A role for the clock period circadian regulator 2 gene in regulating the clock gene network in human oral squamous cell carcinoma cells

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Abstract. Clock genes are the core of the circadian rhythms in the human body and are important in regulating normal physiological functions. To date, research has indicated that the clock gene, period circadian clock 2 (*PER2*), is downregulated in numerous types of cancer, and that it is associated with cancer occurrence and progression via the regulation of various downstream cell cycle genes. However, it remains unclear whether the decreased expression of *PER2* influences the expression of other clock genes in cancer cells. In the present study, short hairpin RNA interference was used to knockdown *PER2* effectively in human oral squamous cell carcinoma SCC15 cells. Quantitative polymerase chain reaction was used to assess the mRNA expression levels of various clock genes and revealed that, following the knockdown of *PER2* in SCC15 cells, the mRNA expression levels of *PER3*, brain and muscle ARNT-like 1, deleted in esophageal cancer *(DEC)1*, *DEC2*, cryptochrome circadian clock (*CRY*)2, timeless circadian clock, retinoic acid receptor-related orphan receptor-alpha and neuronal PAS domain protein 2 were significantly downregulated, while the mRNA expression levels of *PER1* and nuclear receptor subfamily 1 group D member 1 were significantly upregulated. In addition, flow cytometric analysis demonstrated that proliferation was enhanced and apoptosis was reduced following *PER2* knockdown in SCC15 cells ($P<0.05$). To the best of our knowledge, the present study is the first to report that *PER2* is important for the regulation of other clock genes of the clock gene network in cancer cells. This is of great significance in elucidating the molecular function and tumor suppression mechanism of *PER2*.

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Abbreviations: CCGs, clock-controlled genes; shRNA, short hairpin RNA; OSCC, oral squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence

Key words: clock genes, period circadian clock 2, tumor, oral cancer, RNA, gene expression

Introduction

The human body possesses a circadian clock, which serves an important role in regulating normal physiological functions (1). Clock genes are at the core of this circadian clock. To date, 14 clock genes have been identified: Clock circadian regulator (*CLOCK*), brain and muscle ARNT-like 1 (*BMAL1*), period circadian clock (*PER*)1, *PER2*, *PER3*, deleted in esophageal cancer (*DEC*)1, *DEC2*, cryptochrome circadian clock (*CRY*)1, *CRY2*, timeless circadian clock (*TIM*), casein kinase 1 epsilon (*CK1ε*), retinoic acid receptor-related orphan receptor-α (*RORα*), neuronal PAS domain protein 2 (*NPAS2*) and nuclear receptor subfamily 1 group D member 1 (*REV-ERBα*) (2-5), all of which exist in the majority of human cells (6). In mammals, 2-10% of the genome is regulated by these clock genes (7,8), including numerous key cell cycle genes and cancer-associated genes (9-11); these are termed clock-controlled genes (CCGs). Different clock genes regulate different downstream CCGs and, thus, clock genes are important in regulating cell biochemical and physiological functions (12).

PER2 is an important clock gene, and previous research has indicated that *PER2* serves a notable function in the occurrence and progression of cancer (13,14). The expression of *PER2* has been revealed to be downregulated in various types of cancer, including breast cancer, gastric carcinoma, head and neck cancer and oral cancer (15-18). *PER2* regulates a number of downstream cell cycle genes and cancer-associated genes, including cyclin D1, cyclin A, tumor protein p53, v-Myc avian myelocytomatosis viral oncogene homolog, mouse double minute 2 homolog proto-oncogene and B-cell lymphoma 2 apoptosis regulator (18,19). However, it remains unclear whether the decreased expression of *PER2* in cancer cells is able to affect the expression of other clock genes.

The present study used short hairpin RNA (shRNA) interference to knockdown *PER2* in human oral squamous cell

carcinoma (OSCC) SCC15 cells, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to assess the altered mRNA expression of other clock genes. The mRNA expression levels of many clock genes, proliferation and apoptosis of tumor cells were altered. This research demonstrates that the effect of *PER2* on tumor occurrence and progression is induced not only through the regulation of downstream CCGs, but also through the regulation of other clock genes in the network. This is of great significance to further illustrating the molecular functions and tumor-suppressive mechanisms of *PER2*.

