Circadian clock gene Per2 plays an important role in cell proliferation, apoptosis and cell cycle progression in human oral squamous cell carcinoma

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Abstract. Previous studies have shown that the aberrant expression of period circadian clock 2 (Per2) is closely related to the occurrence and development of cancers, but the specific mechanism remains unclear. In the present study, we used shRNA to downregulate Per2 in oral squamous cell carcinoma (OSCC) Tca8113 cells, and then detected the alterations in cell cycle, cell proliferation and apoptosis by flow cytometric analysis and mRNA expression alterations in all the important genes in the cyclin/cyclin-dependent protein kinase (CDK)/cyclin-dependent kinase inhibitor (CKI) cell cycle network by RT-qPCR. We found that in the Tca8113 cells, after Per2 downregulation, the mRNA expression levels of cyclin A2, B1 and D1, CDK4, CDK6 and E2F1 were significantly increased ($P<0.05$), the mRNA expression levels of p53, p16 and p21 were significantly decreased ($P<0.05$), cell proliferation was significantly higher ($P<0.05$), apoptosis was significantly lower ($P<0.05$) and the number of cells in the G1/G0 phase was significantly decreased ($P<0.05$). The present study proves that in OSCC, clock gene Per2 plays an important role in cell cycle progression and the balance of cell proliferation and apoptosis by regulation of the cyclin/CDK/CKI cell cycle network. Further research on Per2 may provide a new effective molecular target for cancer treatments.

Introduction

In mammals, many life activities vary in an approximate 24 h periodic fluctuation, which is called the circadian rhythm (1,2). The circadian rhythm, which is one of the basically intrinsic characteristics of life activities, plays an important role in maintaining complicated life activities in a highly coordinated and orderly manner (3,4). The clock genes, whose rhythmic expression is responsible for circadian rhythms, exist in almost all cells in the body (5,6). To date, at least 14 core clock genes have been described, including Per1, period circadian clock 2 (Per2), Per3, Cry1, Cry2, Tim, Ck1ε, Clock, Bmal1, Rors, Rev-Erbs, Npas2, Dec1 and Dec2 (7,8). In mammals, circadian rhythms play an important role in physiological activities, including cell proliferation, metabolism and hormone secretion (9,10). Aberrant circadian rhythms lead to cardiovascular diseases, gastrointestinal diseases, nervous and mental diseases and cancers (11-13).

Per2 is an important clock gene that works as a pacemaker of circadian rhythms in mammals (14). The absence of Per2 can lead to loss of circadian rhythms (15-17). In recent years, studies have shown that the aberrant expression of Per2 is responsible for not only circadian rhythm alterations but also the occurrence and development of cancers (6,18). Per2 is reduced in various types of solid cancers, including breast and skin cancer, hepatocellular carcinoma, colorectal cancer, renal carcinoma, gastric cancer and head and neck squamous cell carcinoma (11,19-24). Since many downstream cell cycle genes are regulated by Per2, the aberrant expression of Per2 affects cell cycle progression and leads to carcinogenesis (6,22,25).

Imbalance of cell proliferation and apoptosis caused by cell cycle disorder is the main reason for carcinogenesis (26,27). The cell cycle is under the control of the cyclin/cyclin-dependent protein kinase (CDK)/cyclin-dependent kinase inhibitor (CKI) cell cycle network composed of cyclins, CDKs and CKIs (28,29). To date, there have been only dispersed research studies on the Per2 regulation of downstream cell cycle genes, and these are cyclin A, B1, D1 and E, p53, c-myc, Rb, Mdm-2 and Gadd45α, which are mainly focused on the Per2 regulation of cyclins (6,18,25,30). However, the role Per2 plays in CDKs and CKIs, which are the two other important aspects of the cyclin/CDK/CKI network, remains unclear. We speculated that Per2 may regulate numerous molecules in all the three aspects of the cyclin/CDK/CKI cell cycle network. In the present study, we downregulated Per2 in oral squamous

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cell carcinoma (OSCC) cell line Tca8113, and then detected the alterations in the cell cycle, cell proliferation and apoptosis and all the important genes in the cyclin/CDK/CKI network to further illustrate the relationship of Per2 with the occurrence and development of cancers.

