Cryptochrome 2 (CRY2) Suppresses Proliferation and Migration and Regulates Clock Gene Network in Osteosarcoma Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Circadian disruption is a potential cancer risk factor in humans. However, the role of the clock gene, cryptochrome 2 (CRY2), in osteosarcoma (OS) is still not clear.

Material/Methods: To evaluate the potential role of CRY2 in HOS osteosarcoma cells, CRY2-silenced cell lines were established. Furthermore, we investigated the effect of CRY2 knockdown on HOS cells by CCK-8, colony formation, migration assay, and flow cytometry, in vitro.

Results: CRY2 knockdown promoted HOS OS cell proliferation and migration. We used a cell cycle assay to show that CRY2 knockdown increased the S phase cell population and reduced the G1 phase cell population. Western blot analyses showed that CRY2 knockdown decreased P53 expression and increased expression of c-myc and cyclin D1. Simultaneously, CRY2 knockdown increased the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, but did not change the phosphorylation of c-Jun N terminal kinase (JNK) and P38. CRY2 knockdown also increased the expression of matrix metalloproteinase (MMP)-2 and β-catenin, and increased OS cell proliferation and migration by inducing cell cycle progression and promoting mitogen-activated protein kinase (MAPK) and Wnt/β-catenin signaling pathways. Although it has previously been unclear whether the expression of CRY2 affects the expression of other clock genes in the clock gene network, our results show that knockdown of CRY2 significantly increased the mRNA expression of CRY1, Period (PER) 1, PER2, BMAL1, and CLOCK.

Conclusions: Our results suggest that CRY2 may be an anti-oncogene in OS, whose functions involve both downstream genes and other circadian genes.

MeSH Keywords: Cell Migration Assays • Cell Proliferation • Circadian Clocks • Cryptochromes • Osteosarcoma

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Background

The circadian clock is an internal timing system shared by many living organisms, which coordinates nearly all aspects of physiology and behavior to match day/night alternating patterns [1]. Deregulation of circadian rhythm may negatively affect normal cellular functions, potentially leading to increased incidences of several kinds of cancer, such as colorectal cancer [2], breast cancer [3], prostate cancer [4], pancreatic cancer [5], osteosarcoma (OS) [6], and other cancers [7,8]. It is therefore important to characterize the involvement of the circadian clock in cancer development and tumor progression. The circadian clock is controlled by the clock genes, which drive approximately 5–15% of genome-wide mRNA expression. In mammals, the circadian system is based on a central pacemaker located in suprachiasmatic nuclei, and local oscillators in peripheral tissues, which are mainly composed of feedback loops of sets of the following core circadian genes: *Period 1* (*PER1*), *Period 2* (*PER2*), *Period 3* (*PER3*), *CLOCK*, *BMAL1*, *Cryptochrome 1* (*CRY1*), and *Cryptochrome 2* (*CRY2*). *PER* and *CRY* accumulate in the cytoplasm, and enter the nucleus where they disrupt *BMAL1/CLOCK*-associated transcriptional complexes and attenuate *BMAL1/CLOCK* activity, to form the major circadian negative feedback loop [9,10].

The *CRY2* gene, which encodes the *CRY2* protein, is a core component of the circadian clock and is necessary for the stability of circadian rhythms. *CRY2* plays a key role in DNA damage checkpoint control, and regulates important cell cycle progression genes [11]. In addition, *CRY2* modulates the risk of breast cancer, possibly by mediating hormone signaling [12], and *CRY2* degradation is involved in the chemoresistance of colorectal cancer [13]. Importantly, several studies have reported that disruption of circadian rhythms is a potential cancer risk factor in humans [12,13]. However, Ozturk [14] reported that the loss of Cryptochrome reduced cancer risk in p53 mutant mice. These contradictory reports suggest that *CRY2* has a complex function in cancer development.

As the most common primary malignant bone tumor encountered in childhood and adolescence, OS is characterized by its poor prognosis and rapid death resulting from lung metastases [15]. Although advances in treatment have combined surgery and chemotherapy, most OS patients have a poor prognosis [16]. It is therefore important to identify the cellular and molecular mechanisms responsible for the development of OS.

