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Cryptochrome 1 Alleviates the Antiproliferative Effect of Isoproterenol on Human Gastric Cancer Cells

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Abstract

Background: Cryptochrome 1 (CRY1) is a key protein that regulates the feedback loop of circadian clock. The abnormal expression of CRY1 was reported in numerous cancers, and contributed to tumorigenesis and progression. But the underlying mechanism remains undefined.

Methods: *CRY1* overexpression was constructed by lentivirus vector. Gene and protein expression was detected by reverse transcription quantitative polymerase chain reaction and Western blot. Cell proliferation was analyzed by CCK-8 assay. Cell migration ability was analyzed by scratch assay and transwell migration assay. The cAMP concentration was measured by intracellular cAMP assay.

Results: Overexpression of *CRY1* showed slightly effect on the proliferation and migration of HGC-27 cells. Upon exposure to isoproterenol (ISO), a β-adrenergic receptor agonist, cell proliferation, and migration were inhibited while the cAMP/PKA pathway was activated and ERK1/2 phosphorylation was suppressed. *CRY1* overexpression reduced cAMP accumulation, retained ERK1/2 phosphorylation level and alleviated the antiproliferative effect upon exposure to ISO. However, *CRY1* overexpression was inoperative on the antiproliferative effect of forskolin (FSK), a direct activator of adenyl cyclase (AC), or 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase (PDE) inhibitor.

Conclusions: Our results suggest *CRY1* overexpression may protect cells from the antiproliferative effects via activation of the cAMP/PKA pathway through interrupting signal transduction from G protein-coupled receptors to AC.

Keywords

Cryptochrome, isoproterenol, gastric cancer antiproliferation, the cAMP/PKA pathway

Introduction

To adapt to the daily environmental cycles on the Earth, many organisms change their physiology and behavior with a 24-hour period according to their intrinsic circadian clock.[1] Cryptochromes (CRYs) are a class of circadian proteins that play key roles for regulating the circadian clock.[2] Two cryptochrome genes, *CRY1* and *CRY2*, were found to encode CRY1 and CRY2, respectively. CRY/PER dimers were formed with CRYs, PER1, PER2, or PER3 proteins, translocate into the nucleus, and repress the circadian transcription activators CLOCK and BMAL1 mediated transcription of circadian genes (including *CRY1*, *CRY2* and *PERs*), forming a negative feedback loop of the human circadian clock.[3,4] Nevertheless, phosphorylation and ubiquitination-proteasome degradation of CRYs, PERs, and other circadian proteins are also involved in the regulation of the circadian clock.[5]

Considering the importance of the circadian clock, it is not surprising that abnormal expression of the circadian proteins was correlated with numerous diseases, including cancers.

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CRY1 overexpression cases of chronic lymphocytic leukemia were found to have shorter progression free survival, indicated that CRY1 may be a valuable predictor of chronic lymphocytic leukemia progression.[6] Furthermore, clinical analysis showed that *CRY1* expression was correlated with the TNM stage and lymph node metastasis, and high *CRY1* expression was associated with poor prognosis in colorectal patients with cancer.[7] A single nucleotide polymorphism rs1056560 of *CRY1* was reported in gastric cancer, which downregulates *CRY1* expression and then increased overall survival.[8] However, the underlying mechanism of these observations remains to be elucidated.

Other biological roles of CRY1 was reported, such as downregulation of the cAMP/PKA signaling pathway.[9-12] The activation of the cAMP/PKA pathway generally inhibits the MAPK pathway, leading to inhibition of cell growth and proliferation.[13-17] Nevertheless, the levels of intracellular cAMP was altered by changes in β-adrenergic receptors, and then cell growth and differentiation were affected.[18] The β2-adrenergic antagonists was reported to suppress invasion and proliferation in pancreatic cancer cell by inhibiting cAMP/PKA pathway, which could regulate activation of the MAPK pathway.[19] These cues prompted us to study the relationship between CRY1 expression and the cAMP/PKA pathway in gastric cancer cells and the effect on the proliferation. In this study, the proliferation and migration of the human gastric cancer HGC-27 cells were analyzed in normal expression or overexpression of CRY1, and in the absence or the presence of the β-adrenergic receptor agonist isoproterenol (ISO). The contents of intracellular cAMP, the protein and phosphorylation levels of CREB in the cAMP/PKA pathway, and those of ERK1/2 in the MAPK pathway were determined. Furthermore, the other activators of the cAMP/PKA pathway, such as forskolin (FSK), a direct activator of adenyl cyclase (AC), and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase (PDE) inhibitor, were also used to treat the HGC-27 cells of normal expression or overexpression of *CRY1*. Their effects on the proliferation of the cells were recorded and compared to verify the possible interacting target of CRY1 in the cAMP/PKA pathway.