Materials and methods

Cell culture. Normal oral mucosa was collected from the Department of Maxillofacial Plastic Surgery at the Affiliated Hospital of Stomatology, Chongqing Medical University following approval by the local ethics committee. Patient samples were obtained after informed consent following the tenets of the Declaration of Helsinki, and written consent was obtained from all patients prior to surgery. The mucosa was kept in 1.0 U/ml Dispase II (Roche, Indianapolis, IN, USA) at 4˚C overnight. Complete epithelial layer was separated from the mucosa under a microscope (Leica, Wetzlar, Germany). The epithelial layer was digested in 0.25% TrypLE Express (Gibco, Grand Island, NY, USA) at 37˚C, and then inoculated in a 6-well plate paved by rat tail collagen using OKM (ScienCell, San Diego, CA, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The oral mucosal epithelial cells were continuously passaged/4 days. The second generation of cells was used for RNA and protein extraction, and the third generation of cells was used for making cell climbing slices. Tca8113 cells (Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, China) were cultured in RPMI-1640 medium with 10% fetal bovine serum (both from HyClone, Logan, UT, USA) at 37˚C in an atmosphere of 95% humidity and 5% CO_2.

Purity identification of the oral mucosal epithelial cells. Cell climbing slices were fixed by 4% paraformaldehyde, and then incubated for 10 min with 3% H_2O_2 at 37˚C. After permeabilization in 0.1% Triton X-100 for 15 min, the slices were blocked using goat serum for 30 min at 37˚C. The slices were incubated firstly with the rabbit monoclonal anti-keratin antibody (1:100; ZA-0540) overnight at 4˚C, and secondly with the rabbit SP kit (SP-9001) (both from ZSGB-BIO, Beijing, China) for 1 h at 37˚C according to the manufacturer's recommended protocol. Finally, slices were examined under a microscope (Olympus, Tokyo, Japan). In negative controls all reagents were used except the primary antibody.

Downregulation of Per2 in Tca8113 cells by shRNA plasmids. The plasmids pGPU6-Per2-shRNA-I-III and pGPU6-control-shRNA were obtained from Chengdu Biotechnology Co., Ltd. (Table I). The day before transfection, the Tca8113 cells were incubated into a 6-well plate to ensure that by the time of transfection the cells reached ~30% confluency. Approximately 4 μg of the plasmids were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The effect of Per2 downregulation was examined 36-72 h later. There were five groups in our experiment: Per2-shRNA-I, Per2-shRNA-II, Per2-shRNA-III, control-shRNA and Tca8113 group. Per2-shRNA-I, Per2-shRNA-II, Per2-shRNA-III and the control-shRNA group were transfected with pGPU6-Per2-shRNA-I, pGPU6-Per2-shRNA-II,

Table I. The RNA oligos of shRNA and negative control plasmids.

Plasmid	RNA oligos (5'-3')
pGPU6-Per2-shRNA-I	5'-CACCGAAGTACGCCCTCAGGAGCTTCAAGAGAGCTCCTGAGGGCGTACTTCTTTTTTG-3'
	5'-GATCCAAAAAAGAAGTACGCCCTCAGGAGCTCTCTTGAAGCTCCTGAGGGCGTACTTC-3'
pGPU6-Per2-shRNA-II	5'-CACCGTGAAGAATGCCGATATGTTTCAAGAGAACATATCGGCA TTCTTCACTTTTTTG-3'
	5'-GATCCAAAAAAGTGAAGAATGCCGATATGTTCTCTTGAAACATATCGGCATTCTTCAC-3'
pGPU6-Per2-shRNA-III	5'-CACCGAAGTACGCCCTCAGGAGCTTCAAGAGAGCTCCTGAGGGCGTACTTCTTTTTTG-3'
	5'-GATCCAAAAAAGAAGTACGCCCTCAGGAGCTCTCTTGAAGCTCCTGAGGGCGTACTTC-3'
pGPU6-control-shRNA	5'-CCGGGCACTACGAGAAGCTAACTCAGCTCGAGCTTAGTTAGCTCTCGTAGTGTTTTTG-3'
	5'-AATTCAAAAAGCACTACGAGAAGCTAACTCAGCTCGAGCTGAGTTAGCTCTCGTAGTGC-3'

