (Chk2) checkpoint pathway after inducing DNA damage. Per3 was also shown to physically interact with ATM and Chk2, and the overexpression of Per3 induced the activation of Chk2 in the absence of treatment with DNA damaging agents. Our results suggested that Per3 might be an essential component of checkpoint pathway, which plays a distinct role in the checkpoint activation process.

2. Materials and methods

2.1. Cell culture, plasmid construction and transient transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS and antibiotics. All cell culture

Abbreviations: Per, period; Chk, checkpoint kinase; ATM, ataxia telangiectasia mutated

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doi:10.1016/j.febslet.2010.11.003

reagents were purchased from Welgene Inc (Seoul, Korea). Hydroxyurea and caffeine were purchased from Sigma–Aldrich (USA). Per3 siRNAs were chemically synthesized by Samchully Pharm (Daejon, Korea). The sense strand sequences of siRNAs used in this study were as follows: GL2, 5′-AACGUACGCGGAAUACUUCGA-3′; Per3-1, 5′-GGUGUAUAAUUGGAUUCAA-3′; Per3-2, 5′-GCAUCCUCCCUUUGAACAU-3′; ATM, 5′-UAUAUCACCUGUUUGUUAGUU-3′. Transfections of siRNAs (120 nM) and plasmid DNA were performed using oligofectamine and lipofectamine-plus (Invitrogen), respectively, following the manufacturer's instruction.

2.2. Immunoblotting and immunoprecipitation

For immunoblotting, cell lysates were prepared with lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, and protease inhibitors. For immunoprecipitation, total cell extracts were prepared using immunoprecipitation buffer (20 mM Tris–HCl, pH8.0, 100 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM ATP, 5 mM sodium fluoride, 1 mM sodium vanadate, and protease inhibitors). Immunoprecipitations were performed using anti-FLAG M2 agarose beads (Sigma–Aldrich) or anti-Chk2 antibody (Santa-cruz) at 4 °C. The primary antibodies against FLAG, p-Chk1 (Ser317), p-Chk2 (Thr68), p-ATM (Ser1981), Caspase3 and PARP were purchased from Cell Signaling Technology (USA); antibodies against Chk1 and Chk2 were from Abcam (UK); actin and Cdc25A antibodies were from Santa Cruz Biotechnology (USA).

2.3. Colony formation assay

Cells were plated in 100 mm dishes and transfected with 4 μg of plasmid. After the cells were incubated for 24 h, the culture medium was changed with fresh medium. The cells were cultured for 14 days, fixed and stained with coomassie brilliant blue.

2.4. Flow cytometry analysis

Cells were collected after treatment with trypsin, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol in PBS. The cells, resuspended in PBS containing RNase A (2 μg/ml), were incubated at 37 °C for 30 min. After incubation with 10 μg/ml propidium iodide for 10 min at 37 °C, the DNA content in the samples was analyzed using a FACScalibur flow cytometer (Becton Dickinson).

2.5. Bromodeoxyuridine (BrdU) incorporation assay

The BrdU incorporation assay was conducted using a BrdU-Labeling and Detection Kit (Roche) according to the manufacturer's instruction. The nuclei of cells were stained with 0.1 μg/ml DAPI in PBS for 3 min in the dark. The stained cells were imaged by fluorescent microscopy (NIKON, TE2000).

3. Results

3.1. Downregulation of Per3 abolishes the activation of ATM-Chk2 pathway after DNA damages

To examine the possible role of *PER3* in the checkpoint responses, activation of checkpoint pathways in *PER3* depleted cells were examined. As shown in Fig. 1A and B, transfection of two different *PER3* siRNAs, respectively, significantly reduced the level of Per3 in *HeLa* cells. Treatment with etoposide, an inducer of double strand breaks or hydroxyurea, an inhibitor of DNA replication, resulted in the activation of both Chk1 and Chk2 in control siRNA treated cells. However, while activation of Chk1 by etoposide or hydroxyurea was not significantly affected by the depletion of Per3 proteins, Chk2 activation was impaired in Per3 depleted *HeLa* cells. On the other hand, the phosphorylational activation of ATM, an upstream checkpoint kinase of Chk2, was still observed in Per3 depleted cells (Fig. 1A and B), suggesting that Per3 is required for the ATM dependent activation of Chk2 after DNA damage.