Materials and Methods

Cell Culture and Drug Treatments

The human gastric cancer HGC-27 and the human embryonic kidney HEK-293T cells were purchased from Cell Cock Biotech, Guangzhou, China (catalog No. CC0402 and CC4003), which were authenticated by their short tandem repeat profiles. The mycoplasma contamination was verified by using mycoplasma detection kit (Thermo Fisher). The HGC-27 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 ng/mL streptomycin, and 100 U/mL penicillin. For drug treatment, ISO (0-1000 μM), FSK (0-20 μM), or IBMX (0-500 μM) was added into the medium, respectively. The HEK-293T cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. The cells were cultured in a humidified chamber at 37 °C with 5% CO_2.

Plasmid Construction, Lentivirus Production, and Cell Infection

The transfer plasmid pCDH-ECMV-MCS-3×FLAG-EF1α-ZsGreen1-T2A-Puro, the helper plasmids psPAX2 and pMD2.G were purchased from MiaoLing Bio, Wuhan, China. The total RNA was extracted from the HGC-27 cells using Trizol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from the total RNA using Moloney murine leukemia virus reverse transcriptase and random hexamer primers. The *CRY1* gene was obtained by polymerase chain reaction (PCR) using cDNA as the template and the primer pair: pCDH_h*CRY1*_F, 5′-ATTCTCGAGATGGGGGTGAACGCCGTGC-3′, and pCDH_h*CRY1*_R, 5′-ACTGGATCCCTAATTAGTGCTCT GTCTCTGGACT-3′. The gene fragment and the pCDH-ECMV-MCS-3×FLAG-EF1α-ZsGreen1-T2A-Puro were digested by *Xho*I and *Bam*HI, and ligated to obtain the pCDH-*CRY1* transfer plasmid.

To construct the *CRY1* overexpression lentivirus, the pCDH-CRY1 transfer plasmid, the helper plasmids psPAX2, and pMD2.G were transfected into HEK-293T cells by using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, the supernatant of the cell culture was collected and filtered through 0.45 μm filter membrane (Millipore) to obtain the packaged lentivirus particles.

The HGC-27 cells were infected using the packaged lentivirus particles in the presence of 6 μg/mL polybrene (Sigma). After infection for 24 hours, the medium was changed to RPMI-1640 with 4 μg/mL of puromycin. In the next week, the medium was changed to RPMI-1640 with 2.5 μg/mL of puromycin. The *CRY1*-overexpression stable cells (named CRY1o) were obtained after 5 passages. The HGC-27 cells infected with the empty lentivirus were used as the control.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA extraction from cells and cDNA synthesis were performed as above. Quantitative PCR was performed on an ABI Step One real-time PCR system (Applied Biosystems) using cDNA as the template with Forget-Me-Not qPCR Master Mix (Biotium). The conditions were as follows: 95 °C for 2 minutes, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. The following primers were used for detection of the *CRY1* gene, q*CRY1*_F, 5′-CTCCTCCAATGTGGGCATCAA-3′, and q*CRY1*_R, 5′-CCACGAATCACAAACAGACGG-3′; for detection of the β-actin coding gene, qβ-actin_F, 5′-TGTAAGGTTGTCCAGTTCAAAAGACT-3′, and qβ-actin_R5′-CCAGCTCACCATGGATGATG-3′. The β-actin coding gene was used as the reference gene for quantification. Three independent reverse transcription-PCR experiments were performed and each sample was in triplicate in each

experiment. The data were analyzed using the normalized expression ($\Delta\Delta C_T$) analysis method.

Western Blotting Analysis

Total proteins were extracted from the cells and separated by SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 3% bovine serum albumin. Target proteins were detected by incubating the membranes with primary antibodies and the horseradish peroxidase-conjugated secondary antibody (Sigma) followed by the Pierce ECL Western blotting substrate (Thermo Fisher). The following primary antibodies were used for CRY1, sc-393466 (Santa Cruz); for CREB and p-CREB, #9197 and #9198 (Cell Signaling Technology); for ERK1/2 and p-ERK1/2, #4695 and #4370 (Cell Signaling Technology); for β-actin, BM0627 (Boster). Each experiment was repeated at least 3 times.