Table II. Primers used for real-time PCR amplification of gene expression

Gene	Forward primer sequence	Reverse primer sequence
Per2	5'-CGTGTTCCACAGTTTCACCT-3'	5'-GGTAGCGGATTTCATTCTCG-3'
cyclin A2	5'-ATGTCACCGTTCCTCCTTG-3'	5'-AGGGCATCTTCACGCTCTA-3'
cyclin B1	5'-TGGTTGATACTGCCTCTCCA-3'	5'-TGACTGCTTGCTCTTCCTCA-3'
cyclin D1	5'-GTGTATCGAGAGGCCAAAGG-3'	5'-CAACCAGAAATGCACAGACC-3'
cyclin E	5'-CTGGATGTTGACTGCCTTGA-3'	5'-ATGTCGCACCACTGATACCC-3'
c-myc	5'-ATCCTGTCCGTCCAAGCA-3'	5'-CGCACAAGAGTTCCGTAG-3'
p53	5'-GTCCAACAACACCAGCTCCT-3'	5'-CTCTCGGAACATCTCGAAGC-3'
CDK1	5'-GTCCGCAACAGGGAAGAAC-3'	5'-CGAAAGCCAAGATAAGCAACT-3'
CDK2	5'-CAGGATGTGACCAAGCCAGT-3'	5'-TGAGTCCAAATAGCCCAAGG-3'
CDK4	5'-CTGGACACTGAGAGGGCAAT-3'	5'-TGGGAAGGAGAAGGAGAAGC-3'
CDK6	5'-TCTTCATTCACACCGAGTAGTGC-3'	5'-TGAGGTTAGAGCCATCTGGAAA-3'
Rb1	5'-CACAAGCAACCTCAGCCTTC-3'	5'-GCGTTCACAAAGTGTATTTAGCC-3'
E2F1	5'-CCAACTCCCTCTACCCTTGA-3'	5'-GTCTCCCTCCCTCACTTTCC-3'
Wee1	5'-TGTGGTGGTGTGCTGCTTAT-3'	5'-TTCAAAGGGAGGGTATGTCTG-3'
cdc25	5'-TACTCGGCCATGTCACCCTT-3'	5'-GGGTCGTATCGCCCTCATC-3'
p16	5'-ACCAGAGGCAGTAACCATGC-3'	5'-TGATCTAAGTTTCCCGAGGTTT-3'
p21	5'-TTAGCAGCGGAACAAGGAGT-3'	5'-CGTTAGTGCCAGGAAAGACA-3'
GAPDH	5'-ACAACTTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

pGPU6-Per2-shRNA-III and pGPU6-control-shRNA, respectively; the Tca8113 group did not accept any reagents.

Western blotting. The cells were lysed using RIPA + PMSF (Beyotime, Jiangsu, China) and centrifuged for 2 min, at 4˚C and 167.7 x g. The concentration of cell protein was detected using the enhanced BCA protein assay kit (Beyotime). Proteins (50 μg) were seperated by 8% SDS-PAGE using Mini-PROTEAN 3 system (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL, USA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were incubated with mouse monoclonal anti-Per2 antibody (1:500; 19-J6:sc-101105; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal anti-β-actin antibody (1:1,000; 60008-1-lg), respectively, overnight at 4˚C, followed by goat monoclonal anti-mouse IgG (1:1,000) (SA00001-1) (both from ProteinTech, Chicago, IL, USA) for 1 h at 37˚C. Blots were detected using enhanced chemiluminescence reagent (Pierce) under a fluorescent chemiluminescence imaging system (ChemiDoc XRS+; Bio-Rad). The software Quality One (Bio-Rad) was used to analyze the blots. To ensure accuracy the experiment was performed in triplicate.

Flow cytometric analysis. For analysis of the cell cycle, after a 48-h transient transfection, the cells were harvested and fixed using 70% ethanol overnight at 4˚C. The cells were stained with propidium iodide solution (Cell Cycle Detection kit; KGA, China) for 30 min at 4˚C in the dark, and subsequently detected by a flow cytometer (FACSVantage; BD Biosciences, San Jose CA, USA). The following formula was used to calculate the proliferation index (PI) of the cells: PI = (S + G2/M)/(G0/G1 + S + G2/M) x 100% (G0, G1, S, G2 and M represent the corresponding cell cycle phases). To ensure accuracy, the experiment was performed in triplicate. For analysis of apoptosis, after a 48-h transient transfection, the cells were harvested and stained with the Annexin V-FITC cell apoptosis analysis kit (with propidium iodide) (Sungene, Tianjin, China). Apoptotic cells were quantified by a flow cytometer (FACSVantage). The following formula was used to calculate apoptotic index (AI) of the cells: AI = (number of apoptotic cells/number of total detected cells) x 100%. To ensure accuracy, the experiment was performed in triplicate.