To explore the possibility that Per3 is directly involved in the checkpoint activation process, the physical interaction between Per3 and checkpoint proteins was examined. Immunoprecipitation of Per3 using an anti-FLAG antibody resulted in the co-precipitation of ATM and Chk2 proteins (Fig. 1C), and reciprocal immunoprecipitation with an anti-Chk2 antibody also pulled down Per3 proteins (Fig. 1D). In addition, the physical interactions between those proteins were not dependent on DNA damage, because co-precipitations were observed both in the absence and in the presence of DNA damage. These results suggested that the Per3 protein might play a direct role in the activation of Chk2 by physically interacting with checkpoint proteins.

3.2. Ectopic expression of Per3 results in the activation of Chk2 without exogenous DNA damage

Since Per3 interacted with checkpoint proteins, the effect of overexpressing Per3 on the checkpoint pathway was assessed. Interestingly, ectopic expression of Per3 stimulated Chk2 phosphorylation in the absence of exogenous DNA damage while Chk1 phosphorylation was not significantly affected. To test whether the increase in Chk2 phosphorylation led to the activation of effective checkpoint responses, the cellular level of Cdc25A phosphatase, a major target of checkpoint pathway, was examined.

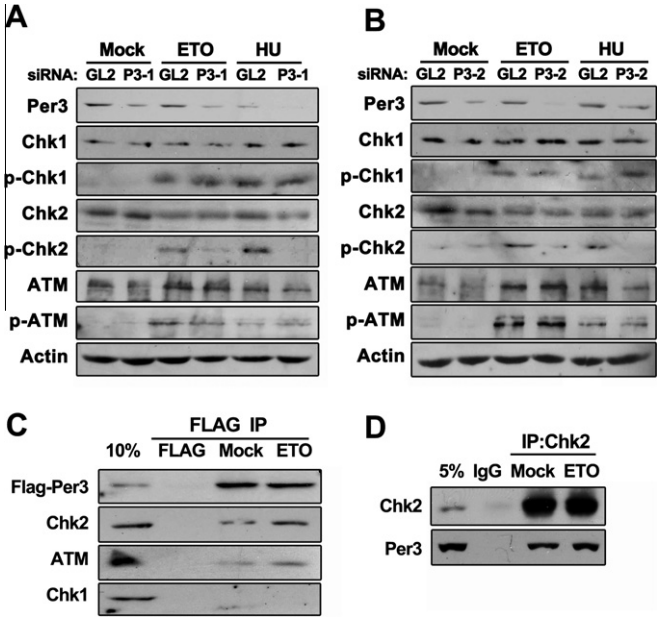

Fig. 1. Per3 is required for the activation of the checkpoint pathway after inducing DNA damage. (A and B) *HeLa* cells were transfected with 120nM siRNA; firefly luciferase (GL2) and Per3 (P3-1 or P3-2). The transfected cells were cultured for 48 h and treated with 20 μM etoposide (ETO) or 5 mM HU for 18 h, and cellular levels of proteins were detected by immunoblotting. Actin was used as a loading control. (C and D) Cells transfected with pCDNA-FLAG-Per3 plasmid were incubated in the absence or presence of 20 μM etoposide for 18 h. Total cell lysates of about 500 μg was used for immunoprecipitation with anti-FLAG M2 agarose beads (C) or anti-Chk2 antibodies (D). FLAG and IgG indicates that the immunoprecipitation was carried out in the presence of 0.2 mg/ml FLAG peptides and using non-specific IgG, respectively. Input represents 10% (C) or 5% (D) of the lysates used for immunoprecipitation.