Cell Proliferation Assay

Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8 kit; Beyotime). In brief, cells were cultured in 96 well culture plates. After certain time periods, 10 μL-aliquots of the CCK-8 reagent were added to the wells and incubated at 37 °C for 2 hours. The absorbance values were then measured at 450 nm using a MultiskanGO microplate reader (Thermo Scientific).

Flow Cytometry Assay

Cells were digested with trypsin, then harvested by centrifugation. The cells were rinsed twice with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C for 24 hours. Ethanol was removed by centrifugation. The fixed cells were resuspension with PBS, treated with RNase A at 37 °C for 5 minutes, and then stained with propidium iodide at 37 °C for 30 minutes in the dark.

ACytoFLEX flow cytometer (Beckman Coulter) was used to analyze the distribution of the cell cycle phases. The proliferation index (PI) was calculated according to the following formula: PI = (S+G2/M)/(G0/G1+S+G2/M). Each experiment was repeated 3 times.

Scratch Assay

Cells in logarithmic phase were seeded into a well of 6-well plates, and cultured at 37 °C overnight. Cells were grown to 95% confluence. A wound was created by scratching cells with a sterile pipette tip. The cells were washed 3 times with PBS to remove the floating cells. Then the fresh medium was added to the well. The drug treatment groups were supplemented with drugs at the original concentrations. After incubation at 37 °C for 24 hours, the migration of cells in each group was observed on an inverted microscope. Image J software was used to measure scratch areas. The migration ability was measured by healing rate (HR) = (scratch area at 0 hours-scratch area at 24 hours)/scratch area at 0 hours. The experiment was repeated for 3 times.

Transwell Migration Assay

Cells in logarithmic phase were digested with trypsin, harvested, and resuspended in serum-free medium with or without 400 μM ISO. Aliquots of 100 μL cell suspension were seeded into the upper Transwell chambers (BD Biosciences). Aliquots of 500 μL medium containing 10% FBS were added into the lower chambers. After incubation at 37 °C for 24 hours, the cells remaining on the upper surface of the membrane were removed. The cells that migrated through the membrane were fixed in 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet for 5 minutes, then imaged and counted. All the experiments were repeated in triplicate.

Intracellular cAMP Assay

Cells were incubated in medium with or without 400 μM ISO for 30 minutes and lysed in lysis buffer, a PDE inhibitor was added to the cell lysate, and the degradation of cAMP was terminated by the PDE while cells were lysed. The instantaneous cAMP concentrations in cell lysates were measured using a cAMP ELISA kit (KGE002B, R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

The results were presented as the mean ± standard error of the mean of 3 independent experiments. A 2-tailed Student t test was used to compare the differences between the groups. A value of $P < .05$ was considered to be statistically significant.

Results

Overexpression of CRY1 Had Little Effect on the Proliferation of the HGC-27 Cells

To evaluate the effect of *CRY1* overexpression on the proliferation of the HGC-27 cells, we overexpressed *CRY1* in the HGC-27 cells using lentivirus vector. The messenger RNA and protein levels of CRY1 in the cells infected with the *CRY1* overexpression lentivirus (named CRY1o) and the HGC-27 control cells were measured. The results showed that *CRY1* was successfully overexpressed in the CRY1o cells (Figure 1A and B). The growth curves of the CRY1o and the control cells were determined, which had no significant difference between them (Figure 1C). Nevertheless, the cell cycle phase distributions and the calculated PI values of the CRY1o and the control cells were almost identical (Figure 2C and D). These results indicated that overexpression of *CRY1* had little effect on the proliferation of the HGC-27 cells.