Materials and methods

Construction of lentiviral shRNA plasmids. The mRNA sequence of *hPER2* was acquired from the GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/; accession no. NM_022817). BLAST screening (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ascertain three interference target sequences of *PER2* (20): PER2-I, CAG AGTCCAGATACCTTTA; PER2-II, ATCCATATTTCA CTGTAAA; and PER2-III, CACACACAAAGAACTGAT A. Based on the design principles of Chernolovskaya and Zenkova (21), three RNA interference target sequences of PER2 were designed and synthesized: PER2-shRNA-I, PER2-shRNA-II and PER2-shRNA-III (Table I). The restriction enzymes *Age*I/*Eco*RI (New England BioLabs, Inc., Ipswich, MA, USA) were used to double digest the lentiviral vector (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the double enzyme-restricted vector was connected with PER2-shRNA-I-III, prior to the double-strand DNA being annealed using T4 DNA ligase in order to construct PER2-shRNA-I-III lentiviral plasmids. Scramble plasmids (Invitrogen; Thermo Fisher Scientific, Inc.), which exerted no interference effects on any genes, served as the controls (control-shRNA). Subsequently, these lentiviral plasmids were transformed into freshly prepared *Escherichia coli* DH5α cells (Sangon Biotech Co., Ltd., Shanghai, China), selected in lysogeny broth medium containing the antibiotic ampicillin and then cultured overnight at 37˚C. Plasmids were extracted using a Qiagen Plasmid Midi kit (Qiagen GmbH, Hilden, Germany). qPCR was performed using a SYBR Premix Ex TaqII kit (Takara Bio, Inc., Otsu, Japan) in a reaction system comprising 5 μl SYBR Premix Ex TaqII, 0.5 μl each of the forward and reverse primer (0.4 μmol/l; Sangon Biotech Co., Ltd.), (forward primer sequence: 5'-CCATGATTCCTT CATATTTGC-3' and reverse primer sequence: 5'-GTAATA CGGTTATCCACGCG-3'), 2 μl DNA template (50 ng/μl) and 2 μl double distilled H_2O (ddH_2O). The ddH_2O is the template for blank control, and the negative control was a template for an empty carrier that is not inserted in the target gene. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 3 min; denaturation for 30 sec at 95˚C; annealing extension for 30 sec at 55˚C and 30 sec at 72˚C, which for 22 cycles. Fluorescence signals were recorded during the 72˚C annealing extension phase. The amplification products from PCR were assessed using a DNA sequencing assay (3730 DNA Analyzer; Applied Biosystems; Thermo Fisher Scientific, Inc.), and the results of DNA sequencing were analyzed using Chromas v2.4 software (Technelysium Pty Ltd., Brisbane, Australia).

Lentiviral PER2-shRNA plasmid packing. Each PER2-shRNA-I-III and scramble plasmid (8 μg) was mixed with 20 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 15 min at room temperature. The mixture was then applied drop-wise to 293 cells (Institute of Life Sciences, Chongqing Medical University, Chongqing, China), which had been cultured to 70-80% growth density, and incubated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Natocor Industria Biologica, Cordoba, Argentina) for 48 h at 37˚C and 5% CO_2. Between 48 and 72 h after transfection, the culture supernatant was collected and centrifuged for 10 min at 4˚C and 12,000 x g to remove the cell debris, prior to being centrifuged again for 1 h at 4˚C and 25,000 x g in overspeed centrifuge tubes. The sediment was then mixed with virus preservation solution and centrifuged for 5 min at 4˚Cand 10,000 x g. The supernatant was divided and four groups of lentiviral plasmids were obtained: PER2-shRNA-I, PER2-shRNA-II, PER2-shRNA-III and control-shRNA.

Cell transfection. SCC15 cells (Institute of Life Sciences, Chongqing Medical University) were cultured in Dulbecco's modified Eagle's medium/F-12 (Gibco; Thermo Fisher Scientific, Inc.) containing FBS and penicillin 100 U/ml and streptomycin 0.1 mg/ml (Yocon Biotechnology Co., Beijing, China) in cell culture flasks, at 37˚C in an atmosphere containing 5% CO_2. Lentiviral plasmids (5 μl) were applied drop-wise to SCC15 cells during the logarithmic growth phase, and 100 μl Polybrene infection reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added. Cells were incubated in DMEM containing 10% FBS without antibiotics for 24 h (37˚C, 5% CO_2), following which the medium was replaced every day with DMEM containing 10% FBS and puromycin (2 μg/ml). Stably transfected SCC15 cells, in which *PER2* was knocked down, were obtained following continuous cultivation for 7 days. The cells were divided into five experimental groups: PER2-shRNA-I, PER2-shRNA-II and PER2-shRNA-III groups (transfected with lentiviral plasmids PER2-shRNA-I to III, respectively); a control group (transfected with control-shRNA, expressing scramble shRNA); and an SCC15 group (untransfected SCC15 cells as a blank control).