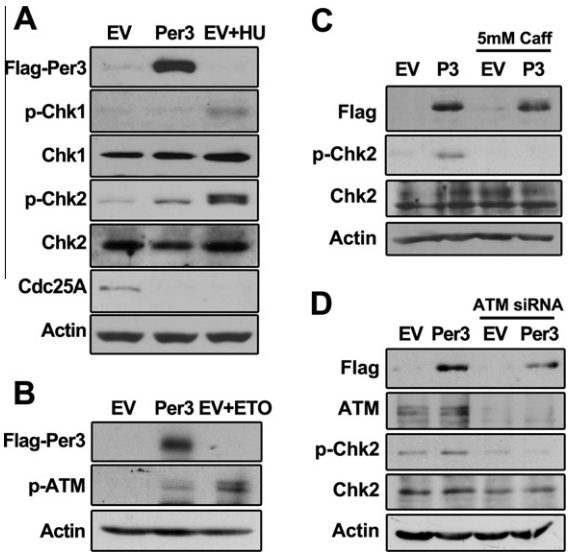

Fig. 2. Per3 overexpression induces Chk2 activation in the absence of DNA damage. (A and B) HeLa cells were transfected with 4 μg plasmid; empty vector (EV) or pCDNA3-FLAG-Per3 (Per3). The transfected cells were incubated for 48 h. The activated forms of Chk1, Chk2 and ATM were detected by Ser^{317}-phospho specific Chk1, Thr^{68}-phospho-Chk2 or Ser^{1981}-phospho-ATM antibodies, respectively. Actin was used as a loading control. (C and D) *HeLa* cells were transfected in the same manner as (A) and simultaneously treated with 5 mM caffeine (C) or ATM siRNA (D). After 48 h, total cell lysates were prepared and immunoblotting was carried out using the indicated antibodies. Actin was used as a loading control.

Indeed, a decrease in Cdc25A was observed in Per3 expressing cells and the level of decrease was comparable to that in hydroxyurea treated cells (Fig. 2A). Phosphorylation of ATM was also observed in Per3 overexpressed cells (Fig. 2B), and depletion of ATM with siRNA or treatment with caffeine, which is an inhibitor of upstream ATM kinase, completely reversed the effects of Per3 overexpression on Chk2 activation (Fig. 2C and D). Taken together, these results suggest that overexpression of Per3 leads to the activation of the ATM-Chk2 pathway by increasing the phosphorylation of Chk2 in the absence of exogenous DNA damage.

3.3. Per3 overexpression leads to the inhibition of cell proliferation and apoptotic cell death

Since Chk2 was activated by ectopic expression of Per3, the effect of Per3 overexpression on the proliferation of *HeLa* cells was examined. Using the colony formation assay, cell proliferation was found to be significantly decreased in cells transfected with Per3 (Fig. 3A). Per3 overexpressing cells also showed a decrease of BrdU incorporation, which suggests that DNA replication was inhibited in those cells (Fig. 3B). Flow cytometry analysis also confirmed that DNA synthesis was inhibited in those cells, which was determined by the complete loss of the G2 peaks in Per3 expressing cells. In addition, the number of sub-G1 cells, which represent cell death, was dramatically increased by Per3 overexpression (Fig. 3C). Furthermore, the products of apoptosis, such as the cleaved forms of caspase 3 and poly (ADP-ribose) polymerase, were detected in Per3 overexpressing cells (Fig. 3D). Taken together,