Quantitative real-time PCR (RT-qPCR). After a 36-h transient transfection, total RNA was isolated from the cells using RNAiso Plus (9109; Takara, Japan). The optical density and concentration of RNA were detected by an ultraviolet spectrophotometer (NanoDrop, USA). RNA was reverse-transcribed with 20 μl of the system with a Prime Script RT reagent kit (RR047A; Takara) on a T100 thermal cycler (Bio-Rad, Singapore) according to the manufacturer's instructions. Oligo 17.0 software was used to design the specific primers of p53, p16, p21, cyclin A2, B1, D1 and E, CDK1, CDK2, CDK4, CDK6, E2F1, c-myc, cdc25, Wee1, Rb1 and GAPDH (endogenous reference) (Table II). According to the manufacturer's instructions, cDNA was used as the template for amplification using SYBR Premix Ex Taq™ II (RR820A; Takara) on a CFX96 Real-Time PCR Detection system (Bio-Rad). The threshold cycle (Ct) value was acquired. The relative expression levels of p53, p16, p21, cyclin A2, B1, D1 and E, CDK1, CDK2, CDK4, CDK6, E2F1, c-myc, cdc25, Wee1 and Rb1 mRNA in cells were calculated using the $2^{-\Delta\Delta Ct}$ method. To ensure accuracy, the experiment was performed in triplicate.

Statistical analysis. The SPSS 17.0 statistical software package was used to analyze and calculate the mean ± SD of the data. The difference in Per2 expression between epithelial

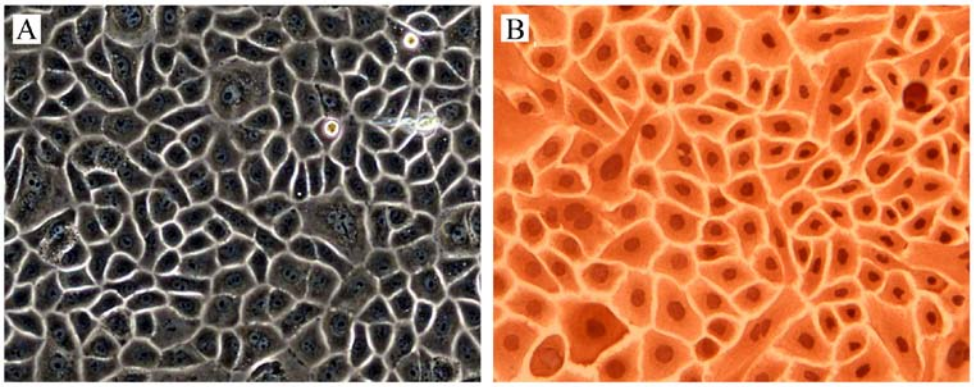

Figure 1. Culture and purity identification of the oral mucosal epithelial cells. (A) Oral mucosal epithelial cells had a paving stone appearance under a microscope (magnification, x100). (B) Keratin (red) was expressed in 100% of the cells under a microscope (magnification, x100).

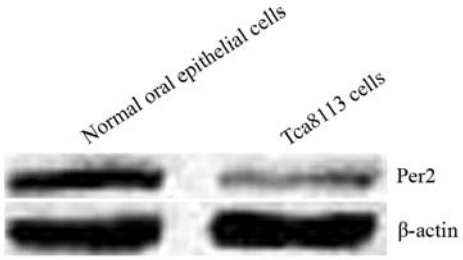

Figure 2. Expression of Per2 protein in oral mucosal epithelial and Tca8113 cells. In the oral mucosal epithelial and Tca8113 cells the expression levels of Per2 protein were 2.87±0.26 and 1.11±0.13, respectively. The expression level of Per2 protein in the Tca8113 cells was significantly lower than that in the oral mucosal epithelial cells (P<0.05). β-actin served as a loading control. The result is representative of three separate experiments.

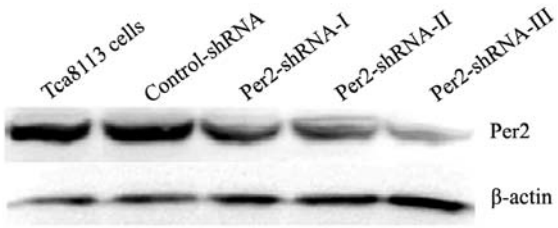

Figure 3. Expression of Per2 protein in the Tca8113 cells after transfection. In the Tca8113, control-shRNA, Per2-shRNA-I, Per2-shRNA-II and Per2-shRNA-III groups, the expression levels of Per2 protein were 3.21±0.42, 3.18±0.52, 1.52±0.11, 1.22±0.15 and 0.87±0.21, respectively. There was no significant difference in Per2 protein expression between the control-shRNA and Tca8113 group (P>0.05). Per2 mRNA and protein expression levels in the Per2-shRNA-III group were significantly lower than those in the control-shRNA and Tca8113 group (P<0.05). β-actin served as a loading control. The result is representative of three separate experiments.

and Tca8113 cells was analyzed using group t-test. In addition, one-way ANOVA was used to analyze the differences between the various groups transfected with the different plasmids. A value of P<0.05 was considered statistically significant.