However, studies about the relationship of *CRY2* expression with the development of OS have rarely been reported. In the present study, we showed that knockdown of the clock gene, *CRY2*, promoted proliferation and migration of human OS cell lines via the protein kinase RNA-like endoplasmic reticulum kinase (PERK) and β-catenin signaling pathways. Moreover, *CRY2* knockdown

affected the clock gene expression network. Finally, we showed that *CRY2* is a novel regulator of OS development, as well as a possible therapeutic target for the treatment of OS patients.

Material and Methods

Cell culture

HOS human OS cell line and 293T cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 mg/mL streptomycin (Invitrogen), and 100 IU/mL penicillin (Invitrogen). The cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Plasmid construction, lentivirus production, and transfection

The lentivirus of *CRY2* short hairpin RNAs (shRNAs) and their control were purchased from Hanbio (Shanghai, China). The lentiviral vector was pHBLV-U6-ZsGreen-Puro. The shRNA sequences were as follows: *CRY2*, shRNA-1, 5'-TCAACCGATGGAGGTTCCTACTTCA-3' and *CRY2*, shRNA-2, 5'-GTGGAAGTAGTGACGGAGAAT-3'. The target cells were infected with 5 μL of lentivirus in 10 μg/mL polybrene for 48 h. According to the virus infection instructions, puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added to the target cells at a concentration of 3 μg/mL to select cells for 7 days before being used for further experiments.

Western blotting

The cells were washed with ice-cold phosphate-buffered saline and solubilized in radioimmunoprecipitation lysis buffer (Merck Millipore, Darmstadt, Germany). The concentration of protein lysate was determined using the BCA Protein Assay Kit (Beyotime, Nanjing, China). Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corp, Billerica, MA). The membrane was then blocked with 5% skim milk for 2 h and incubated with primary antibody overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Then, the bound antibody was visualized using the FluoChem E system (Bio-Techne, Minneapolis, MN, USA). The antibodies used were as follows: rabbit anti-*CRY2* antibody (1: 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-β-catenin antibody (1: 1000, Santa Cruz Biotechnology), mouse anti-P53 antibody (1: 1000, Santa Cruz Biotechnology), rabbit anti-cyclin D1 antibody (1: 1000, Santa Cruz Biotechnology), rabbit

anti-matrix metalloproteinase (MMP) 2 antibody (1: 1000, Santa Cruz Biotechnology), mouse anti-β-actin antibody (1: 1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-c-myc antibody (1: 1000, Cell Signaling Technology), rabbit anti-extracellular signal-regulated kinase (ERK) 1/2 antibody (1: 1000, Cell Signaling Technology), rabbit anti-phospho-ERK1/2 antibody (1: 1000, Cell Signaling Technology), rabbit anti-c-Jun N terminal kinase (JNK) antibody (1: 1000, Cell Signaling Technology), rabbit anti-phospho-JNK antibody (1: 1000, Cell Signaling Technology), rabbit anti-p38 antibody (1: 1000, Cell Signaling Technology), and rabbit anti-phospho-p38 antibody (1: 1000, Cell Signaling Technology).

Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8) assay. In brief, 3×10^3 cells/well were plated in 96-well culture plates (Corning, NY, USA) in 100 μL DMEM and cultured for 4 days. Then, 10 μL of the CCK reagent (Dojindo) was added to each well and the plate was incubated at 37°C for 2 h. The absorbance was then measured at 450 nm using a microplate reader (Tecan Ltd., Switzerland).

Colony forming assay

Cells were seeded at 500 cells/6-cm dish and incubated for 2 weeks at 37°C in a 5% CO_2 atmosphere. The cells were then fixed and stained with crystal violet. Only colonies containing >50 cells were manually counted. Each experiment was repeated 3 times in duplicate.

