β-adrenergic receptor activation promotes the proliferation of HepG2 cells via the ERK1/2/CREB pathways

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Abstract. Primary liver cancer is one of the most frequently diagnosed malignant tumors seen in clinics, and typically exhibits aggressive invasive behaviors, a poor prognosis, and is associated with high mortality rates. Long-term stress exposure causes norepinephrine (NE) release and activates the β-Adrenergic receptor (β-AR), which in turn exacerbates the occurrence and development of different types of cancers; however, the molecular mechanisms of β-AR in liver cancer are not fully understood. In the present study, reverse transcription (RT)-PCR and RT-quantitative PCR showed that β-AR expression was upregulated in human liver cancer cells (HepG2) compared with normal liver cells (LO2). Moreover, NE treatment promoted the growth of HepG2 cells, which could be blocked by propranolol, a β-AR antagonist. Notably, NE had no significant effect on the migration and epithelial-mesenchymal transition in HepG2 cells. Further experiments revealed that NE increased the phosphorylation levels of the extracellular signal-regulated kinase 1/2 (ERK1/2) and cyclic adenosine monophosphate response element-binding protein (CREB), while inhibition of ERK1/2 and CREB activation significantly blocked NE-induced cell proliferation. In summary, the findings of the present study suggested that β-adrenergic receptor activation promoted the proliferation of HepG2 cells through ERK1/2/CREB signaling pathways.

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Introduction

Liver cancer is the sixth most common disease (906,000 cases), accounting for 830,000 cancer deaths globally (1). The prevalence and mortality of this malignancy have increased globally and are particularly high in North African and Asian nations (1). Currently, treatments include surgery, transcatheter arterial chemoembolization, liver transplant, radiotherapy, and biotherapy (2,3). Unlike other malignancies, liver cancer is frequently discovered only when it has progressed to the point that liver transplant, surgical treatments, and resection are no longer feasible (4). As a result of the genetic, metabolic, and inflammatory heterogeneity of liver cancer, the development of therapies is difficult; chemotherapy (such as cisplatin, gemcitabine, or doxorubicin) or treatment with multikinase inhibitors (such as first-line sorafenib, second-line regorafenib, Lenvatinib, or third-line cabozantinib) only slightly prolongs overall survival (5). Thus, there is an urgent need for new molecular therapeutic and diagnostic targets to raise the standard of care and survival for people with liver cancer.

Acute stress and chronic stress are two different types of stress (6). Acute stress can help the body adapt to harsh environments and generally have a beneficial effect on the physiological status of a body. Yet, growing epidemiological research indicates that long-term stress exposure may cause various physiological issues, including the emergence and growth of tumors (7,8). Recent work suggests that stress hormone receptors, specifically the Catecholamine hormone receptor and β-adrenergic receptor (β-AR), play an essential role in tumor genesis and development, and are considered to be an important target for cancer therapy (9,10). Specifically, epinephrine and norepinephrine (NE) via β-adrenergic receptors modulate the immune response, angiogenesis, invasion, and inflammation to promote tumor development (11). In fact, numerous studies have shown that the AR pathway plays a role

in promoting a variety of tumor types, including melanoma, leukemia, breast, cervical, liver, lung, gastric, oral, and pancreatic cancer (12,13). Although it has been noted that liver cancer cells produce more β-ARs, the exact molecular mechanism by which β-AR regulates the development, invasion, and metastasis of liver cancer is yet unknown (14).

β-AR belongs to the G protein-coupled receptor (GPCR) family, which can regulate multiple malignant biological processes, including tumor cell proliferation, angiogenesis, epithelial-mesenchymal transition (EMT), invasion, metastasis, and anti-apoptotic mechanisms (13). Previous studies have shown that β-AR can activate adenylate cyclase (AC), which in turn activates the cAMP/PKA signaling pathways and cAMP/EPAC/Rap1/MEK1/2/extracellular signal-regulated kinase 1/2 (ERK1/2), and promotes downstream transcription factor expression, including the NF-κB, CREB, AP1, and Ets family of proteins (15,16). For example, it has been reported that NE enhances the invasion and proliferation of oral squamous cell carcinoma (OSCC) through activating β_2-AR and inducing activation of ERK and cAMP response element binding protein (CREB). Simultaneously, NE can promote a cancer stem cell-like phenotype and increase the expression of stem cell markers (13). It has been shown that psychological stress can activate EMT, promote tumor growth, and enhance radiation resistance via β-AR (17). By downregulating PPAR expression, chronic stress, and hormone-induced β_2-AR activation can enhance breast cancer development and VEGF/FGF2-mediated angiogenesis (18). Isoproterenol, a β-AR agonist, has been shown to play critical roles in regulating VEGF production via β-AR receptors, enhancing vascular distribution in mouse femurs, and the release of the proinflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6), altering endothelial cell adhesion and promoting cancer cell bone metastasis (19). By stimulating the Notch 1 pathway, NE promotes tumor cell activity and invasion while inhibiting tumor cell death in pancreatic ductal adenocarcinoma (20). Chronic stress may also enhance gastric cancer (GC) cell proliferation and metastasis by stimulating the production of NE and its binding to AR, as well as upregulating NF-κB, CREB, and STAT3 expression (12). Furthermore, chronic stress-induced activation of the miR-337-3P/STAT3 axis may increase breast cancer metastasis (21). Notably, there is limited research on unraveling the role of β-AR signaling in liver cancer. For example, current research has revealed that the sympathetic nervous system (SNS)/β-ARs/CCL2 alleviates immunosuppression in liver cancer cells and overcomes PD-L1 immunotherapy resistance (22). Additionally, β-AR promotes liver cancer growth by increasing YB-1 phosphorylation at S102 via β-arrestin-1-dependent activation of the PI3K/AKT pathway (23). However, the molecular mechanism by which β-AR is activated in liver cancer cells and its downstream signaling pathways governing the occurrence and progression of liver cancer cells are not fully understood.