Results

Culture and purity identification of the oral mucosal epithelial cells. Under microscope, the oral mucosal epithelial cells had a paving stone appearance (Fig. 1A), and keratin was expressed in 100% of the cells (Fig. 1B), which identified the purity of the oral mucosal epithelial cells.

Expression of Per2 mRNA and protein in the oral mucosal epithelial and Tca8113 cells. In the oral mucosal epithelial and Tca8113 cells, the expression of Per2 mRNA was 2.41±0.21 and 1.00±0.12, respectively; and the expression of Per2 protein was 2.87±0.26 and 1.11±0.13, respectively (Fig. 2). The expression levels of Per2 mRNA and protein in the Tca8113 cells were significantly lower than those of the oral mucosal epithelial cells (P<0.05), indicating that Per2 expression is reduced in OSCC.

Alterations of Per2 mRNA and protein expression after transfections in the Tca8113 cells. In the Tca8113, control-shRNA, Per2-shRNA-I, Per2-shRNA-II and Per2-shRNA-III groups, the expression levels of Per2 mRNA were 3.20±0.52, 3.01±0.11, 1.67±0.30, 1.45±0.34 and 1.00±0.13, respectively; and the expression levels of Per2 protein were 3.21±0.42, 3.18±0.52, 1.52±0.11, 1.22±0.15 and 0.87±0.21, respectively (Fig. 3). Per2 mRNA and protein expression showed no significant difference between the control-shRNA and Tca8113 groups (P>0.05). However, Per2 mRNA and protein expression levels were significantly lower in the Per2-shRNA-III group than those noted in the control-shRNA and Tca8113 groups (P<0.05). Therefore, Per2 was effectively reduced in the Per2-shRNA-III group, and the Per2-shRNA-III group was adopted for subsequent experiment.

Effects of Per2 downregulation on cell cycle distribution, PI and AI in the Tca8113 cells. The cells from the different groups were collected and their cell cycle distribution (Fig. 4A; Table III) and apoptosis (Fig. 4B; Table III) were analyzed, and then PI and AI were calculated (Table III). Compared with those of the Tca8113 and control-shRNA groups, the Per2-shRNA-III group had a significantly decreased number of cells in the G1/G0 phase (P<0.05), significantly increased PI (P<0.05), and significantly decreased AI (P<0.05). The number of cells in the G1/G0 phase, and PI and AI showed no significant difference between the Tca8113 and control-shRNA group (P>0.05).