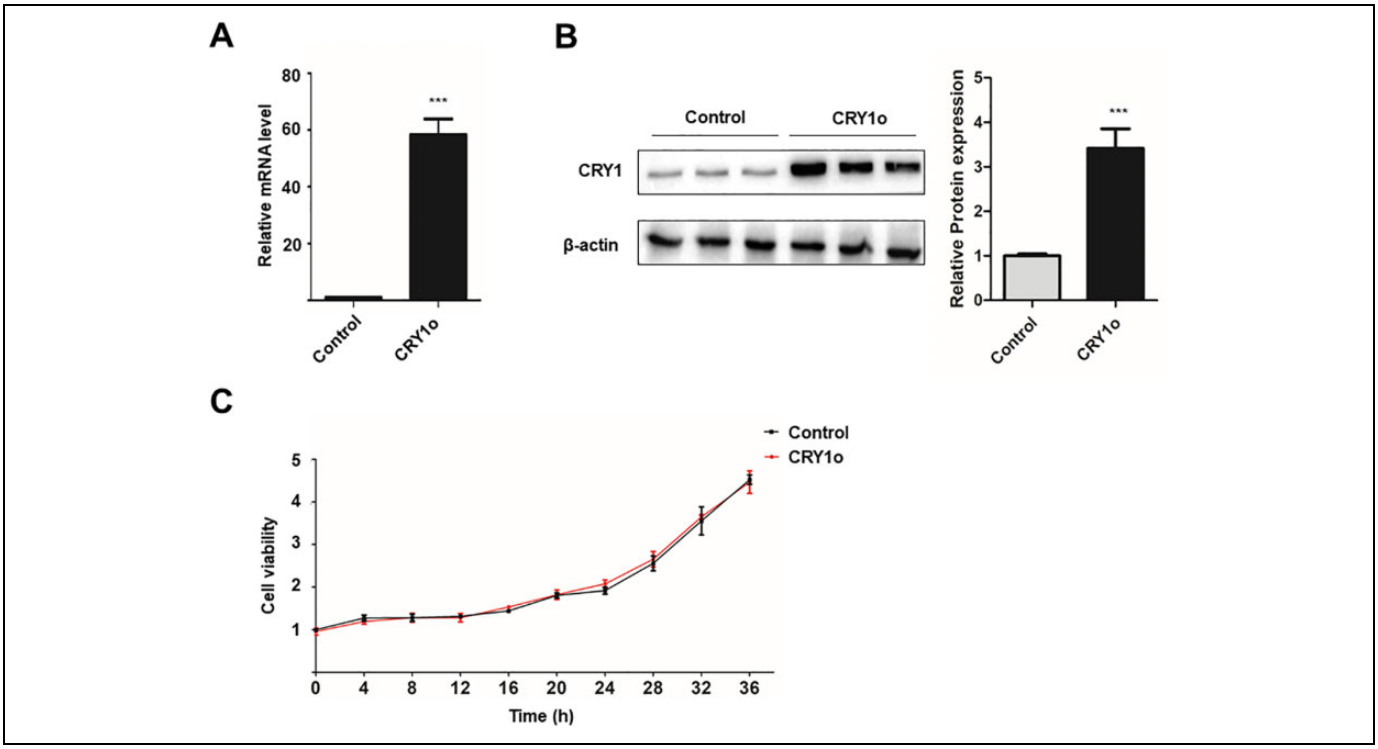

Figure 1. Overexpression of *CRY1* in the HGC-27 cells and its effect on the cell proliferation. A, The messenger RNA level of *CRY1*. B, The protein level of CRY1. C, The growth curves of the cells. CRY1o, the *CRY1*-overexpressionstable cells derived from the HGC-27 cells. Control, the HGC-27 cells infected with the empty lentivirus. ***$P < .001$ versus the control, $n = 3$.

Overexpression of CRY1 in the HGC-27 Cells Alleviated the Antiproliferative Effect of ISO

The HGC-27 control cells, which express β-adrenergic receptors,[20] were treated with 0 to 1000 mμ ISO for 24 hours. It was found that the cell proliferation was severely inhibited when the cells were treated with ISO at the concentrations >100 μM (Figure 2A, black line). The antiproliferative effect of ISO on the CRY1o cells was significantly alleviated compared with that on the control cells at the ISO concentrations of 200 to 500 μM (Figure 2A, red line). The most prominent alleviation was observed when the cells were treated with 400 μM ISO. Next, we determined and compared the growth curves of the CRY1o and the control cells after supplement of ISO at 400 μM (Figure 2B). It was found that the CRY1o and the control cells were still growing at 0 to 4 hours after ISO supplement. Then the viability of both kinds of cells dramatically declined at 4 to 12 hours after ISO supplement. After 12 hours of ISO supplement, the proliferation of the CRY1o cells was recovered, but that of the control cells was still suppressed. The cell cycle phase distributions of the CRY1o and the control cells were monitored after ISO treatment for 24 hours (Figure 2C). The PI values of both kinds of cells after ISO treatment were lower than those of the untreated ones. However, the PI value of the ISO treated CRY1o cells was significantly higher than that of the ISO treated control cells, which also indicated that the antiproliferative effect of ISO was significantly alleviated by *CRY1* overexpression (Figure 2D).

Overexpression of CRY1 in the HGC-27 Cells Relieved the Inhibitory Effect of ISO on Cell Migration

The effects of ISO and *CRY1* overexpression on the migration of the HGC-27 cells were investigated by scratch assay (Figure 3A and B). The results showed that the migration of the CRY1o and the control cells has no significant difference, indicating that overexpression of *CRY1* did not affect cell migration. The treatment of ISO inhibited the migration of the HGC-27 control cells. However, the migration ability of the CRY1o cells was retained under the treatment of ISO. The Transwell experiments were performed which gave similar results (Figure 3C and D). These results revealed that *CRY1* overexpression also relieved the inhibitory effect of ISO on cell migration.