The aim of the present study was to explore the capacity of β-AR in increasing HepG2 hepatoma cell proliferation, migration, and epithelial cell transformation, as well as the underlying molecular processes. The results showed that β-AR was abundantly expressed in HepG2 cells, and that NE can boost HepG2 cell proliferation via activation of β-AR and its downstream ERK1/2/CREB signaling pathways.

Materials and methods

Antibodies and reagents. Tocris Bioscience supplied the NE and propranolol (PRO). Western blotting antibodies, including phospho-ERK1/2 (Thr202/Tyr204)) (cat. no. 4370; 1:2,000), ERK1/2 (cat. no. 4695; 1:2,000), phospho-CREB (Ser133) (cat. no. 9198; 1:2,000), CREB (cat. no. 9197; 1:2,000), β-actin (cat. no. 4970; 1:5,000), and anti-rabbit IgG HRP-linked antibody (cat. no. 7074, goat anti-rabbit, 1:5,000) were purchased from Cell Signaling Technology, Inc. ADRB1 (cat. no. 28323-1-AP; 1:2,000) and ADRB2 (cat. no. 29864-1-AP; 1:2,000) antibodies were purchased from ProteinTech Group, Inc. All cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc.). SYBR® Premix Ex Taq™ II was purchased from Takara Bio, Inc. MTT was purchased from MilliporeSigma.

Cell culture and transfection. HepG2 (hepatoblastoma), Huh7 (hepatoma), and LO2 (normal liver) cells were purchased from iCell Bioscience Inc. Both cell lines were cultured in DMEM supplemented with 4.5 g/l glucose, 10% FBS, 50 μg/ml streptomycin, and 50 IU/ml penicillin. The cells were regularly tested for mycoplasma and authenticated using Short Tandem Repeat (STR) analysis to confirm their identity. The STR profiles of both cell lines matched the reference profiles provided by the cell bank. Additionally, their growth characteristics and morphology were consistent with the reported characteristics of HepG2 and LO2 cells. Cells were maintained at 37˚C in an incubator supplied with 5% CO_2 air. Small interfering (si) RNAs were used to knock down CREB expression in HepG2 cells. The cells were transfected for 48 h using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) with siRNAs against CREB (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions.

Measurement of cell viability. Cells were added to 24-well plates with culture media and incubated at 37˚C for 24 h. After the cells had adhered, the cells were serum-starved overnight and treated for 48 h with various treatments. The culture media was then replaced with supplemented medium containing 50 μl MTT reagent (5 mg/ml) in each well. After a further 3 h of incubation at 37˚C, the supernatant was removed, and 500 μl dimethyl sulfoxide was added to each well and shaken for 10 min to dissolve the precipitate. The optical density (OD) at 490 nm was then measured. The cell viability is represented as a percentage of the control (100%).

Western blotting. Cells were serum-starved overnight at 37˚C prior to treatment. After treatment, the cells were lysed with RIPA buffer on ice to extract total protein. A total of 10 μg protein/lane was loaded and resolved using 10% SDS-PAGE before transfer to a PVDF membrane and blocked for 2 h at room temperature in 5% fat-free milk. Subsequently, the membrane was treated with one of the primary antibodies against ADRB1, ADRB2, phospho-ERK1/2, ERK1/2, phospho-CREB, CREB, or β-actin overnight at 4˚C, followed by incubation with the HRP-conjugated anti-rabbit IgG secondary antibody for 2 h at room temperature. Signals were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Densitometry analysis was

performed using ImageJ (version 1.47t; National Institutes of Health).

Wound-healing assays. Wound healing assays were used to assess cell migration. Briefly, HepG2 cells were seeded into a 6-well plate with complete growth medium containing 10% FBS and incubated until they reached 100% confluence. The cells were then cultured in serum starved DMEM (2% FBS) for 12 h. Subsequently, a sterile yellow pipette tip was used to generate a wound, after which, the cells were washed with PBS to remove any floating cells and debris. After adding NE, the cells were cultured in DMEM containing 2% FBS at 37˚C. The width of the wound was measured at 0, 12, and 24 h post-scratch, and images of randomly selected fields from each group were captured using a phase-contrast microscope (Olympus Corporation).

RNA extraction and reverse transcription (RT)-PCR. Using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was isolated from HepG2 and LO2 cells. The RNA purity and concentration were measured according to the ratio of absorbance at 260 and 280 nm, using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA was then reverse-transcribed into cDNA using the PrimeScript® RT reagent (Takara Bio, Inc.) according to the manufacturer's protocol. The thermocycling protocol was as follows: Amplification step, 94˚C for 5 min; followed by 35 cycles of denaturation for 1 min at 94˚C; 1 min of annealing at 55˚C; elongation at 72˚C for 1 min; with a final extension step at 72˚C for 1 min. The sequences of the primers are shown in Table SI. Standard electrophoresis was performed on a 1.2% agarose gel at 100 V for 40 min. The bands in the gels were imaged using an UV light transilluminator (ChemiDoc XRS+ system; Bio-Rad Laboratories, Inc.). β-actin was used as the housekeeping gene.

Quantitative (q)PCR. The total RNA and cDNA were obtained as above and qPCR was performed on a ViiA7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). A total of 20 μl qPCR reaction system contained 6 μl nuclease-free water, 10 μl SYBR Premix Ex Taq II (2X), 0.4 μl ROX Reference Dye II, 2 μl cDNA, 0.8 μl forward primer (10 μM) and 0.8 μl reverse primer (10 μM). The thermocycling conditions were: 95˚C for 30 sec; followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 34 sec, elongation at 95˚C for 15 sec, and extension at 60˚C for 1 min. The $2^{-\Delta\Delta Cq}$ method was used to analyze the expression levels of β_1-AR and β_2-AR (24); β-actin was used as the housekeeping gene. Each sample was assessed in triplicate.