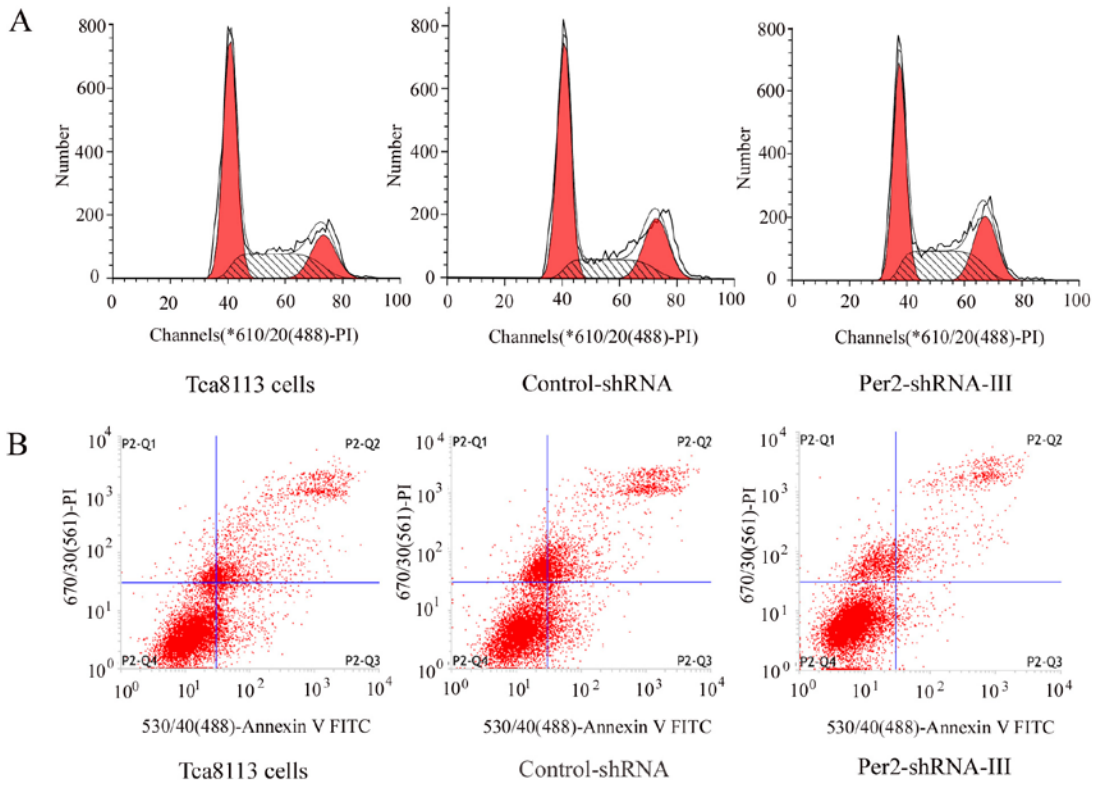

Figure 4. Effects of Per2 downregulation on cell cycle distribution and apoptosis in the Tca8113 cells. (A) Flow cytometric analysis of the cell cycle. Compared with those of the Tca8113 and control-shRNA groups, the Per2-shRNA-III group had a significantly decreased number of cells in the G1/G0 phase (P<0.05). The number of cells in the G1/G0 phase showed no significant difference between the Tca8113 and control-shRNA group (P>0.05). (B) Flow cytometric analysis of apoptosis. Compared with those of the Tca8113 and control-shRNA groups, the number of apoptotic cells was significantly decreased in the Per2-shRNA-III group (P<0.05). The number of apoptotic cells showed no significant difference between the Tca8113 and control-shRNA group (P>0.05).

Table III. Effects of shRNA on cell cycle distribution, PI and AI in Tca8113 cells (mean ± SD).

Index	Tca8113	Control-shRNA	Per2-shRNA-III	P	P_1	P_2	P_3
G1/G0 (%)	51.00±2.12	51.83±2.27	46.04±1.76	0.028	0.026	0.014	0.642
S (%)	29.23±1.10	27.41±4.19	30.93±1.94	0.355	0.476	0.167	0.447
G2/M (%)	19.77±2.75	20.76±3.17	23.03±0.71	0.319	0.155	0.301	0.638
PI (%)	49.00±2.12	48.17±2.27	53.96±1.77	0.028	0.026	0.014	0.642
AI (%)	14.66±1.93	14.13±0.94	10.26±1.02	0.015	0.008	0.014	0.651

The cells from different groups were collected and their cell cycle distribution, PI and AI were analyzed. Compared with those of the Tca8113 and control-shRNA groups, in the Per2-shRNA-III group the number of cells at the G1/G0 phase was significantly decreased (P<0.05), PI was significantly increased (P<0.05) and AI was significantly decreased (P<0.05). There was no significant difference in the number of cells at the G1/G0 phase, PI and AI between the Tca8113 and control-shRNA group (P>0.05). P represents one-way ANOVA among the three groups. P_1, P_2 and P_3 represent the pairwise comparison with the Per2-shRNA-III and Tca8113 group, Per2-shRNA-III and control-shRNA group and control-shRNA and Tca8113 group, respectively. A value of P<0.05 represents a significant difference. PI, proliferation index; AI, apoptosis index; G0/G1, cell number at the G0 and G1 phase; G2/M, cell number at the G2 and M phase.

Effects of Per2 downregulation on mRNA expression of cell cycle-related genes in the Tca8113 cells. Results of the RT-qPCR are shown in Table IV. Compared with those of the Tca8113 and control-shRNA groups, the Per2-shRNA-III group had a significantly decreased mRNA expression of p53, p16 and p21 (P<0.05), significantly increased mRNA expression of cyclin A2, B1 and D1, CDK4, CDK6, E2F1 (P<0.05), and a similar mRNA expression of c-myc, cyclin E, CDK1, CDK2, cdc25, Wee1 and Rb1 (P>0.05). Between the Tca8113 and control-shRNA group, there was no significant difference in mRNA expression of p53, p16, p21 cyclin A2, B1 and D1, CDK4, CDK6 and E2F1 (P>0.05).

Discussion

Previous studies have shown that Per2 expression is reduced in various types of solid cancers, including breast and skin cancer, hepatocellular carcinoma, colorectal cancer, renal

Table IV. Effects of shRNA on the mRNA expression of cell cycle-related genes in the Tca8113 cells (mean ± SD).