CRY1 Downregulated the cAMP/PKA Pathway by Interrupting the Signal Transduction From the G Protein-Coupled Receptors to AC

As a classical β-adrenergic receptor agonist, it is known that ISO can stimulate the production of intracellular cAMP and activate the cAMP/PKA pathway. The cAMP contents in the CRY1o and the HGC-27 control cells were measured without or with ISO treatment. It was found that both kinds of cells contained low levels of cAMP without ISO treatment, which had little difference between them. After ISO treatment, the levels of cAMP in both kinds of cells were elevated; but the

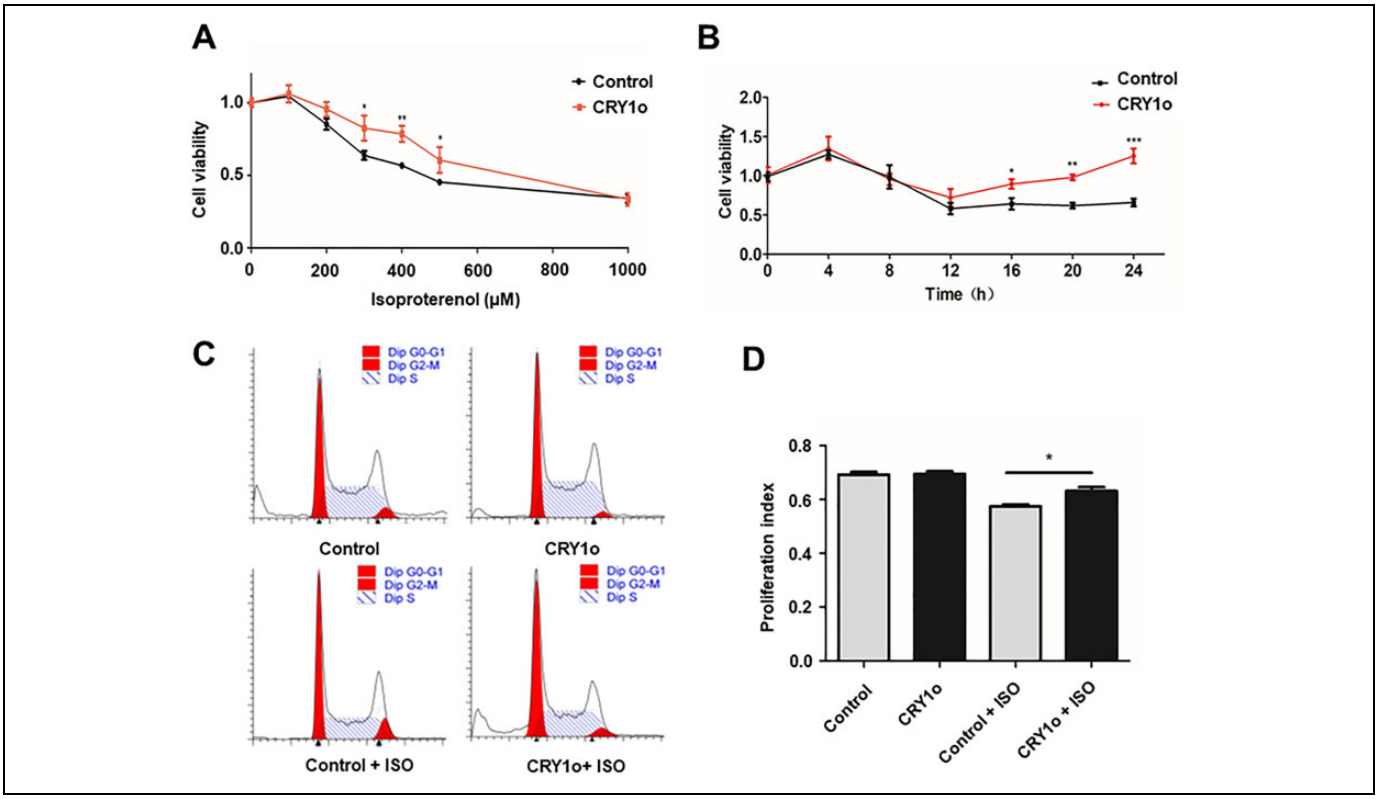

Figure 2. Overexpression of *CRY1* in the HGC-27 cells alleviated the antiproliferative effect of ISO. A, The cell viability determined after treatment with 0-1000 μM ISO for 24 hours. B, The growth curves of the cells after treatment with 400 μM ISO. C, The cell cycle phase distributions without or with 400 μM ISO treatment for 24 hours determined by flow cytometry. D, The calculated proliferation index (PI) values from the flow cytometry results. CRY1o, the CRY1-overexpression stable cells derived from the HGC-27 cells. Control, the HGC-27 cells infected with the empty lentivirus. *$P < .05$, **$P < .01$, and ***$P < .001$, $n = 3$.