EMT. The HepG2 cells were seeded into 6-well plates ($1x10^6$ cells) in supplemented media. Following adherence of cells, NE was added, and the cells were cultured in DMEM without FBS for 96 h. Random field images were selected and analyzed for morphological changes using ImageJ software (version 1.47t; National Institutes of Health).

Statistical analysis. Data are presented as the mean ± SEM of at least three separate experiments and were analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc.).

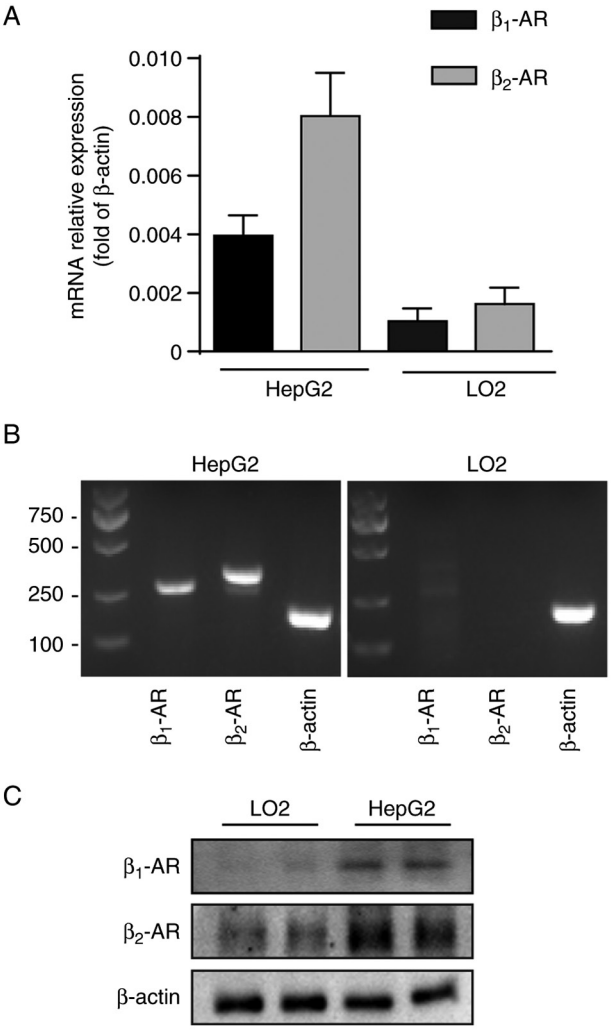

Figure 1. Expression of β_1-AR and β_2-AR in HepG2 and LO2 cells. (A) mRNA expression levels of β_1-AR, β_2-AR, and β-actin in HepG2 and LO2 cells were determined using (A) RT-qPCR and (B) RT-PCR. Data are presented as the mean ± SEM. (C) Western blot analysis was performed to assess the expression of β_1-AR and β_2-AR in LO2 and HepG2 cells. β_2-AR expression was higher in HepG2 cells compared with LO2 cells. RT-PCR, reverse transcription-PCR; RT-qPCR, reverse transcription-quantitative PCR; β-AR, β-Adrenergic receptor.

Data were compared using a Student's t-test (2 groups) or a one/two-way ANOVA with Bonferroni's Multiple Comparison Corrections (>2 groups). $P<0.05$ was considered to indicate a statistically significant difference.

Results

β_1-AR and β_2-AR expression in HepG2 and LO2 cells. To investigate the expression of β_1-AR and β_2-AR in HepG2 and LO2 cells and compare the differences in the expression of these receptors between the cell lines, RT-PCR and RT-qPCR assay was used. The results indicated that β_1-AR and β_2-AR expression was detectable in HepG2 and LO2 cells (Fig. 1A). Western blotting was further performed to confirm the results (Fig. 1B and C). Notably, the expression of β_2-AR was higher in HepG2 cells than that in the LO2 cells, and the expression of β_2-AR was evidently higher than that of β_1-AR in HepG2. These findings showed that β-AR, particularly β_2-AR, may