Western blot analysis. Cells in the logarithmic growth phase were lysed at 4˚C in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) for 30 min and collected by a cell scraper, following which the cell suspension was centrifuged for 5 min at 4˚C and 12,000 x g to collect the supernatant. Protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocols. Subsequently, the sample solution, containing 50 μg/lane protein, was subjected to SDS-PAGE (12% gel). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA), which were subsequently blocked with 5% dried skimmed milk for 1 h at room temperature. Following blocking, the membranes were

Table I. Seque nces of PER2-shRNAs.

Group	Sense strand	Antisense strand
PER2-shRNA-I	5'-CCGGGCCAGAGTCCAGATACCTTTACTCG AGTAAAGGTATCTGGACTCTGGCTTTTTG-3'	5'-AATTCAAAAAGCCAGAGTCCAGATACCTT TACTCGAGTAAAGGTATCTGGACTCTGGC-3'
PER2-shRNA-II	5'-CCGGGCATCCATATTTCACTGTAAACTCG AGTTTACAGTGAAATATGGATGCTTTTTG-3'	5'-AATTCAAAAAGCATCCATATTTCACTGTAA ACTCGAGTTTACAGTGAAATATGGATGC-3'
PER2-shRNA-III	5'-CCGGGACACACACAAAGAACTGATACTC GAGTATCAGTTCTTTGTGTGTGTCTTTTTG-3'	5'-AATTCAAAAAGACACACACAAAGAACTG ATACTCGAGTATCAGTTCTTTGTGTGTGTC-3'

PER2, period circadian clock 2; shRNA, short hairpin RNA.

probed with a rabbit polyclonal anti-hPER2 antibody (1:300; ab180655; Abcam, Cambridge, UK) and a mouse monoclonal anti-hGAPDH antibody (1:1,000; 51332; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C, followed by three washes in PBS. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology, Inc.) for 2 h at room temperature, followed by three further washes in PBS. Under dark conditions, the enhanced chemiluminescent color-substrate solution (Beyotime Institute of Biotechnology) was applied to the PVDF membranes and exposed using chemiluminescence apparatus. A gel imaging system GelDoc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for visualization and protein detection. The ratio of the grey value of each PER2 band to that of GAPDH was calculated by ImageJ (version 1.48u; National Institutes of Health, Bethesda, MA, USA). All assays were performed in triplicate.

RT-qPCR. RT-qPCR was performed using the PrimeScript RT Reagent kit (cat no. RR037A; Takara Biotechnology Co., Ltd.) according to the manufacturers' protocols. Total RNA was extracted from cells using RNAiso Plus (Takara Biotechnology Co., Ltd.), prior to the RNA concentration and quality being determined using a UV/Visible spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, UK) by measuring absorbance at 260 and 280 nm. RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocols. The reaction system comprised 5X Primer Script Buffer (4 μl), Primer Script RT Enzyme mix (1 μl), Oligo-dT Primers (1 μl), Random Hexamers (1 μl) and RNase Free dH_2O (13 μl). The reverse transcription reaction conditions were 37˚C for 15 min and 85˚C for 5 sec. The qPCR primers for *CLOCK, BMAL1, PER1, PER2, PER3, DEC1, DEC2, CRY1, CRY2, TIM, CKIε, RORα, NPAS2* and *REV-ERBα* were designed using Oligo 7.0 primer analysis software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA) and are listed in Table II. β-actin served as the normalization control. The reaction system comprised 5 μl SYBR Premix Ex TaqII, 0.5 μl each forward and reverse primer (0.4 μmol/l), 2 μl cDNA template (50 ng/μl) and 2 μl double-distilled H_2O. The reaction conditions were as follows: Pre-denaturation at 95˚C for 1.5 min, followed by 40 cycles of denaturation for 10 sec at 95˚C and annealing extension for 30 sec at 60˚C. Fluorescence signals were recorded during the 60˚C annealing extension phase. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (22). All assays were performed in triplicate.