Gene	Tca8113	Control-shRNA	Per2-shRNA-III	P	P_1	P_2	P_3
Cyclin A2	0.99±0.02	0.96±0.07	3.36±0.53	0.000	0.000	0.000	0.911
Cyclin B1	0.97±0.35	1.02±0.22	2.77±0.83	0.01	0.006	0.007	0.926
c-myc	1.10±0.17	1.21±0.37	1.39±0.33	0.536	0.286	0.512	0.653
Cyclin D1	0.86±0.13	1.01±0.47	3.11±0.55	0.001	0.001	0.001	0.672
Cyclin E	1.45±0.39	1.31±0.53	1.27±0.47	0.884	0.651	0.924	0.719
p53	2.24±0.46	2.38±0.47	0.57±0.02	0.002	0.002	0.001	0.667
CDK1	1.14±0.24	1.04±0.11	1.07±0.13	0.771	0.648	0.815	0.496
CDK2	1.10±0.13	1.23±0.40	1.08±0.10	0.731	0.906	0.477	0.549
CDK4	1.06±0.28	0.87±0.22	1.79±0.33	0.016	0.019	0.007	0.447
CDK6	1.00±0.00	1.00±0.25	2.74±0.74	0.005	0.003	0.003	0.997
p16	1.60±0.13	1.70±0.08	0.92±0.14	0.000	0.000	0.000	0.354
p21	3.88±0.21	4.05±0.60	1.10±0.26	0.000	0.000	0.000	0.633
cdc25	1.46±0.40	1.32±0.55	1.62±0.54	0.775	0.709	0.493	0.747
Wee1	1.38±0.86	1.11±0.20	1.32±0.66	0.867	0.917	0.700	0.627
Rb1	1.16±0.39	1.31±0.27	1.25±0.25	0.83	0.735	0.802	0.560
E2F1	0.92±0.56	1.13±0.54	3.46±0.87	0.006	0.004	0.005	0.710

mRNA expression of p53, p16, p21, cyclin A2, B1 and D1, CDK4, CDK6, E2F1, c-myc, cyclin E, CDK1, CDK2, cdc25, Wee1 and Rb1 in the Tca8113, control-shRNA and Per2-shRNA-III groups was calculated using the $2^{-\Delta\Delta Ct}$ method (mean ± SD). P represents one-way ANOVA in the three groups. P_1, P_2 and P_3 represent the pairwise comparison with the Per2-shRNA-III and Tca8113 group, Per2-shRNA-III and control-shRNA group and control-shRNA and Tca8113 group, respectively. A value of $P<0.05$ represents a significant difference.

carcinomas, gastric cancers and head and neck squamous cell carcinomas (11,19-24). The alteration in Per2 expression has a close relationship with the occurrence and development of cancers (6,18). The present study found that Per2 expression in OSCC Tca8113 cells was significantly lower than that in the oral mucosal epithelial cells; in Tca8113 cells, downregulation of Per2 significantly increased PI, decreased AI and altered the cell cycle distribution by significantly decreasing the number of cells in the G1/G0 phase, which suggested that the clock gene Per2 has a tumor-suppressor role in OSCC.

Cell cycle disorder is the main reason for carcinogenesis (26,27). Normal cell cycle strictly and chronologically progresses along the G1, S and G2 M phase under the precise control of the cyclin/CDK/CKI cell cycle molecular network (29). CDKs are the core of the cell cycle, to which cyclins and CKIs are the positive and negative regulators, respectively (27,29). Cyclin A2, B1, D1 and E play an important role in cyclins; and CDK1, CDK2, CDK4 and CDK6 play an important role in CDKs (27,29). CKIs contain the Ink4 family and Cip/Kip family, in which p16 and p21 play an important role, respectively (27,29). In the different cell cycle phases, cyclins, CDKs and CKIs vary. Cyclin/CDK complex formed by combinations of cyclins and CDKs can promote orderly cell cycle progression by activating CDKs. While CKIs inhibit CDKs by combining with the corresponding CDKs or the cyclin/CDK complex, which may inhibit the transformation of the cell cycle phase (27,29). To date, studies have confirmed that Per2 can regulate cyclins and p53 which is a regulator of the cell cycle checkpoint, and the mutation of Per2 expression is responsible for the aberrant expression of cyclin A, B1, D1 and E, and p53 (6,18,25,31). Both changes in cell cycle progression and imbalance of cell proliferation and apoptosis induce cancers. Previous studies have mainly focused on the role of Per2 in cyclins (6,18,25,31), but there is little research concerning the role of Per2 in the other two important aspects of the cyclin/CDK/CKI network.