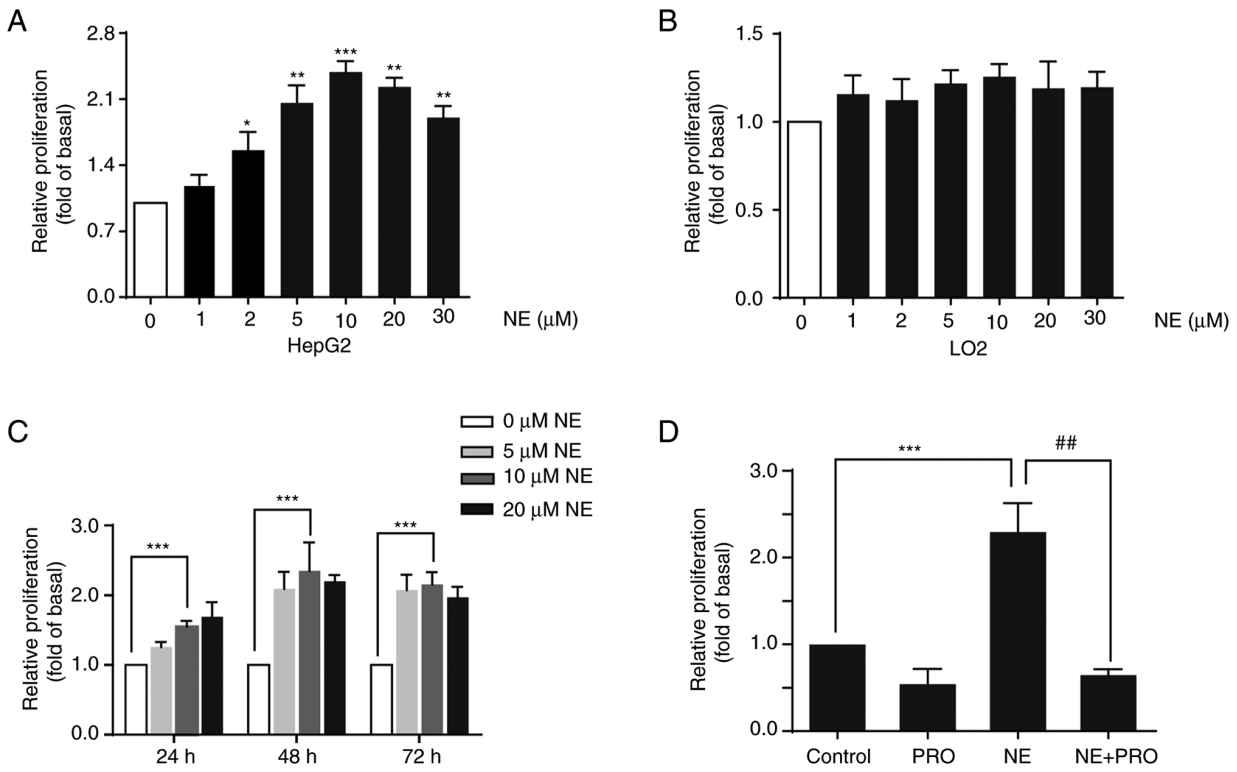

Figure 2. Effect of NE on the proliferation of cells. (A and B) Dose-dependent effects of NE on the proliferation of HepG2 cells. After HepG2 and LO2 cells were treated with increasing concentrations of NE for 48 h, cell viability was examined using an MTT assay. (C) Time-dependent effects of NE on the proliferation of HepG2 cells. Cells were treated with NE for 24, 48, or 72 h, after which the cell viability was examined using an MTT assay. (D) HepG2 cells were treated with 10 μM NE for 48 h in the absence or presence of 20 μM PRO, and cell viability was measured using an MTT assay. Data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#\#}$P<0.01 vs. NE. NE, norepinephrine; PRO, propranolol.

play an important role in the tumorigenesis and development of liver cancer.

Effect of NE on the proliferation of HepG2 and LO2 cells. To further investigate the activity of the β-ARs in HepG2 and LO2 cells, cells were treated with the β-AR agonist NE. Cells were treated with different doses of NE for 48 h, and cell viability was assessed using an MTT assay. The results demonstrated that NE significantly promoted the proliferation of HepG2 cells in a dose-dependent manner, with 10 μM concentration showing the most pronounced effect. However, NE did not notably affect the proliferation of LO2 cells (Fig. 2A and B). To confirm the effect of NE on liver cancer, Huh7 liver cancer cells were treated with different doses of NE, and a significant increase in the proliferation of these cells was observed (Fig. S1). Next, the effect of NE on cell viability after treatment of HepG2 cells with NE for 24, 48, or 72 h was assessed. NE increased HepG2 cell growth in a dose and time-dependent manner (Fig. 2C). Additionally, HepG2 cells were co-treated with the non-selective β-AR blocker PRO and NE for 48 h. The results indicated that PRO blocked NE-induced cell growth, suggesting that the proliferative effect of NE may be mediated through the activation of β-AR (Fig. 2D).

β-AR activation induces ERK1/2 and CREB phosphorylation in HepG2 cells. As it was demonstrated that β-AR activation may have exerted pro-proliferative effects of NE in HepG2, the specific molecular mechanisms were next explored. It has been reported that the MAPK/ERK1/2 pathway is crucial in regulating key processes, such as cell proliferation, survival, and metastatic progression (25). To determine whether ERK1/2 and CREB were implicated in β-AR activation, HepG2 cells were treated with NE, and then, the phosphorylation levels of ERK1/2 and CREB were measured by western blot analysis. NE induced a brief and transitory increase in ERK1/2 phosphorylation but had no effect on ERK1/2 expression levels (Fig. 3A and B). The phosphorylation of ERK1/2 peaked at 10 min and then decreased. Similarly, the transcription factor CREB, which affects processes such as cell cycle, apoptosis, and cellular metabolism, was also transiently phosphorylated and peaked within 10 min (Fig. 3C).

To confirm that ERK1/2 and CREB phosphorylation was a result of β-AR activation, HepG2 cells were pre-treated with PRO and then stimulated with NE. PRO inhibited the phosphorylation of ERK1/2 and CREB (Fig. 3D-F). As CREB is a key downstream target of ERK1/2, whether NE-induced CREB phosphorylation was mediated by ERK1/2 was assessed. The MEK1/2 inhibitor U0126 was used to treat HepG2 cells. Inhibiting the MAPK pathway significantly inhibited NE-induced ERK1/2 and CREB phosphorylation, suggesting that NE stimulation of β-AR promoted ERK1/2 phosphorylation, which then activated CREB (Fig. 3G-I).