Flow cytometry assay. To prepare a single-cell suspension, cells in the logarithmic growth phase were harvested by 0.25% trypsinization and centrifuged for 5 min at 800 x g and 4˚C, following which the supernatant was discarded, and the cell pellets were washed twice with pre-cooled PBS and were resuspended in PBS at a density of $1x10^6$ cells/ml. The suspension was then divided into 1-ml aliquots in EP tubes. For the detection of cell cycle distribution and proliferation, 1-ml single-cell suspensions were centrifuged for 5 min at 800 x g and 4˚C) and the supernatants were discarded. Subsequently, 70% pre-cooled ethyl alcohol (-20˚C, 1 ml) was added, mixed repeatedly and incubated overnight at 4˚C. The cells were resuspended in 1 ml PBS, and 1 ml RNase ribonuclease was added to the cell suspensions, followed by incubation for 40 min at 37˚C. Propidium iodide solution (1 ml) was added and the mixture was kept in the dark for 10 min at 4˚C. Proliferation was analyzed using a FACS Vantage flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the ModFit LT 2.0 program (Verity Software House, Inc., Topsham, ME, USA) was used to analyze the proportion of cells in each phase of the cell cycle. The tumor cell proliferation index (PI) was calculated as follows: PI=[S + (G2 and M)]/[(G0 and G1) + S + (G2 and M)] x100.

For the detection of cell apoptosis, the single-cell suspensions (1 ml) were fixed in PBS for 2 h at 4˚C and were then resuspended in Binding Buffer (0.5 ml). The suspensions were incubated with Annexin V-FITC (20 μg/ml) reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min at 4˚C in the dark according to the manufacturer's protocols, and were then stained with 1 ml propidium iodide solution for 2 min at room temperature in the dark. Apoptosis was analyzed using the FACS Vantage flow cytometer (BD FACS Aria II software; BD Biosciences, Franklin Lakes, NJ, USA). The tumor cell apoptotic index (AI) was calculated as follows: AI=(number of apoptotic cells/total number of cells) x100. Each experiment was performed in triplicate.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Comparisons between multiple groups were made using one-way analysis of variance, followed by the least significant difference test. Data are expressed as the mean ± standard

Table II. Primer sequences for clock genes used for reverse transcription-quantitative polymerase chain reaction.

Gene abbreviation	Gene name	Forward primer sequence	Reverse primer sequence
PER1	Period circadian clock 1	5'-CTGCTACAGGCACGTTCAAG-3'	5'-CTCAGGGACCAAGGCTAGTG-3'
PER2	Period circadian clock 2	5'-TTGGACAGCGTCATCAGGTA-3'	5'-TCCGCTTATCACTGGACCTT-3'
PER3	Period circadian clock 3	5'-GCAGGTCTATGCCAGTGTGA-3'	5'-ACCACCACCATTCGGTTCT-3'
CLOCK	Clock circadian regulator	5'-CAGCCAGTGATGTCTCAAGC-3'	5'-ATGCGTGTCCGTTGTTCC-3'
BMAL1	Brain and muscle ARNT-like 1	5'-TGCCACCAATCCATACACAG-3'	5'-TTCCCTCGGTCACATCCTAC-3'
DEC1	Deleted in esophageal cancer 1	5'-CAGCTTTCGGATGATGAAGG-3'	5'-GCTGAAGGTGGGATCAGGTA-3'
DEC2	Deleted in esophageal cancer 2	5'-GGGACCAACTGCTTCACACT-3'	5'-TAATCTGTGGGACGGTAGGC-3'
CRY1	Cryptochrome circadian clock 1	5'-TGTGATTCGTGGACAACCAG-3'	5'-TAGCTGCGTCTCGTTCCTTT-3'
CRY2	Cryptochrome circadian clock 2	5'-AGGAGAACCACGACGAGA-3'	5'-TCCGCTTCACCTTTTTATAC-3'
CKIε	Casein kinase 1 epsilon	5'-TGAGTATGAGGCTGCACAGG-3'	5'-CTTCCCGAGATGGTCAAATG-3'
TIM	Timeless circadian clock	5'-GATAGAGGCCCATTCCTGCAT-3'	5'-GAAGGGCTGGGGAACTTAGAC-3'
NPAS2	Neuronal PAS domain protein 2	5'-AACCTCGGCAGCACTTTAAC-3'	5'-GGTTCTGACATGGCTGTGTG-3'
RORα	Retinoic acid receptor-related orphan receptor-α	5'-CTATCCCTCCAAGGCACAAG-3'	5'-AACACAAGACTGACGAGCACA-3'
REV-ERBα	Nuclear receptor subfamily 1 group D member 1	5'-ACAGAATCGAACTCTGCACTTCT-3'	5'-GGGGAGGGAGGCAGGTATT-3'
β-actin	β-actin	5'-AGCGAGCATCCCCCAAAGTT-3'	5'-GGGCACGAAGGCTCATCATT-3'

Table III. Sequences of PER2-shRNA interference.