At the G1 phase, the p16/p21-cyclin D1-CDK4/6-Rb1-E2F1 pathway which is an important transduction pathway of molecular information is related to the occurrence and development of tumors (32). E2F1 plays an important role in promoting the transition of cells from the G1 to the S phase (33). At the G0 and the early G1 phase, the transcriptional activity of E2F1 is inhibited by combining with unphosphorylated Rb1 at specific binding sites. At the late G1 phase, the cyclin D1/CDK4 and cyclin D1/CDK6 complex, formed by combination of cyclin D1 and CDK4/6, phosphorylate Rb1 from which E2F1 is consequently released to start DNA biosynthesis and promote cells into the S phase (34). p16 and p21, as CDKIs, can inhibit the activities of CDK4/6 by competing with cyclin D1 for CDK4/6 binding (35). The present study found that downregulation of Per2 in Tca8113 cells significantly reduced the expression of p16 and p21, significantly increased the expression of cyclin D1, CDK4, CDK6 and E2F1 and significantly reduced the number of cells at the G1 phase. These results illustrate that in Tca8113 cells, Per2 downregulation decreases the expression of p16 and p21, and consequently increases the binding of cyclin D1 and CDK4/6, which can phosphorylate Rb1 to release more E2F1 from the Rb1/E2F1 complex, resulting in promotion of the cell transformation during the G1/S phase. Fu *et al* (6) and Yang *et al* (36) both reported that the downregulation of Per2 increased the expression of cyclin D1. In the present study, there was no significant difference in the expression of Rb1

mRNA, and a difference in phosphorylated Rb1 and unphosphorylated Rb1 was not detected.

p53 is an important regulator of the G1/S cell cycle checkpoint in the cyclin/CDK/CKI network (37). In the G1/S checkpoint, p53 is activated by damaged DNA to stagnate the progression of the cell cycle, leading to either repair of the damaged DNA or apoptosis (14,27). Meanwhile p53 in the cytoplasm can directly react with the BCL-2 family to promote cell permeabilization of mitochondria and apoptosis (18). The present study found that Per2 downregulation in the Tca8113 cells significantly reduced the expression of p53, the number of cells at G1 and AI. This suggests that in Tca8113 cells, Per2 downregulation reduces the expression of p53, leading to a reduction in the repair of damaged DNA in the G1/S phase checkpoint and a decreased ability to induce apoptosis, resulting in the damaged DNA being translated into the S phase. This can destroy the integrity and stability of the cell genome, which promotes cell malignant transformation. Meanwhile, p53 can reduce the expression of cyclin B1 (25). During the G2/M phase, the absence of cyclin B1 could block cells at the G2 phase, resulting in the inability to enter into the M phase (25). Thus, in the present study, Per2 downregulation significantly reduced the expression of p53, and subsequently significantly increased the expression of cyclin B1, which accelerated mitosis and significantly increased the PI. Gotoh *et al* reported that Per2 is at the key site of the transcription mediated by p53, and Per2 downregulation reduces p53 expression (31). Sun *et al* reported that in leukemic K562 cells Per2 downregulation decreased p53 expression, and Per2 overexpression increased p53 expression (25). Hua *et al* reported that Per2 overexpression increased p53 expression in Lewis lung cancer cells (LLCs), decreased cell proliferation and accelerated apoptosis in LLCs and breast cancer cells (EMT6) (18). The present study was in accordance with the above reports. The present study also proved that Per2 downregulation significantly increased and decreased the expression of E2F1 and p21, respectively, which are the dual-directional regulators of apoptosis (38,39), resulting in a worsening of the imbalance of cell proliferation and apoptosis.

The present study found that circadian clock gene Per2 was reduced in OSCC. In the Tca8113 cells, Per2 downregulation significantly increased the mRNA expression of cyclin A2, B1 and D1, CDK4, CDK6 and E2F1, while significantly decreased the mRNA expression of p53, p16 and p21. Cell proliferation was significantly higher, apoptosis was significantly lower, and progression of the cell cycle was altered. The present study represents the first demonstration that in OSCC, the clock gene Per2 plays an important role in the G1/S checkpoint and the three aspects of the cyclin/CDK/CKI network at the transcriptional level. On this basis, further research of Per2 at the protein level and the modification level after protein translation may further define the interaction of the circadian rhythm and the cell cycle, and their relationship with carcinogenesis. This may provide effective new molecular targets for the treatment of cancers.

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