increase of cAMP level in CRY1o cells was significantly lower than that in the control cells (Figure 4A). CREB is a downstream component of the cAMP/PKA pathway. The phosphorylation level of CREB in the control cells was dramatically increased after ISO treatment. However, the phosphorylation of CREB was compromised in the CRY1o cells under the same conditions (Figure 4B).

The activation of the cAMP/PKA pathway generally leads to the suppression of the MAPK pathway.[13-17] And the phosphorylation of ERK1/2 is in line with the activation of the MAPK pathway. In our experiments, it was observed that the phosphorylation level of ERK1/2 was obviously decreased in the HGC-27 control cells after treatment of ISO, meanwhile the phosphorylation level of ERK1/2 was retained in the CRY1o cells (Figure 4B). These results demonstrated that the antiproliferative effect of ISO on the HGC-27 cells was due to the activation of the cAMP/PKA pathway and the suppression of the MAPK pathway; and overexpression of *CRY1* alleviated the antiproliferative effect of ISO by downregulating the cAMP/PKA pathway.

Then, we investigated the effects of the other 2 activators of the cAMP/PKA pathway, FSK and IBMX, on the proliferation of the HGC-27 cells. Forskolin directly stimulates AC to produce cAMP, and IBMX is a PDE inhibitor that prevent the degradation of cAMP. Both of the activators inhibited the proliferation of the HGC-27 control cells in dose-dependent manners. Nevertheless, overexpression of *CRY1* in the CRY1o cells did not alleviate the antiproliferative effects of these 2 activators (Figure 4C and D). These results suggested that the inhibitory target of CRY1 in the cAMP/PKA pathway should be upstream of AC, such as the Gs protein or G protein-coupled receptors.

Discussion

CRY1 is one of the core components of the circadian clock that participates in the regulation of the circadian rhythm of human.[1-5] In line with the importance of CRY1, the dysfunctional expression of *CRY1* is associated with many diseases, including cancers. For a number of cancers, including gastric cancer, it was observed that the expression level of CRY1 was negatively correlated with the prognosis of the patients.[6-8] However, we found that overexpression of CRY1 in the human gastric cancer HGC-27 cells had little effect on cell proliferation. This implied that CRY1 is not a common oncogene that directly promotes cell proliferation.

Recently, several works reported that CRY1 also had an inhibitory effect on the cAMP/PKA pathway that played roles