Group	Sense strand
PER2-shRNA-I	5'-CCGGGCCAGAGTCCAGATACCTTTACTCGAGTAAAGGTATCTGGACTCTGGCTTTTTG-3'
PER2-shRNA-II	5'-CCGGGCATCCATATTTCACTGTAAACTCGAGTTTACAGTGAAATATGGATGCTTTTTG-3'
PER2-shRNA-III	5'-CCGGGCACACACACAAAGAACTGATACTCGAGTATCAGTTCTTTGTGTGTGTCTTTTTG-3'

PER2, period circadian clock 2; shRNA, short hairpin RNA.

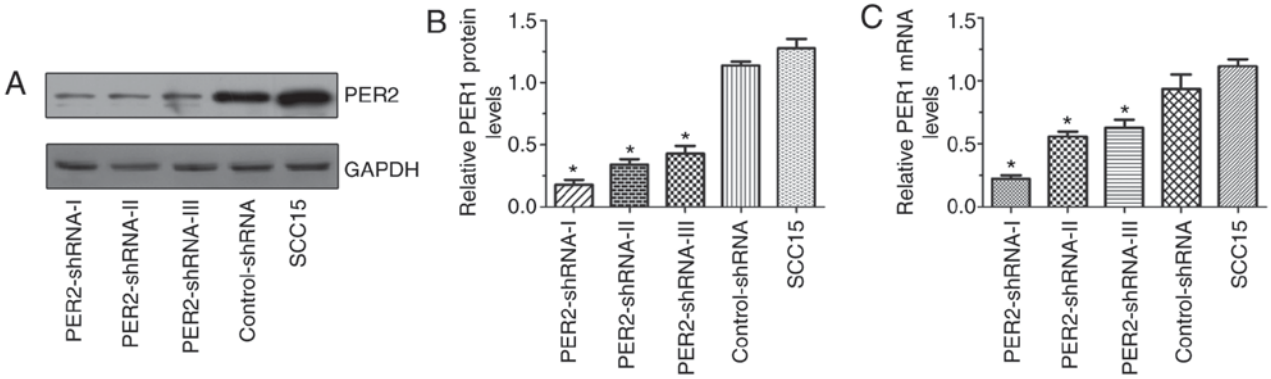

Figure 1. *PER2* is most significantly knocked down in the PER2-shRNA-I group among the five groups of SCC15 cells. (A) Protein expression of PER2 in the PER2-shRNA-I-III, control-shRNA and SCC15 groups. (B) Protein expression of PER2 was significantly reduced in PER2-shRNA-I-III groups compared with in the control-shRNA and SCC15 groups. (C) mRNA expression of PER2 was significantly reduced in the PER2-shRNA-I-III groups compared with in the control-shRNA and SCC15 groups, and this difference was largest for the PER-shRNA-1 group. Data are presented as the mean ± standard deviation. *P<0.05. *PER2*, period circadian clock 2; shRNA, short hairpin RNA.

deviation. P<0.05was considered to indicate a statistically significant difference.

Results

Construction and sequencing of shRNA lentiviral plasmids. The sequencing results obtained from the lentiviral plasmids, PER2-shRNA-I-III, are presented in Table III. These sequences exactly matched the interference target sequences of the positive-sense strands of PER2-shRNA-I-III, indicating that three interference target sequences of *PER2* were successfully constructed.

mRNA and protein expression of PER2 in SCC15 cells following transfection. The mRNA and protein expression of *PER2* in the PER2-shRNA-I-III, control-shRNA and untransfected SCC15 groups are presented in Fig. 1. The *PER2* mRNA and protein expression in the PER2-shRNA-I group was significantly lower than in the other groups (P<0.05), indicating that the *PER2* knockdown was most effective in the PER2-shRNA-I group. Therefore, this shRNA was selected for use in the subsequent experiments.