Migration assay

Cells (4×10^5) were seeded in 6-well plates and incubated overnight. When approximately 90% confluency was reached, a wound was made by scratching a pipette tip across the cell monolayer. The distance migrated by the cell monolayer to the wounded area was observed and measured. The migration index was the ratio of the cell migration distance at 48 h compared to that at 0 h. The assay was repeated in triplicate.

Cells (8×10^4) in 200 μL of serum-free medium were seeded into the top chamber of cell culture inserts (24-well, 8 μm pore size; BD Biosciences, San Jose, CA, USA). After incubation at 37°C for 48 h, the cells that passed through the membrane were fixed in 100% methanol for 20 min and stained with 0.1% crystal violet for 30 min, then imaged and counted. All the above experiments were repeated in triplicate.

Cell cycle assay

The cells were harvested by centrifugation ($400 \times g$, 5 min at room temperature), fixed in 70% ice-cold ethanol at −20°C overnight, and stained with propidium iodide (BD Biosciences) containing RNase A at room temperature for 30 min in the dark. Samples were then analyzed by flow cytometry (Beckman, Brea, CA).

Quantitative real-time polymerase chain reaction (RT-PCR) analysis

The medium was removed from the culture plates, and total RNA was isolated from cell lines using TRIzol reagent (Takara, Shiga, Japan). Reverse transcription was performed using the PrimeScript™ RT Master Mix (Takara). qRT-PCR was performed using an ABI 7500 Realtime PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green Premix Ex Taq™ (Takara). The conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s.
The following primers were used:
CRY1, forward primer, 5'-CTCCTCCAATGTGGGCATCAA-3', reverse primer, 5'-CCACGAATCACAAACAGACGG-3';
CRY2, forward primer, 5'-TCCCAAGGCTGTTCAAGGAAT-3', reverse primer, 5'-TGCATCCCGTTCTTTCCCAAA-3';
PER1, forward primer, 5'-AGTCCGTCTTCTGCCGTATCA-3', reverse primer, 5'-AGCTTCGTAACCCGAATGGAT-3';
PER2, forward primer, 5'-GACATGAGACCAACGAAAACTGC-3', reverse primer, 5'-AGGCTAAAGGTATCTGGACTCTG-3';
BMAL1, forward primer, 5'-TGCAACGCAATGTCCAGGAA-3', reverse primer, 5'-GGTGGCACCTCTTAATGTTTTCA-3';
CLOCK, forward primer, 5'-TGCGAGGAACAATAGACCCAA-3', reverse primer, 5'-ATGGCCTATGTGTGCGTTGTA-3'; and
β-*actin*, forward primer, 5'-TGTAAGGTTGTCCAGTTCAAAAGACT-3', reverse primer, 5'-CCAGCTCACCATGGATGATG-3'.
β-*actin* was used as the reference gene for quantification. The data were analyzed using the relative standard curve method and the delta-delta Ct method. The assays were done in triplicate.

Statistical analysis

The results were presented as the mean ± standard deviation of 3 independent experiments. A two-tailed Student's *t*-test was used to compare the differences between different groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