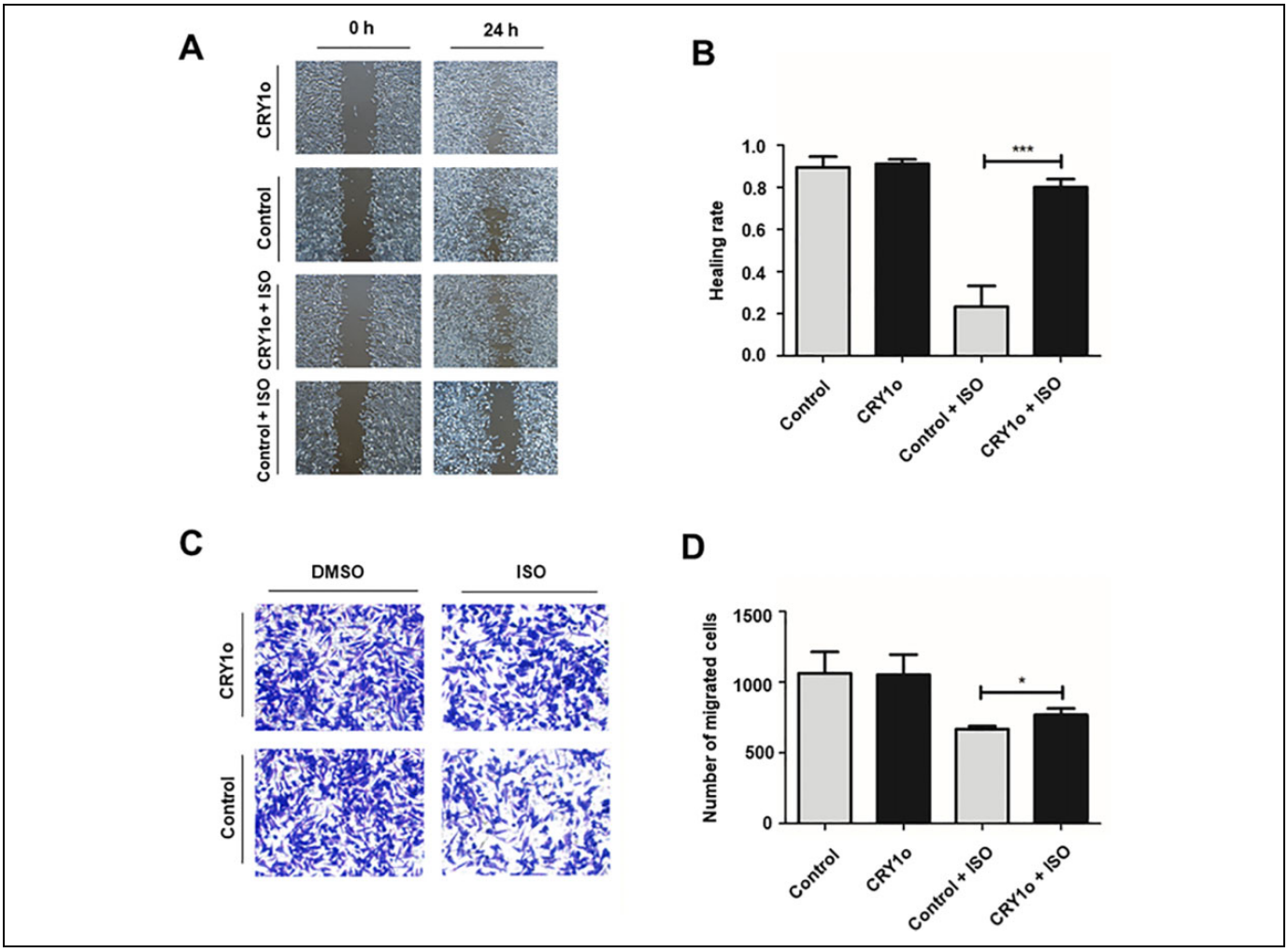

Figure 3. Overexpression of *CRY1* in the HGC-27 cells relieved the inhibitory effect of ISO on cell migration. A, The migration rates of the cells without or with 400 μM ISO treatment for 24 hours detected by scratch assay. B, The calculated healing rate (HR) values from the scratch assay results. C, The transwell migration assay of the cells without or with 400 μM ISO treatment for 24 hours. D, The quantitative results of the Transwell migration assay. CRY1o, the *CRY1*-overexpression stable cells derived from the HGC-27 cells. Control, the HGC-27 cells infected with the empty lentivirus. *$P < .05$, and ***$P < .001$, $n = 3$.

in inflammation and metabolism.[9-12] In this study, we also showed that overexpression of *CRY1* could alleviate the antiproliferative effect of ISO on the HGC-27 cells by downregulating the cAMP/PKA pathway. In physiological conditions, there are many hormones that activate the cAMP/PKA pathway, such as adrenaline, glucagon, prostaglandin E, and vasoactive intestinal peptide. We speculate that overexpression of *CRY1* may protect the cancer cells from the antiproliferative effects of the hormones that activate the cAMP/PKA pathway. This may partially explain the negative correlation of the expression level of CRY1 and the prognosis of the patients.

The inhibitory target of CRY1 in the cAMP/PKA pathway is controversial. In one study, it was shown that CRY1 interacted with Gsα subunit.[9] Another work reported that CRY1 directly interacted with AC III and inhibited its activity.[10] In this study, it was found that *CRY1* overexpression did not alleviate the antiproliferative effect of FSK and IBMX, suggesting that inhibitory target of CRY1 in the cAMP/PKA pathway should be upstream of AC. This research revealed the potential relationship of CRY1, gastric cancer, and the cAMP/PKA pathway, which might provide theoretical basis for CRY1 as a molecular target for tumor treatment and prognosis. However, the exact interacting target of CRY1 in the cAMP/PKA pathway and the interaction pattern need to be elucidated in the future.