Inhibiting ERK1/2 and CREB abrogates NE-mediated proliferation in HepG2 cells. Considering the potential of NE to significantly increase HepG2 cell proliferation and activate ERK1/2 and CREB phosphorylation, HepG2 cells

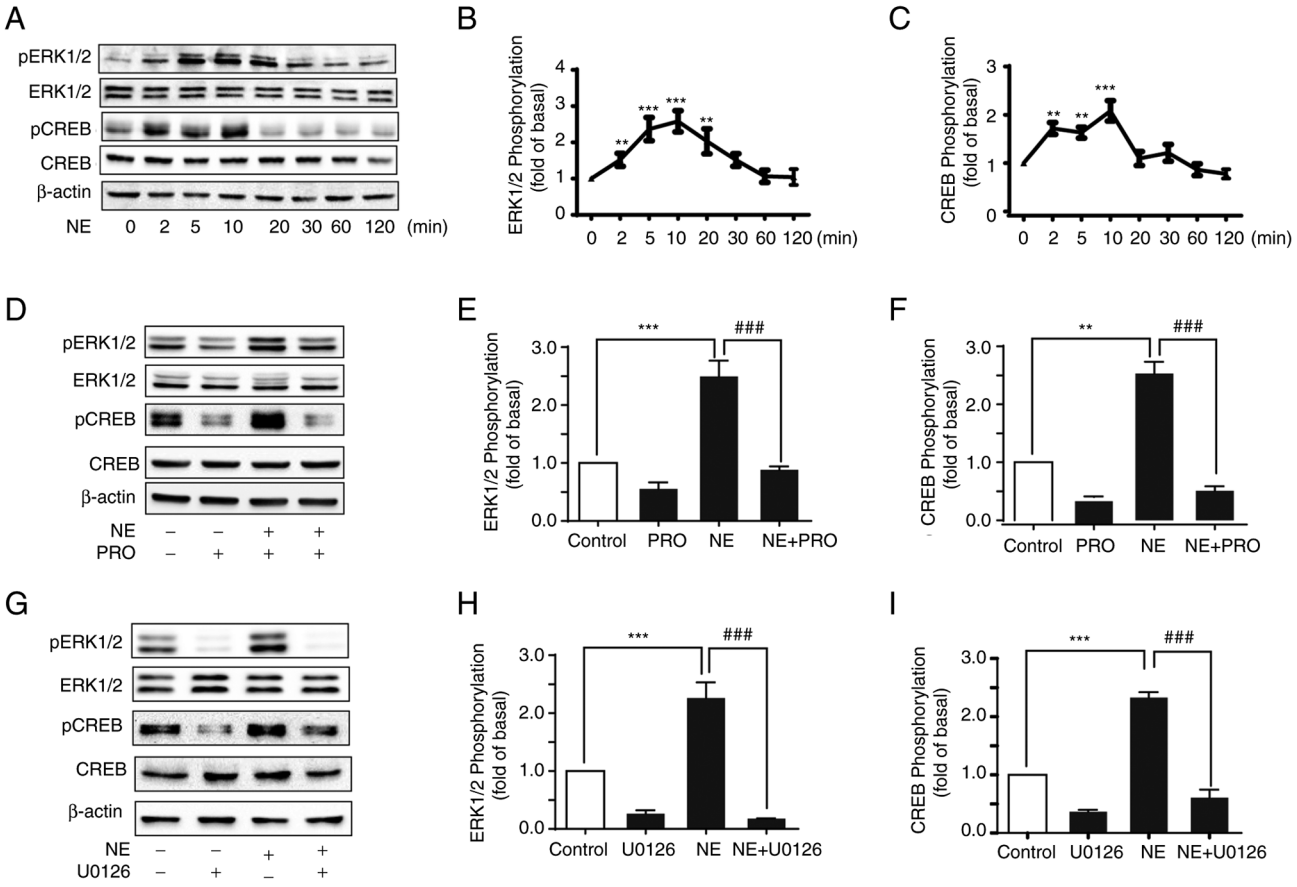

Figure 3. β-AR activates the ERK1/2/CREB pathway in HepG2 cells. (A-C) HepG2 cells were stimulated with 10 μM NE for different lengths of time, and the phosphorylation levels of ERK1/2 and CREB were detected by western blotting. (D-F) Effects of PRO on ERK1/2 and CREB phosphorylation levels in HepG2 cells treated with NE. Cells were pretreated with 20 μM PRO for 30 min and subsequently treated with NE for 10 min. (G-I) Effects of 20 μM U0126 on ERK1/2 and CREB phosphorylation levels in HepG2 cells treated with NE. Cells were pretreated with 20 μM U0126 for 30 min and subsequently treated with NE for 10 min. Data are presented as the mean ± SEM. **P<0.01, ***P<0.001 vs. control; $^{###}$P<0.001 vs. NE. NE, norepinephrine; PRO, propranolol; ERK1/2, extracellular signal-regulated kinase 1/2; CREB, cyclic adenosine monophosphate response element-binding protein; p, phospho.