CRY2 knockdown promoted HOS cell proliferation and migration

To evaluate the potential role of *CRY2* in HOS osteosarcoma cells, *CRY2*-silenced cell lines were established. The knockdown of *CRY2* by 2 specific shRNAs (shRNA1 and 2) in HOS cells was assayed using Western blotting (Figure 1A) and
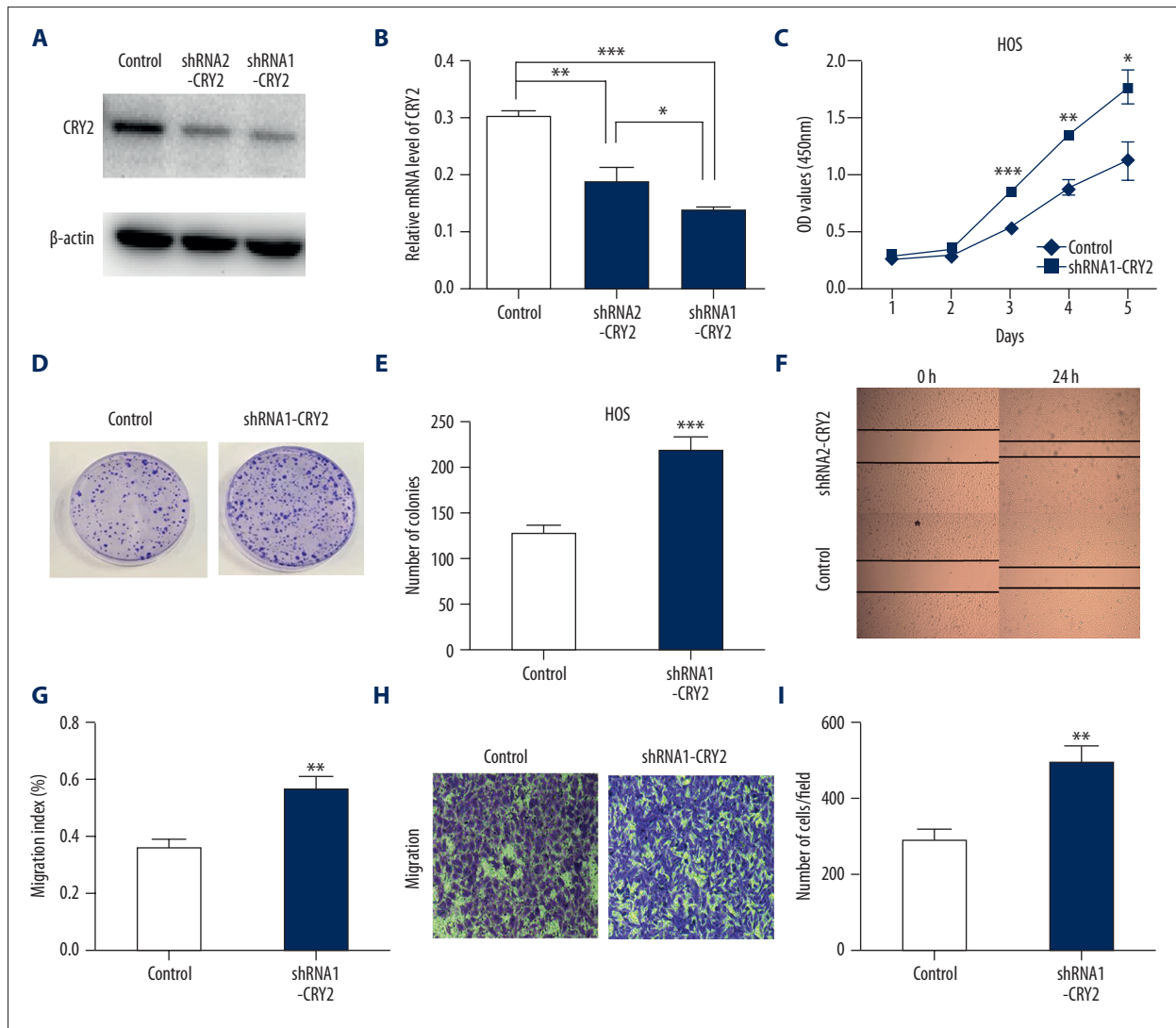
Figure 1. *Cryptochrome 2* (*CRY2*) knockdown promoted HOS cell proliferation and migration. (**A**) The protein level of CRY2 was determined by Western blotting. (**B**) RT-PCR confirmed *CRY2* mRNA expression after transfection. (**C**) Cell proliferation was measured by the CCK-8 assay. (**D**) Images of cell colony formation. (**E**) Quantitative analyses of the relative colony number. (**F**) Detection of migration of HOS cells after wounding. (**G**) Quantitative analyses of the above-mentioned migrated cells. (**H**) HOS cells detected by crystal violet staining. (**I**) Quantitative analyses of the above-mentioned migrated cells. Data are presented as the mean ± standard deviation (SD) from 3 independent experiments (*** $P<0.001$; ** $P<0.01$, and * $P<0.05$).