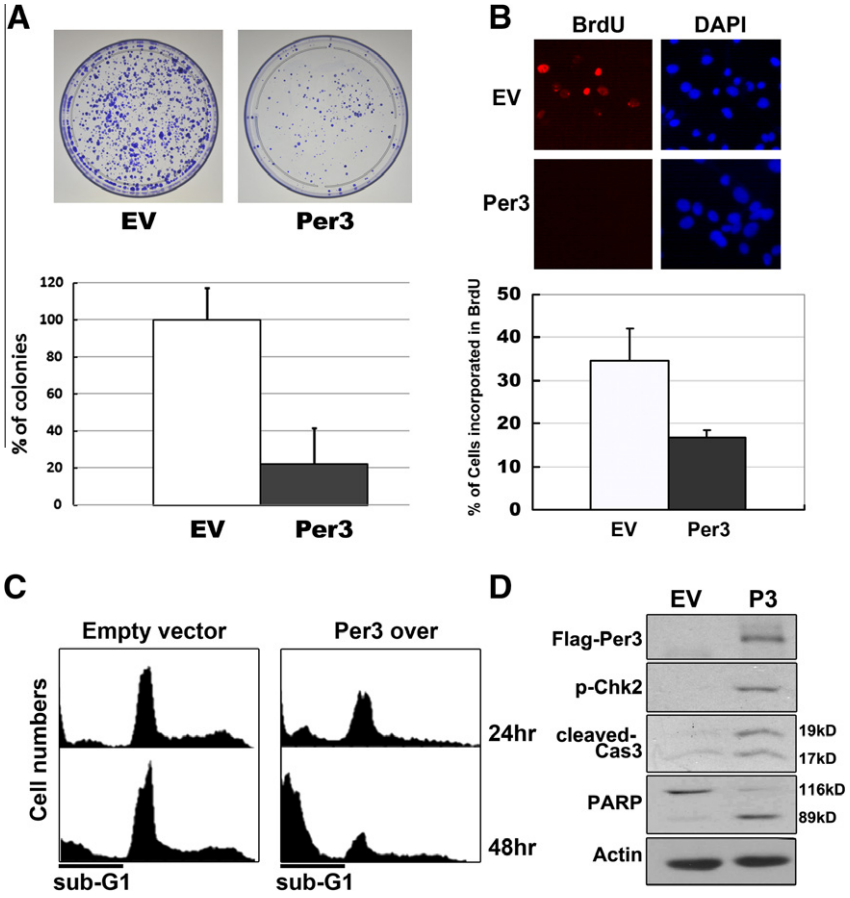

Fig. 3. Per3 overexpression leads to the inhibition of cell proliferation and apoptotic cell death. *HeLa* cells were transfected with 4 μg of empty vector (EV) or pCDNA3-FLAG-Per3 (Per3), and cell proliferation and DNA synthesis were measured using the colony formation assay (A) and the BrdU incorporation assay (B), respectively. Error bars indicate the standard error from three independent experiments. (C) Cell cycle profiles were examined by Flow cytometry analysis 24 or 48 h after transfection with the empty vector or pCDNA3-FLAG-Per3. (D) The induction of apoptotic cell death was detected using the immunoblotting assay. The visualized 19- and 17-kDa protein bands were cleaved products of caspase 3. The 116 kDa band represents the full length PARP, while the 89 kDa band represents the cleaved PARP product.

Per3 overexpression leads to the inhibition of cell proliferation and apoptotic cell death in *HeLa* cells in the absence of exogenous DNA damage.

4. Discussion

PER3 is a member of the circadian clock gene family, Period, but dose not play important roles in circadian clock rhythmicity. Here, *PER3* was shown to be involved in the checkpoint pathway after inducing DNA damage. Participation of circadian genes in cell cycle regulation is not a unique property of Per3. There are a large number of studies that have demonstrated a functional link between the circadian clock and cell cycle control [10,11]. Especially, Per1, Per2 and Tim were known to be involved in checkpoint responses to DNA damage. While Tim is involved in the activation of the ATR-Chk1 pathway in mammalian cells [12], Per1 [13] and Per3 are required for the activation of the ATM-Chk2 pathway. In *Drosophila*, Timeless and Period both play critical roles in controlling the circadian clock. On the other hand, neither Tim nor Per3 in mammalian cells appear to play important roles in the circadian clock. Per1 was shown to play a role in both the circadian clock and checkpoint pathway.

All core circadian clock proteins have the potential to play important roles in controlling the cell cycle because expression of cell cycle related genes such as Wee1, c-Myc, Cyclin D1, and p21 is regulated by the circadian clock [10]. However, the role of Per3 in the checkpoint activation process appears to be more direct. First, Per3 physically interacts with both ATM and Chk2. If the activation of the checkpoint pathway is mediated by the expression of other circadian target genes, the physical interactions between those proteins would not be necessary. Second, Per3 does not affect the expression of circadian clock proteins. Therefore, expression of cell cycle related circadian target genes was not changed by the depletion of Per3 in our study.

Since Per1 and Per3 form a dimer and nuclear localization of those proteins depends on dimerization [14], it is possible that the depletion of Per3 inhibited checkpoint activation by preventing the nuclear import of Per1 proteins or vice versa. There are several contradictory reports regarding the nuclear import of Per1 and Per3 proteins. While a few reports suggested that the nuclear import of Per1 requires dimerization with Per3, other reports have suggested that Per1 is required for the nuclear import of Per3 [14–16]. Thus, the protein that is more critical for the nuclear import and checkpoint activation is still largely controversial and remains to be solved. It is also possible that the dimer of Per1 and Per3 acts together to activate checkpoint pathway.