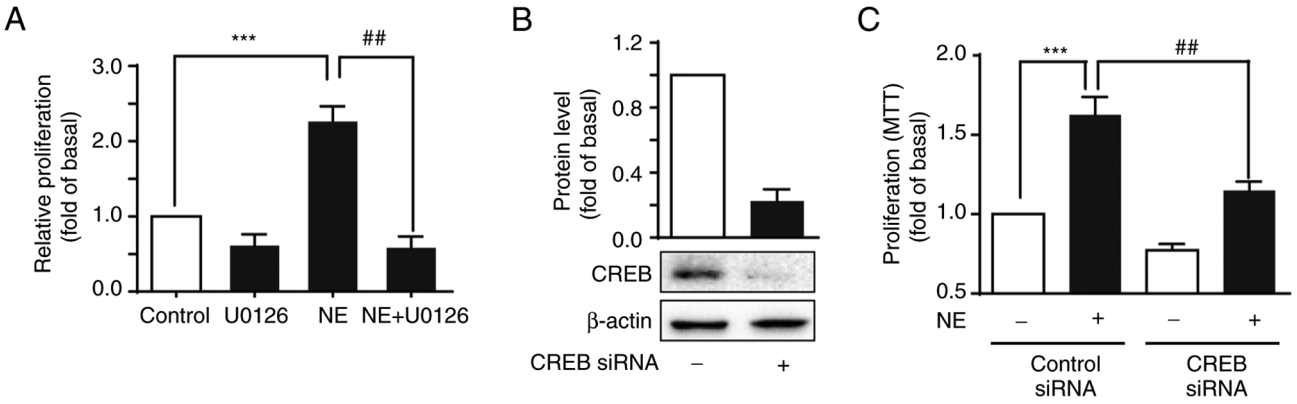

Figure 4. β-AR activation promotes the proliferation of HepG2 cells via the ERK1/2/CREB pathway. (A) Effects of U0126 on NE-enhanced cell proliferation in HepG2 cells. Cells were pretreated with 20 μM U0126 for 30 min and then treated with 10 μM NE for 48 h, after which, cell viability was measured using an MTT assay. (B and C) Effects of siRNA-mediated knockdown of CREB on the proliferation of cells treated with NE. Cells were transfected with siRNA against CREB and then treated with NE for 48 h. The expression levels of CREB and β-actin were tested by western blot analysis and cell viability was assessed using an MTT assay. Data are presented as the mean ± SEM. ***P<0.001 vs. control group; $^{##}$P<0.01 vs. NE-treated cells transfected with control siRNA. β-AR, β-Adrenergic receptor; ERK1/2, extracellular signal-regulated kinase 1/2; siRNA, small interfering RNA.

were pre-treated with U0126 (a selective inhibitor of ERK) and then treated with NE to further demonstrate whether NE-mediated ERK1/2 and CREB activation were involved in cell proliferation. U0126 inhibited the proliferative effects of NE (Fig. 4A). Additionally, knocking down CREB expression using specific siRNAs significantly reduced HepG2 cell growth compared with the control siRNA-transfected cells (Fig. 4B and C). Together, this suggested that ERK1/2 was

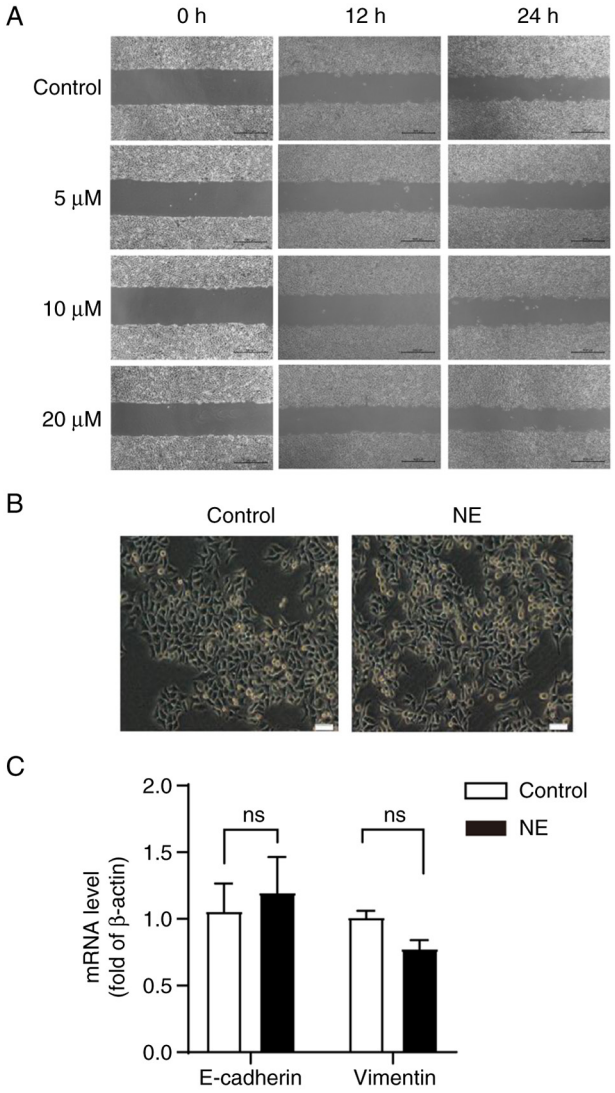

Figure 5. Effect of β-AR activation on HepG2 cell migration and EMT. (A) Cells were treated with NE (0, 5, 10, or 20 μM) for 24 h and the effect of NE on the migration of HepG2 cells was measured using a wound-healing assay. Scale bar, 500 μM. (B) Effects of β-AR activation on the EMT of HepG2 cells. Cells were cultured with NE for 96 h and the morphological changes of NE-induced cells were observed. Scale bar, 100 μM; Magnification, x10. (C) Effects of NE on E-cadherin and Vimentin in HepG2 cells. EMT, epithelial-mesenchymal transition; NE, norepinephrine; β-AR, β-Adrenergic receptor; ns, not significant.

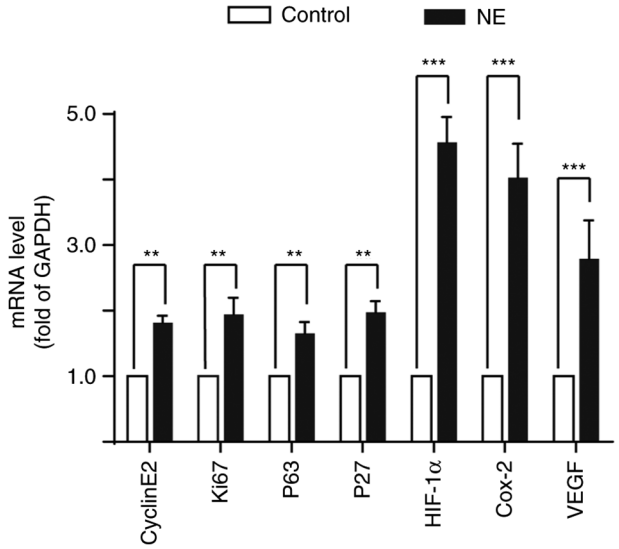

Figure 6. CyclinE2, Ki67, P53, P27, HIF-1α, Cox-2, and VEGF mRNA expression levels following treatment of HepG2 cells with 10 μM NE. Data are presented as the mean ± SEM. **P<0.01, ***P<0.001 vs. control group. NE, norepinephrine.

involved in NE-mediated proliferation in HepG2 cells via CREB phosphorylation.