RT-qPCR (Figure 1B). The knockdown efficiency of shRNA1-*CRY2* was greater than shRNA2-*CRY2*, so further studies only used shRNA1-*CRY2*. *CRY2*-silenced HOS cells grew faster than control cells (Figure 1C). Compared with control cells, the cell colony assay showed that the number of colonies formed by *CRY2*-silenced cells was significantly increased (Figure 1D, 1E). We found that the migration ability of the shRNA1-*CRY2* group was increased when compared with the control group after 24 h of culture (Figure 1F, 1G). In addition, the Transwell® migration assay (without Matrigel) showed that the migration of the shRNA1-*CRY2* group was also increased compared with the control group after 48 h of culture (Figure 1H, 1I). Taken

together, the results showed that *CRY2* knockdown promoted cell migration when compared with control cells.

CRY2 knockdown induced cell cycle progression and promoted PERK and β-catenin signaling pathways in HOS cells

As shown in Figure 2A, *CRY2* knockdown increased the S phase population and reduced the G1 phase population. Subsequent results indicated that *CRY2* knockdown decreased the expression of P53 protein and increased the expression of c-myc and cyclin D1 (Figure 2B). These data suggest that *CRY2* knockdown
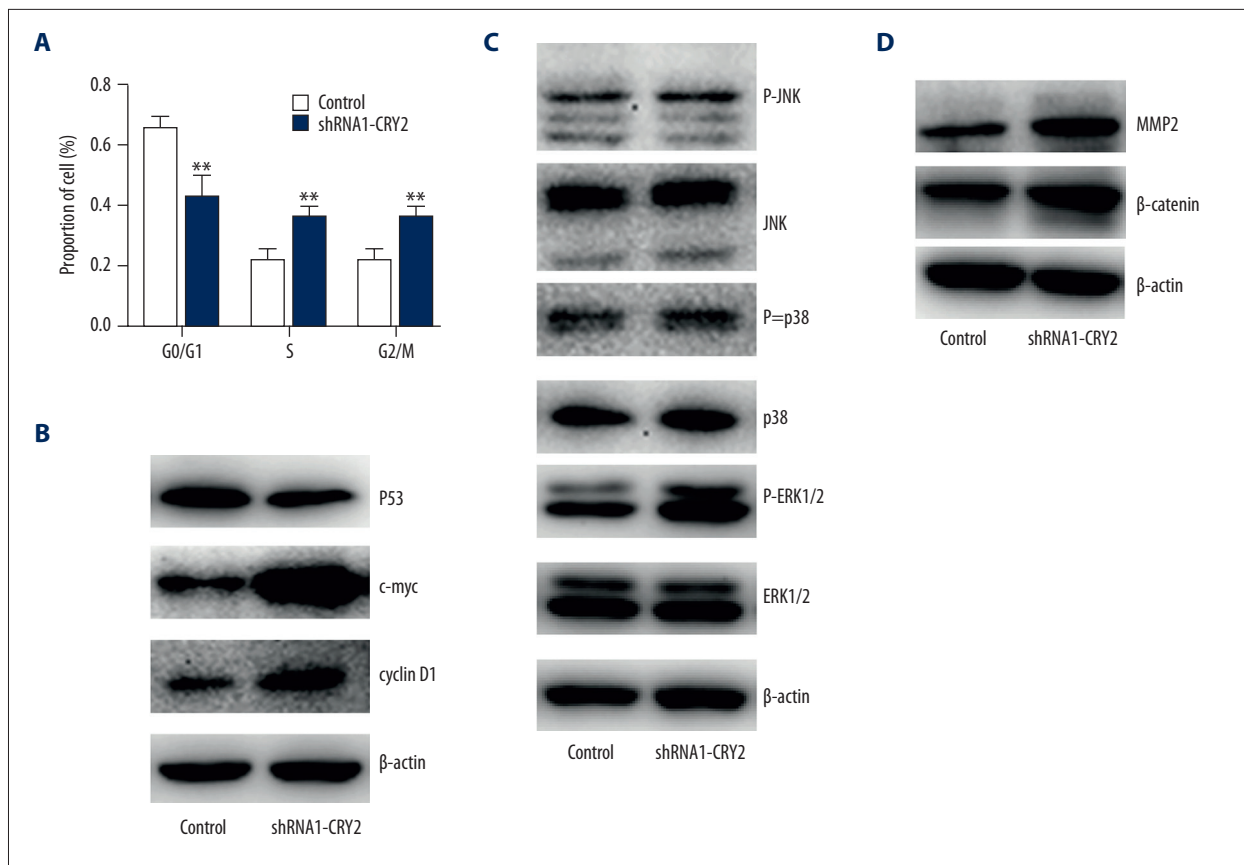
Figure 2. *CRY2* knockdown induced cell cycle progression and promoted protein kinase RNA-like endoplasmic reticulum kinase (PERK) and β-catenin signaling pathways in HOS cells. (**A**) The distribution of cell cycle was determined by flow cytometry. (**B**) The expression of P53, cyclin D1, and c-myc were determined by Western blotting. (**C**) Western blot analyses of the phosphorylation of ERK 1/2, JNK, and P38. (**D**) Western blot analyses of MMP-2 and β-catenin. Data are presented as the mean ±SD from 3 independent experiments (** P<0.01 and * P<0.05).