mRNA expression of clock genes in SCC15 cells following PER2 knockdown. RT-qPCR analysis demonstrated that the mRNA expression levels of *PER1* and *REV-ERBα* were significantly upregulated in the PER2-shRNA-I group compared with in the control-shRNA and SCC15 groups (P<0.05), while the mRNA expression levels of *PER3*, *BMAL1*, *DEC1*, *DEC2*, *CRY2*, *TIM*, *RORα* and *NPAS2* were significantly downregulated (P<0.05). There was no significant difference between mRNA expression levels in the control-shRNA and the SCC15 groups (P>0.05). In addition, there was no significant difference in the mRNA expression levels of *CLOCK*, *CRY1* or *CKIε* among the three groups (P>0.05; Fig. 2).

Proliferation and apoptosis of SCC15 cells following PER2 knockdown. Flow cytometric analysis indicated that the cell PIs of the PER2-shRNA-I group, control-shRNA group and untreated SCC15 group were 43.55±2.64, 35.32±2.64 and 35.36±2.46%, respectively. The cell AIs were 15.94±1.36, 21.85±1.90 and 22.13±2.29%, respectively. The cell PI of the PER2-shRNA-I group was markedly higher than that of the other two groups, while the cell AI was significantly lower than that of the other two groups (P<0.05). There was no significant difference between the control-shRNA group and the SCC15 group with regards to PI or AI (P>0.05; Fig. 3).

Discussion

Previous studies have indicated that different clock genes regulate various downstream CCGs, and therefore clock genes are considered to be important for regulating cell biochemical and physiological functions (12). The clock gene, *PER2,* is downregulated in various types of cancer, and is associated with cancer occurrence and progression via the regulation of numerous downstream cell cycle genes (15-18). Although *PER2* is known to be an important

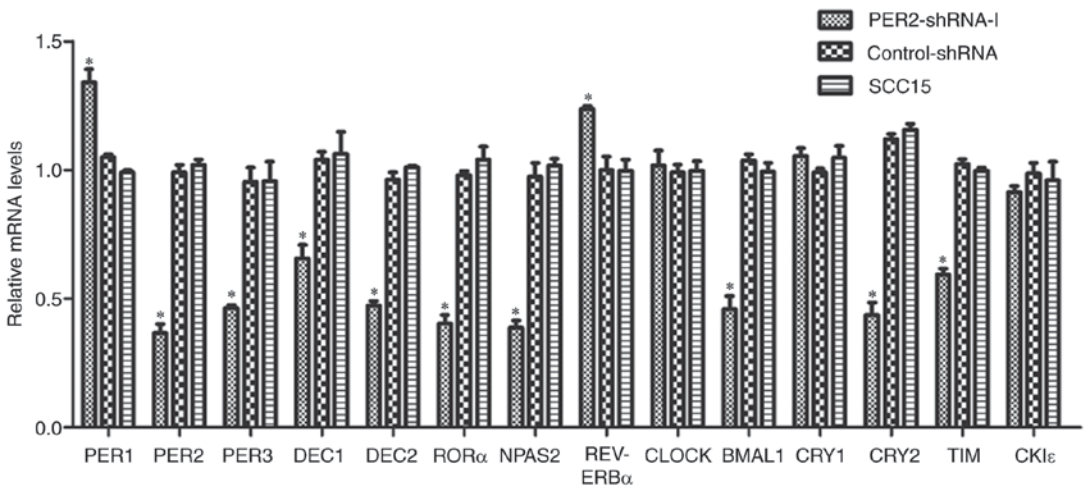

Figure 2. Alteration of mRNA expression of clock genes in SCC15 cells following *PER2* knockdown. mRNA expression levels of *PER1* and *REV-ERBα* were significantly upregulated in the PER2-shRNA-I group when compared with in the control-shRNA and SCC15 groups, while the mRNA expression of *PER3*, *BMAL1*, *DEC1*, *DEC2*, *CRY2*, *TIM*, *RORα* and *NPAS2* was significantly downregulated. There was no notable difference between the control-shRNA and SCC15 groups. The mRNA expression levels of *CLOCK*, *CRY1* and *CKIε* were not significantly different among the three groups. Data are presented as the mean ± standard deviation. *P<0.05. *PER*, period circadian clock; *REV-ERBα*, nuclear receptor subfamily 1 group D member 1; shRNA, short hairpin RNA; *BMAL1*, brain and muscle ARNT-like 1; *DEC*, deleted in esophageal cancer; *CRY*, cryptochrome circadian clock; *TIM*, timeless circadian clock; ROR*α*, retinoic acid receptor-related orphan receptor-α; *NPAS2*, neuronal PAS domain protein 2; *CLOCK*, clock circadian regulator; *CKIε*, casein kinase 1 epsilon.

