PI3K/AKT phosphorylation activates ERRα by upregulating PGC-1α and PGC-1β in gallbladder cancer

LEI WANG[1*], MENGMENG YANG[2*] and HUIHAN JIN[1]

[1]Department of Hepatobiliary Surgery, The Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University; [2]Department of Malaria Control and Prevention, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology (Jiangsu Institute of Parasitic Diseases), Wuxi, Jiangsu 214002, P.R. China

Received February 27, 2021; Accepted May 24, 2021

DOI: 10.3892/mmr.2021.12252

Abstract. The nuclear estrogen-related receptor-α (ERRα) is an orphan receptor that has been identified as a transcriptional factor. Peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1-α (PGC-1α) and PPARγ coactivator-1-β (PGC-1β) act as the co-activators of ERRα. Our previous study reported that activated ERRα promoted the invasion and proliferation of gallbladder cancer cells by promoting PI3K/AKT phosphorylation. Therefore, the aim of the current study was to investigate whether PI3K/AKT phosphorylation could enhance ERRα activity in a positive feedback loop. LY294002 and insulin-like growth factor I (IGF-I) were used to inhibit and promote PI3K/AKT phosphorylation, respectively. A 3X ERE-TATA luciferase reporter was used to measure ERRα activity. The present study found that LY294002 inhibited PI3K/AKT phosphorylation, decreased the proliferation and invasion of NOZ cells and suppressed the activity of ERRα. Conversely, IGF-I induced PI3K/AKT phosphorylation, promoted the proliferation and invasion of NOZ cells and enhanced the activity of ERRα. The protein expression levels of PGC-1α and PGC-1β were elevated and reduced by IGF-I and LY294002, respectively. Moreover, knockdown of PGC-1α and PGC-1β antagonized ERRα activation, which was enhanced by PI3K/AKT phosphorylation. Taken together, the present study demonstrated that PI3K/AKT phosphorylation triggered ERRα by upregulating the expression levels of PGC-1α and PGC-1β in NOZ cells.

Correspondence to: Professor Huihan Jin, Department of Hepatobiliary Surgery, The Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University, 68 Zhongshan Road, Wuxi, Jiangsu 214002, P.R. China
E-mail: jinhuihan@njmu.edu.cn

*Contributed equally

Key words: gallbladder cancer, peroxisome proliferator-activated receptor-γ coactivator-1-α, peroxisome proliferator-activated receptor-γ coactivator-1-β, estrogen-related receptor-α, PI3K/AKT phosphorylation

Introduction

The incidence of gallbladder and biliary tract cancer has increased by 76% between 1990 and 2017 on a global scale (1). Due to the low early detection rate, gallbladder cancer (GBC) often undergoes local invasion and lymph node metastasis (2). Most patients with GBC are diagnosed at advanced stages and are unresectable (3). These patients tend to relapse despite having received standard chemotherapy and radiotherapy. Therefore, the overall survival of GBC is extremely low, ranging from 13.2-19 months (4,5). A recent study revealed that a value of 65 IU/ml CA 19-9 may be helpful in evaluating the prognosis of GBC (6). Currently, there is no effective chemotherapy or targeted therapy for the treatment of GBC. Novel immunotherapeutic drugs, such as immune checkpoint inhibitors of anti-programmed cell death protein-1 antibody and anti-programmed cell death-ligand 1 antibody, have shown limited efficacy in the clinical intervention of GBC (7,8). Therefore, additional efforts should be made to identify novel targets and to determine the in-depth mechanism to advance the understanding and the curative effect of GBC.

Estrogen-related receptor-α (ERRα) is a member of the orphan nuclear receptors (9) and belongs to the ERR family, which consists of ERRα, ERRβ and ERRγ (10). ERRα was identified on the basis of the structural similarity between its DNA binding domain and human estrogen receptor (ER) α; however, ERRα does not bind to natural estrogens or estrogen-like molecules (11). ERRα is involved in various biological processes and activities, including energy metabolism and cell proliferation and invasion, by binding to estrogen-related response elements and estrogen response elements (EREs) (12). A number of orphan nuclear receptors are activated by the peroxisome proliferator-activated receptor γ (PPARγ) coactivator (PGC) family, including PGC-1α, PGC-1β and PRC (13). In the absence of specific ligands, ERRα can be activated by PGC-1 family members, such as PGC-1α (14) and PGC-1β (15). Moreover, as wild-type PGC-1α (PGC-1α WT) can activate other receptors, such as ERRβ and ERRγ, researchers have reported that some peptides (such as L3-09) can bind to ERRα specifically. Herein, the investigators replaced L2 and L3 motifs with L3-09 peptides to generate PGC-1α 2x9, in an attempt to selectively activate

ERRα (16). Moreover, a 3X ERE-TATA luciferase reporter was applied to measure the activity of ERs and ERRs, including ERRα (12).

As one of the most important signaling transduction pathways in mammalian cells, the PI3K/AKT signaling pathway functions to inhibit cellular apoptosis and promote proliferation by interacting with multiple downstream effectors (17). LY294002 has been proved to specifically inhibit the activity of the PI3K (18,19), whereas recombinant human insulin-like growth factor-I (IGF-I) can be applied to activate the PI3K/AKT signaling pathway (20). The binding of IGF-I to IGF-I receptor (IGF-IR) functions to induce receptor autophosphorylation and to elevate the tyrosine kinase activity of IGF-IR, thereby leading to the activation of the 85-kDa subunit of PI3K by recruiting and phosphorylating intracellular insulin receptor substrate-1 (21-23). AKT is then activated via recruitment to cellular membranes by the PI3K lipid (24). Previous studies have reported that ERRα triggered PI3K/AKT phosphorylation by enhancing the transcription of Nectin-4, thereby promoting the growth and metastasis of GBC (25,26).