Effects of β-AR activation on the migration and EMT of HepG2 cells. Previous studies have reported that NE promotes tumor metastasis in colon cancer and other types of cancer (26,27). Therefore, the role of β-ARs in cell migration in HepG2 cells was investigated. The wound healing assays showed that NE had no significant effect on HepG2 cell migration compared with the control group (Fig. 5A).

It has also been shown that β-AR is involved in the EMT process in oral squamous cell carcinoma and glioma cells (28,29). To determine whether β-ARs were involved in the EMT process of HepG2 cells, the morphological changes of the cell nuclei in NE-treated HepG2 cells were observed under a phase-contrast microscope. The results showed that NE treatment did not induce significant morphological changes in HepG2 cells (Fig. 5B). Subsequently, cellular RNA was extracted, and RT-qPCR was used to evaluate the expression of the EMT markers, E-cadherin, and Vimentin. The results demonstrated that NE had no effect on the expression of E-cadherin and Vimentin in HepG2 cells (Fig. 5C).

Activation of β-AR increases the expression of genes related to cell proliferation and cycle regulation. There is growing evidence that NE treatment can regulate the expression of genes related to the cell cycle and proliferation, thereby regulating the occurrence and development of tumors (30). To determine the effect of β-AR on gene expression in HepG2 cells, HepG2 cells were treated with NE, and the expression of cyclinE2, Ki67, P53, P27, HIF-1α, COX-2, and VEGF in HepG2 cells was detected. The results showed that β-AR activation significantly increased the expression of these genes (Fig. 6).

Discussion

In the past decade, despite the continuous improvements in understanding the etiology of liver cancer and techniques for diagnosing liver cancer, the prognosis of patients has remained poor (31,32). Therefore, there is an urgent need to identify novel and effective treatment methods. Chronic stress can activate AR through catecholamine neurotransmitters to mediate tumorigenesis; in addition, β-AR is crucial in the link between tumor development and psychological stress (33,34). Although the activated β-AR can regulate the proliferation of various types of cancer, including lung cancer, gastric cancer, pancreatic cancer, prostate cancer, and glioma, amongst others., there are still relatively fewer studies on the regulation of β-AR in the occurrence of hepatocellular carcinoma (12,16,34-36). In the present study, the possible mechanisms by which β-AR regulated the proliferation of