increased c-myc and cyclin D1 protein expression, thereby inducing cell cycle progression in HOS cells.

Figure 2C and 2D show that MAPK and Wnt/β-catenin signaling pathways may be involved in *CRY2*-mediated functions. *CRY2* knockdown increased the phosphorylation of ERK1/2, but the phosphorylation of JNK and P38 showed no changes (Figure 2C). *CRY2* knockdown also increased the expression of MMP-2 and β-catenin (Figure 2D). Together, the results suggest that *CRY2* knockdown promoted PERK and β-catenin signaling pathways in HOS cells.

CRY2 knockdown affected the expression of mRNA clock genes

As shown in Figure 3A, *CRY2* knockdown cells had significantly increased mRNA expression of *CRY1*, *PER1*, *PER2*, *BMAL1*, and *CLOCK* compared with control cells.

Discussion

Increasing evidence has shown that circadian disruption may be a cancer risk in humans. A previous study reported that the circadian clock gene, *BMAL1*, acted as a potential anti-oncogene in pancreatic cancer by activating the P53 tumor suppressor pathway [17]. Gery et al. reported that the circadian gene, *PER1*, played an important role in cell growth and DNA damage control in human cancer cells [18], and overexpression of *PER2* in breast cancer cells led to significant growth inhibition, loss of clonogenic ability, and apoptosis [19]. Hoffman et al. reported that hypermethylation in the *CLOCK* promoter reduced the risk of breast cancer, and lower levels of *CLOCK* expression were reported in healthy controls relative to normal or tumor tissue from breast cancer patients [20]. Another study reported that higher expression of *CRY1* was positively correlated with the aggressive phenotype of colorectal cancer and predicted poor patient outcomes [21]. Hoffman et al. reported that the core circadian gene, *CRY2*, affected the risk of breast cancer, possibly by mediating hormone signaling [22].
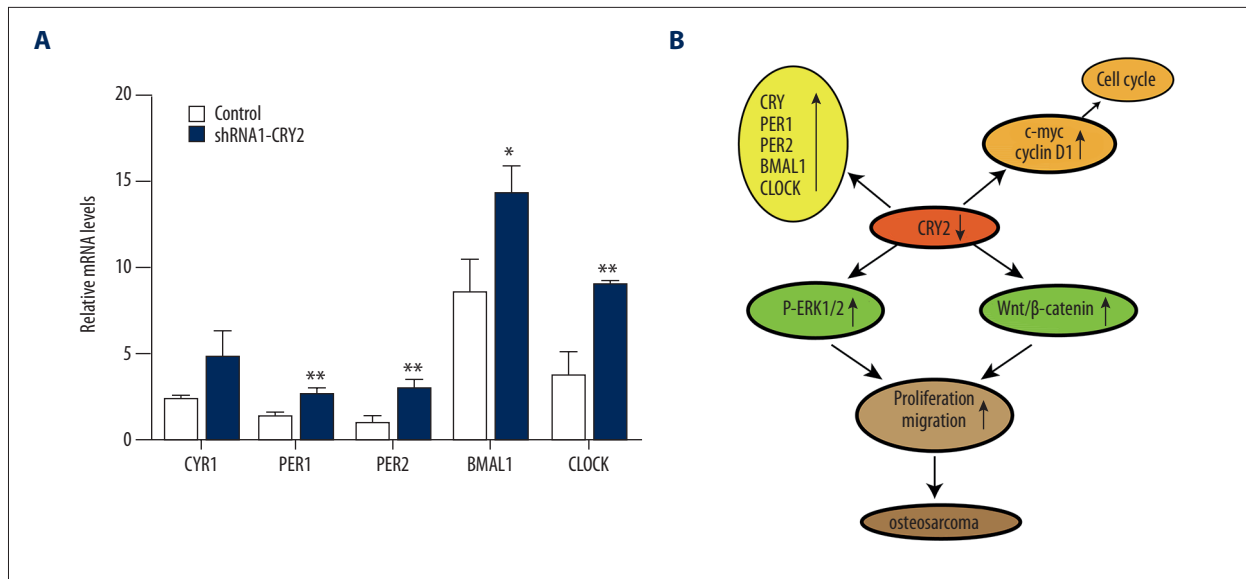
Figure 3. *CRY2* knockdown affected the mRNA expression of clock genes. (**A**) The mRNA expression of clock genes in HOS cells after *CRY2* knockdown. (**B**) A summary diagram describing the role of *CRY2* in osteosarcoma cells. Data are presented as the mean ±SD from 3 independent experiments (** P<0.01 and * P<0.05).