In this study, we also showed that overexpression of Per3 led to the inhibition of cell proliferation and apoptotic cell death in *HeLa* cells. Overexpression of Per1 was shown to sensitize human cancer cells to DNA damage induced apoptosis [13], and overexpression of Per2 was also reported to induce apoptosis in mouse cancer cell lines [17]. De-regulation of the expression of many cell cycle and apoptosis related genes was also reported in these studies. Since many of these genes are clock controlled genes [18,19], de-regulated expression of clock-controlled genes by Per overexpression might have been the reason for apoptotic cell death. This explanation is reasonable because overexpression of Per1 or Per2 significantly alters molecular rhythmicity and Bmal1/Clock activity. However, the molecular mechanism of apoptotic cell death by Per3 overexpression might be different from those induced by Per1 or Per2 overexpression. Per3 did not play any significant roles in the circadian clock. Therefore, expression of circadian clock controlled genes would not be affected by Per3 expression. However, we could not rule out the possibility that the defects in expression of a few important factors in the control of the cell cycle and apoptosis were responsible for the apoptotic cell death of Per3 overexpressing cells.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2008-C00255), and National R&D Program for Cancer Control, Ministry of Health & Welfare, Korea (0620260). JS Im, BH Jung, and SE Kim were supported by the Brain Korea 21 project in 2010.

References

[1] Albrecht, U. and Eichele, G. (2003) The mammalian circadian clock. Curr. Opin. Genet. Dev. 13, 271–277.
[2] Barnard, A.R. and Nolan, P.M. (2008) When clocks go bad: neurobehavioral consequences of disrupted circadian timing. PLoS Genet. 4, e1000040.
[3] Ko, C.H. and Takahashi, J.S. (2006) Molecular components of the mammalian circadian clock. Hum. Mol. Genet. 15, Spec. No. 2 2, R271–R277.
[4] Albrecht, U., Sun, Z.S., Eichele, G. and Lee, C.C. (1997) A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell 91, 1055–1064.
[5] Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., Maebayashi, Y., Okamura, K., Takekida, S., Yamamoto, S., Yagita, K., Yan, L., Young, M.W. and Okamura, H. (1998) A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. EMBO J. 17, 4753–4759.
[6] Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C. and Bradley, A. (1999) The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400, 169–173.
[7] Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., et al. (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105, 683–694.
[8] Shearman, L.P., Jin, X., Lee, C., Reppert, S.M. and Weaver, D.R. (2000) Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. Mol. Cell. Biol. 20, 6269–6275.
[9] Bae, K., Jin, X.W., Maywood, E.S., Hastings, M.H., Reppert, S.M. and Weaver, D.R. (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30, 525–536.
[10] Sancar, A., Lindsey-Boltz, L.A., Kang, T.H., Reardon, J.T., Lee, J.H. and Ozturk, N. (2010) Circadian clock control of the cellular response to DNA damage. FEBS Lett. 584, 2618–2625.
[11] Kondratov, R.V. and Antoch, M.P. (2007) Circadian proteins in the regulation of cell cycle and genotoxic stress responses. Trends Cell Biol. 17, 311–317.
[12] Unsal-Kacmaz, K., Mullen, T.E., Kaufmann, W.K. and Sancar, A. (2005) Coupling of human circadian and cell cycles by the timeless protein. Mol. Cell Biol. 25, 3109–3116.
[13] Gery, S., Komatsu, N., Baldjyan, L., Yu, A., Koo, D. and Koeffler, H.P. (2006) The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol. Cell 22, 375–382.
[14] Yagita, K., Yamaguchi, S., Tamanini, F., van Der Horst, G.T., Hoeijmaker, J.H., et al. (2000) Dimerization and nuclear entry of mPER proteins in mammalian cells. Genes Dev. 14, 1353–1363.
[15] Loop, S. and Pieler, T. (2005) Nuclear import of mPER3 in Xenopus oocytes and HeLa cells requires complex formation with mPER1. FEBS J. 272, 3714–3724.
[16] Lee, C., Weaver, D.R. and Reppert, S.M. (2004) Direct association between mouse PERIOD and CKIepsilon is critical for a functioning circadian clock. Mol. Cell Biol. 24, 584–594.
[17] Hua, H., Wang, Y., Wan, C., Liu, Y., Zhu, B., Yang, C., et al. (2006) Circadian gene mPer2 overexpression induces cancer cell apoptosis. Cancer Sci. 97, 589–596.
[18] Duffield, G.E., Best, J.D., Meurers, B.H., Bittner, A., Loros, J.J. and Dunlap, J.C. (2002) Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. Curr. Biol. 12, 551–557.
[19] Kornmann, B., Preitner, N., Rifat, D., Fleury-Olela, F. and Schibler, U. (2001) Analysis of circadian liver gene expression by ADDER, a highly sensitive method for the display of differentially expressed mRNAs. Nucleic Acids Res. 29, e51.