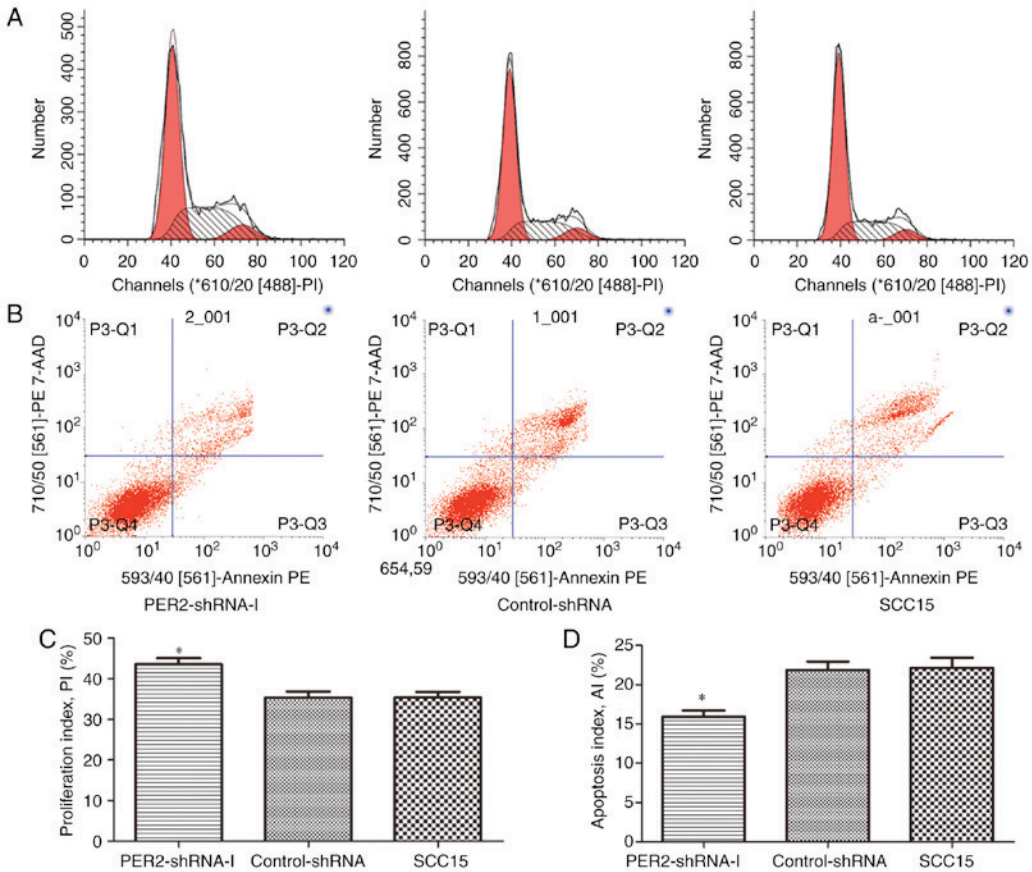

Figure 3. *PER2* knockdown enhances proliferation and reduces apoptosis of SCC15 cells. (A) Flow cytometry results of the proliferation of cells in the SCC15, control-shRNA and PER2-shRNA-I groups. (B) Flow cytometry results of the apoptosis of cells in the SCC15, control-shRNA and PER2-shRNA-I groups. (C) The proliferation index was significantly increased in the PER2-shRNA-I group. (D) The apoptosis index was significantly reduced in the PER2-shRNA-I group. Data are presented as the mean ± standard deviation. *P<0.05. *PER*, period circadian clock; shRNA, short hairpin RNA.

clock gene (23), whether its reduced expression in cancer may lead to the alteration of other clock genes remains unclear at present.

Research has demonstrated that there is a network structure, including three feedback loops, between clock genes at the translation-transcription levels in normal cells (4,24-26).