However, relevant studies of OS have been rare, so we characterized the role of *CRY2* in OS in the present study to determine how *CRY2* influenced the clock gene network.

We found that *CRY2* knockdown promoted HOS cell proliferation and migration by inducing cell cycle progression and promoting the MAPK and Wnt/β-catenin signaling pathways. *CRY2* knockdown decreased P53 expression and increased expression of c-myc and cyclin D1. Our study shows that *CRY2* is involved in regulating cell cycle progression. *CRY2* knockdown increased the phosphorylation of ERK1/2, and increased the expression of MMP-2 and β-catenin. The MAPK and Wnt/β-catenin pathways play critical roles in the pathogenesis of OS. Two previous studies reported extensive crosstalk between Wnt/β-catenin and MAPK signaling in cancers [23], but the relationship between Wnt/β-catenin and MAPK signaling remains unclear. Whether the 2 pathways activate or inhibit one another, and how they are regulated by *CRY2* in osteosarcoma cells, needs to be investigated further.

Our results showed for the first time that *CRY2* plays a critical role in regulating the clock gene network. Knockdown of *CRY2* resulted in significantly increased mRNA expression of

CRY1, *PER1*, *PER2*, *BMAL1*, and *CLOCK* compared with control cells. Moreover, growing evidence has shown that the expression of clock genes regulates cell cycle genes and other tumor-associated genes [24]. Abnormal expression of the clock genes could therefore induce the development and metastasis of tumors. Based on our results, we speculate that the function of *CRY2* in tumorigenesis not only involves downstream genes such as cell cycle and tumor-associated genes, but also involves other circadian genes. However, the specific molecular mechanisms of *CRY2* involvement in tumor formation and the development of OS requires further study.

Conclusions

In summary, our study shows, for the first time, that CRY2 suppresses proliferation and migration, and the regulates clock gene network in osteosarcoma cell (Figure 3B). Further studies are needed to confirm these results.

Conflict of interest

None.

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