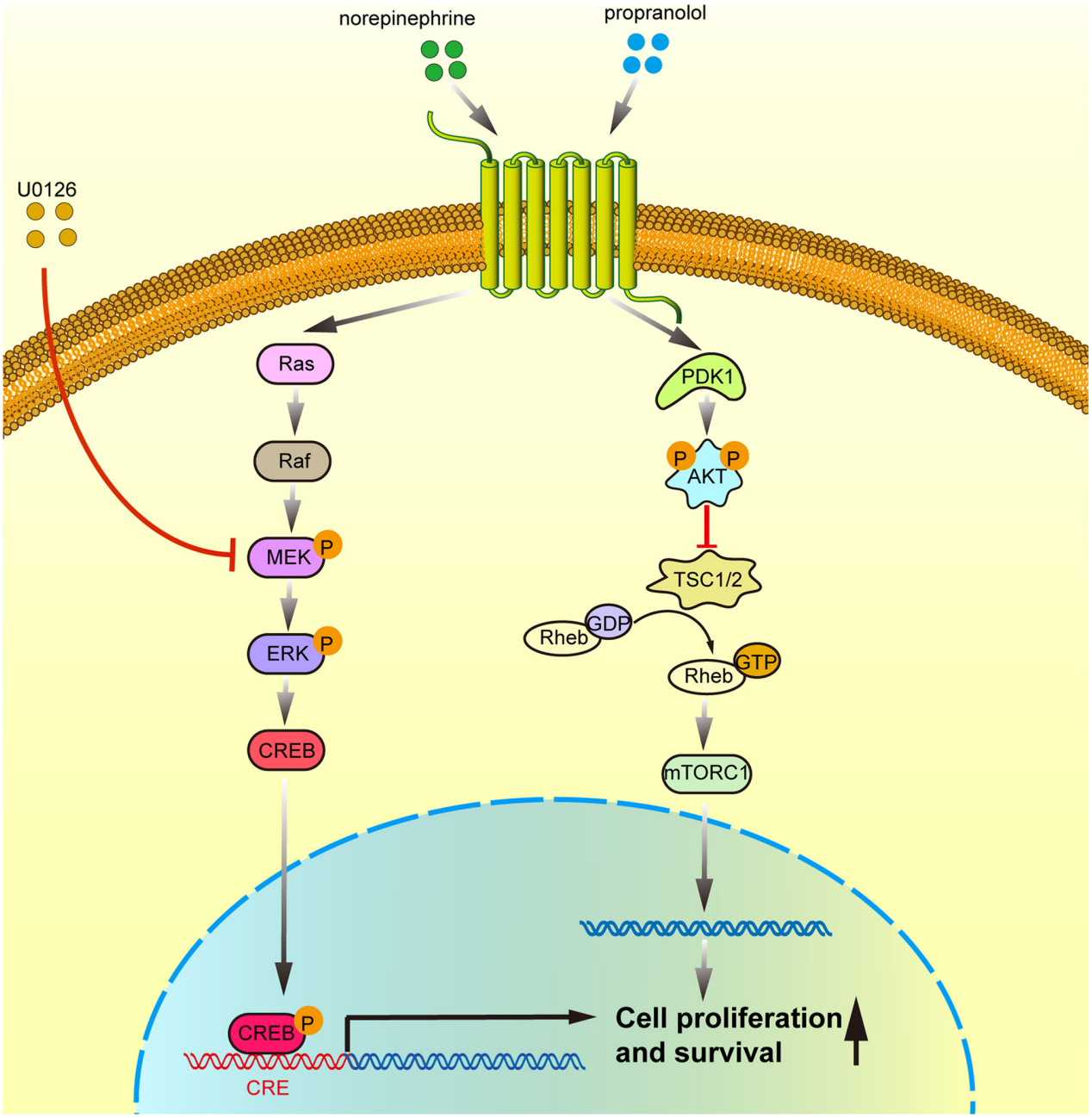

Figure 7. Proposed signaling pathway by which NE mediates HepG2 cell proliferation. NE-induced activation of the β-adrenergic receptor via ERK1/2/CREB and PDK1/AKT signaling pathways, increasing the viability of HepG2 cells. NE, norepinephrine; ERK1/2, extracellular signal-regulated kinase 1/2; CREB, cyclic adenosine monophosphate response element-binding protein; p, phospho.

hepatoma HepG2 cells were explored. First, it was shown that both β_1-AR and β_2-AR were expressed in hepatoma HepG2 cells, and the expression levels were higher than those in normal hepatocytes. Second, NE stimulated HepG2 cell proliferation in a dose-and time-dependent manner. The NE-induced pro-proliferative effect could be inhibited after the application of the β-AR blocker PRO, suggesting that the NE-induced pro-proliferative effect was mediated through β-AR. However, NE has no obvious pro-proliferative effect on normal hepatocytes, which is consistent with the expression of β-AR in normal hepatocytes.

β-AR-mediated cAMP/PKA and MAPK signaling pathways are essential signaling pathways regulating tumorigenesis and development (15). Previous studies have found that activation of β-AR by isoproterenol upregulates the phosphorylation of ERK1/2 and CREB in neuroblastoma (36,37). In the present study, NE administration significantly enhanced ERK1/2 and CREB phosphorylation levels in HepG2 cells; the pro-proliferative effect induced by NE was inhibited by treatment with the β-AR blocker PRO; this suggested that NE promoted HepG2 cell proliferation through β-AR, However, the role of α-AR was not determined, nor was the β-AR subtype involved, and subsequent studies should specifically activate α-AR, β_1-AR, or β_1-AR to address these shortcomings.

Since CREB is a key downstream target of ERK1/2, U0126, a selective inhibitor of ERK1/2, was used to pretreat HepG2 cells to detect changes in CREB protein expression and cell viability. The results showed that NE treatment induced

CREB phosphorylation and the increase in HepG2 cell viability was significantly inhibited. Moreover, similar conclusions were obtained after knocking down CREB expression. These findings suggest that NE regulates the ERK1/2/CREB signaling pathway by activating β-AR, which in turn promotes HepG2 cell proliferation. PDK1 has been linked to several pathological traits, including uncontrolled cell reproduction, apoptosis resistance, invasion, dissemination, metastasis, metabolic reprogramming, and aberrant angiogenesis (38). Increased PDK1 expression can increase PI3K/AKT/MTOR signaling, resulting in a radiation-resistant and dedifferentiated phenotype of liver cancer (31). As a consequence, the effect of NE on the phosphorylation levels of PDK1 and AKT proteins was assessed, and the results suggested that the NE-induced increase in proliferation may also be mediated via the PDK1/AKT signaling pathway (Fig. S2). These findings highlight the critical role of β-AR in hepatocarcinogenesis.

EMT is a key step for tumor cell invasion and migration. Tumor cells acquire mesenchymal and fibroblast-like phenotypes during EMT, and this promotes cell migration and spread to tissues distant from the site of origin (39,40). Simultaneously, several studies have shown that NE may also be involved in the metastatic process of various types of cancer (26). However, the effect of NE on cell migration and EMT has not been demonstrated in HepG2 cells. Here, it was found that NE had a negligible effect on the EMT of HepG2 cells. Furthermore, β-AR has been shown to regulate the expression of genes involved in cell proliferation and cycle regulation, thereby regulating the occurrence and development of tumors (30). Consistent with these findings, the results of the present study showed that β-AR activation significantly enhanced the expression of CyclinE2, Ki67, P53, P27, HIF-1α, Cox-2, and VEGF genes in HepG2 cells.

While the current study has revealed the crucial role of NE/AR signaling in regulating the cell proliferation of HepG2 and Huh7 cells, some important questions remain to be addressed. For example, it is unknown whether NE has a broad-spectrum pro-proliferative effect on hepatocellular carcinoma. Additional types of hepatoma cell lines, with different degrees of malignancy, such as SMMC7721 and HCCLM3, will be used to further confirm the role of β-AR/ERK1/2/CREB signaling cascade in hepatoma cell proliferation. Second, *in vivo* experiments based on animal models of liver cancer should be performed to determine the effect of β-AR-ERK1/2-CREB signaling on tumor growth. Answering these questions is expected to expand our understanding of the role of β-AR in controlling the occurrence and development of hepatocellular carcinoma.

In conclusion, the results of the present study indicate that β-adrenergic receptor activation promotes the proliferation of HepG2 cells by activating the ERK1/2/CREB signaling pathway (Fig. 7), which highlights the significance of β-AR activation in hepatocarcinogenesis and provides a theoretical basis for the development of novel therapeutic approaches that target the ERK1/2/CREB signaling pathway for the management of liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH, PH, XLiu and XLin designed the study. JH and XLin performed the experiments. FL, XLiu and LL analyzed the data. HX, LS, LN and YZ analyzed the western blotting data. JH, XLin, XLiu and PH wrote the manuscript. XLiu and PH were responsible for ensuring that any issues concerning the accuracy or integrity of the work were properly addressed and resolved. PH and XLin confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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