To begin with, BMAL1 combines with CLOCK or NPAS2 to form heterodimers, and acts as a transcription factor to activate the expression of *PER* genes (*PER1, PER2* and *PER3*), *CRY* genes (*CRY1* and *CRY2*), *DEC* genes (*DEC1* and *DEC2*), *TIM*, *RORα* and *REV-ERBα*. PER and CRY proteins then combine to form heterodimers and translocate into the cell nucleus to repress the activity of CLOCK/BMAL1 or NPAS2/BMAL1, thereby generating the first negative feedback loop, which is the primary feedback loop in the network (24,25). In the same way, DEC1 and DEC2 proteins form dimers or heterodimers and translocate to the nucleus to inhibit the activity of CLOCK/BMAL1, thereby comprising the second negative feedback loop (26). REV-ERBα and RORα proteins translocate to the nucleus to suppress and promote the expression of *BMAL1*, respectively, thereby generating the third feedback loop (4). In normal feedback loops involving the aforementioned clock genes, *PER2* is a component of the first, main negative feedback loop, serving as a negative regulatory factor. When the mRNA expression of *PER2* is downregulated, its inhibitory effect on the positive-regulatory factors, CLOCK/BMAL1 and NPAS2/BMAL1, is reduced; therefore, *PER2* downregulation may lead to higher mRNA expression levels of negative regulatory genes, including *PER1*, *PER3*, *CRY1*, *CRY2*, *DEC1*, *DEC2*, *TIM*, *REV-ERBα* and *RORα*. However, in the present study, following *PER2* knockdown, the mRNA expression levels of *NPAS2* and *BMAL1* were significantly downregulated, suggesting that the transcription-activating effect of the positive regulatory factor, CLOCK/BMAL1, is suppressed. Among the negative regulatory genes, only the mRNA expression levels of *PER1* and *REV-ERBα* were significantly upregulated, which is consistent with the features of circadian feedback regulation in normal cells. By contrast, the mRNA expression of remaining negative regulatory genes, including *PER3*, *DEC1*, *DEC2*, *CRY2*, *TIM* and *RORα*, was downregulated, and the mRNA expression of *CRY1* was not significantly altered, which was not consistent with the features of circadian feedback regulation in normal cells. We hypothesized that the reason for this difference may be that the three known feedback loops have been researched in normal cells (4,24-26), while the present study was conducted using cancer cells. Therefore, the regulatory effects of circadian positive- and negative-feedback networks in cancer cells may have been altered, or there may exist other compensatory regulatory approaches. However, this requires further investigation.

In the present study, the mRNA expression levels of the *CLOCK* gene were not notably altered following *PER2* knockdown in human OSCC cells. A previous study by Lee *et al* (27), conducted in normal mouse liver cells, demonstrated that *CLOCK* was not changed by the alteration of *PER2* mRNA expression. Additionally, Shearman *et al* (3) reported that, in normal mouse liver cells, *PER2* knockdown did not alter the expression of CLOCK protein, but instead promoted the nuclear translocation of the CLOCK/BMAL1 heterodimer to act as a transcription factor. Therefore, we hypothesized that the regulatory effect of silencing *PER2* on the positive regulatory factor *CLOCK* may result from changes in its intracellular distribution. In addition, *PER2* may exert no regulation on *CLOCK* at the transcriptional level.

In summary, the clock gene, *PER2,* has previously been reported to be downregulated in various types of cancer, and to be involved in the occurrence and progression of cancer via the regulation of downstream cell cycle genes (18,19). The present study has demonstrated that *PER2* is also important in the regulation of the clock gene network, which contributes to the previously established knowledge regarding *PER2* function. To the best of our knowledge, the present study is the first to report that the reduced expression of *PER2* significantly decreases the mRNA expression levels of *PER3*, *BMAL1*, *DEC1*, *DEC2*, *CRY2*, *TIM*, *RORα* and *NPAS2* in cancer cells, while the mRNA expressions of *PER1* and *REV-ERBα* were significantly upregulated. These observations also indicated that that *PER2* knockdown enhances the proliferation and reduces the apoptosis of OSCC cells. In conclusion, *PER2* serves an important role in regulating other clock genes in the clock gene network in cancer cells. This is of great importance in further illustrating the molecular functions and tumor-suppressor mechanisms of *PER2*. However, the results of the present study identified the effects on expression at the transcriptional level, and further studies at the translational level are required in the future.

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