It is worth noting that the cAMP/PKA pathway does not always inhibit the MAPK pathway and inhibit cell proliferation.[21] In some cell types, the activating of the cAMP/PKA pathway could stimulate the MAPK pathway with the participation of Rap1 and B-Raf, and promote cell proliferation.[22-24] Nevertheless, there is a report that *CRY1* knockdown enhanced proliferation and migration of osteosarcoma cells.[25] Therefore, the roles of CRY1 may not be universal in all cancers, which warrant further detailed investigations.

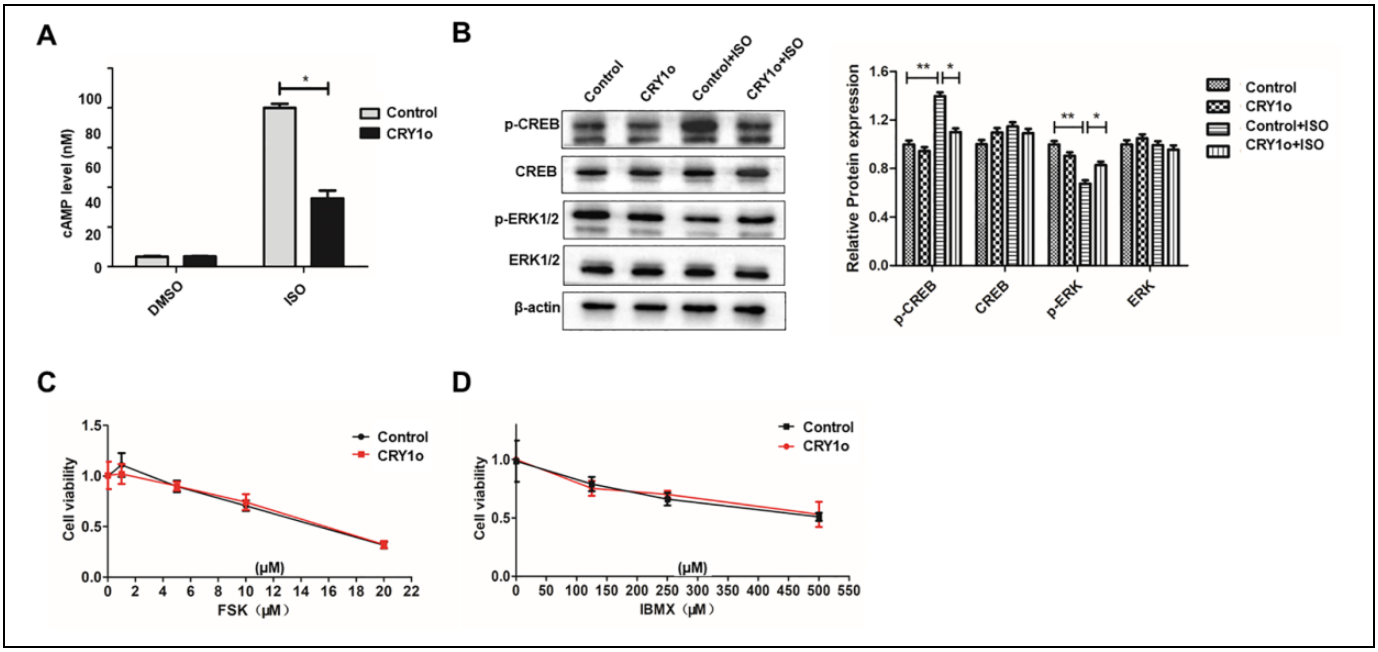

Figure 4. ISO treatment activated the cAMP/PKA pathway and the suppressed the MAPK pathway, and *CRY1* overexpression alleviated the effects of ISO by interrupting the signal transduction of the cAMP/PKA pathway from the G protein-coupled receptors to AC. A, ISO treatment stimulated the production of cAMP in the HGC-27 cells, and *CRY1* overexpression reduced the cAMP stimulating effect of ISO. B, ISO treatment elevated the phosphorylation levels of CREB and suppressed the phosphorylation of ERK1/2; and *CRY1* overexpression alleviated the effects of ISO. C and D, The cell viability determined after treatment with FSK (0-20 μM), or 3-isobutyl-1-methylxanthine (IBMX; 0-500 μM) for 24 hours, respectively. CRY1o, the *CRY1*-overexpression stable cells derived from the HGC-27 cells. Control, the HGC-27 cells infected with the empty lentivirus. *$P < .05$, $n = 3$.

Authors' Note

Q.H. and J.L. contributed equally to this work. M.W., L. X., and Q. H. designed the research. Q. H., M.W., and J. L. conducted experiments. Q. H., L. X., and T. D. analyzed the data. M.W. and Q. H. wrote the manuscript. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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