The present study aimed to investigate whether PI3K/AKT phosphorylation could positively activate the activity and expression of the PGC-1/ERRα axis. To that end, LY294002 and IGF-I were used to specifically inhibit and trigger PI3K/AKT phosphorylation, respectively. Moreover, a 3X ERE-TATA luciferase reporter was applied to measure the degree of ERRα activation. XCT-790 is a specific inverse agonist of ERRα. PGC-1α 2x9 and XCT-790 were used to specifically enhance and inhibit the activity of ERRα, respectively.

Materials and methods

Cell culture. The NOZ human GBC cell line was purchased from Shanghai Key Laboratory of Biliary Tract Diseases, and was cultured in William's medium E (Genom Biotech Pvt., Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in the humidified incubator containing 5% CO_2 at 37˚C.

Chemicals. LY294002 (cat. no. S1105) was purchased from Selleck Chemicals to inhibit PI3K phosphorylation. Recombinant human IGF-I (cat. no. 291-G1) was acquired from R&D Systems, Inc. to promote PI3K phosphorylation. XCT-790 (cat. no. HY-10426) was purchased from MedChemExpress to inhibit the activity of ERRα. The concentration gradient was set to detect the values of IC_{50} or half maximal effective concentration (EC_{50}) of the chemicals in NOZ cells. Inhibition curves with concentration gradients ranging from 0.1-20 μM (treatment for 72 h at 37˚C) and 0.6-40 μM (treatment for 72 h at 37˚C) for LY294002 and XCT-790, respectively, were drawn to determine the IC_{50} values of LY294002 and XCT-790 in NOZ cells. Based on the IC_{50} values, the final concentrations of 7 μM LY294002 and 6 μM XCT-790 were cocultured with NOZ cells at 37˚C for 72 h in indicated experiments. The activation curve with concentration gradients ranging from 0.1-100 ng/ml (treatment for 72 h at 37˚C) for IGF-I was drawn to determine the EC_{50} value. Based on the EC_{50} value, 13 ng/ml IGF-I was cocultured with NOZ cells at 37˚C for 72 h in indicated assays.

Cell Counting Kit (CCK)-8 assay. The viability of GBC cells was determined using a CCK-8 assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. The cells were seeded into the 96-well plate at the density of $1x10^3$ cells each well. Then, 24 h later, 10 μl CCK-8 solution and 90 μl complete medium were co-cultured with NOZ cells for 2 h at 37˚C. The absorbance value (optical density) of NOZ cells was detected on a microplate reader at the wavelength of 450 nm (Bio-Tek Instruments, Inc.).

Colony formation assay. The biological effects of LY294002 and IGF-I on the colony formation ability of NOZ cells were tested. In brief, the NOZ cells were seeded into 6-well plate at a density of 500 cells each well. After 6 h, 7 μM LY294002 and 13 ng/ml IGF-I were added into the medium for co-incubation with NOZ cells for 72 h at 37˚C. Subsequently, LY294002 and IGF-I were removed, leaving the NOZ cells cultured at 37˚C with the medium for 1 week. The cloning foci were fixed using 4% PFA (paraformaldehyde) for 20 min and were stained using 0.1% crystal violet for 20 min, both at room temperature. The colonies with >50 cells were counted under a light microscope (magnification, x20).

Transwell invasion assay. The 8-μm Transwell filters (BD Biosciences) and 24-well Transwell chambers were used to detect the invasive capacity of cells. In total, 70 μl 1 mg/ml Matrigel (BD Biosciences) was added onto the upper chamber at 37˚C overnight. Then, the upper chamber with Matrigel-coated membrane was seeded with $4x10^4$ NOZ cells in 200 μl serum-free medium. Moreover, 500 μl basal medium containing 15% FBS was added into the lower chamber. Following the 20-h co-culturing in an incubator containing 5% CO_2 at 37˚C, the cells that invaded to the lower layer were fixed using 4% paraformaldehyde for 20 min and were then stained using crystal violet for another 20 min, both at room temperature. In total, five random fields were chosen to count the invaded cells using a light microscope (magnification, x20) in order to determine the invasive capacity of NOZ cells. The assays were carried out in triplicate.

Antibodies and western blot analysis. Primary antibodies, including rabbit anti-PI3K p85 (1:1,000; cat. no. 4257), anti-AKT (1:1,000; cat. no. 4691) and anti-phosphorylated (p)-AKT (Ser473; 1:2,000; cat. no. 4060) were purchased from Cell Signaling Technology, Inc. Rabbit anti-ERRα primary antibody (1:500; cat. no. NBP1-47254) was purchased from Novus Biologicals, LLC. Rabbit anti-PGC-1α (1:500; cat. no. ab191838), PGC-1β (1:1,000; cat. no. ab176328) and p-PI3K p85α (p-Y607; 1:1,000; cat. no. ab182651) were purchased from Abcam. Goat anti-rabbit HRP-conjugated secondary antibody (1:5,000; cat. no. S0001) was obtained from Affinity Biosciences.

Total proteins were extracted from each group of cells using RIPA lysis buffer (Cell Signaling Technology, Inc.), and a BCA protein quantification kit (Thermo Fisher Scientific, Inc.) was used to quantify the concentration of protein. A total of 30 μg protein was separated via 10-15% SDS-PAGE and the proteins were then transferred onto PVDF membranes (MilliporeSigma). For the testing of non-phosphorylated antibody, 5% non-fat dry milk was used to block the PVDF membrane at room temperature for 1 h; for the testing of phosphorylated antibody, 5% BSA (Suzhou Yacoo Science Co., Ltd.) was used to block the membranes

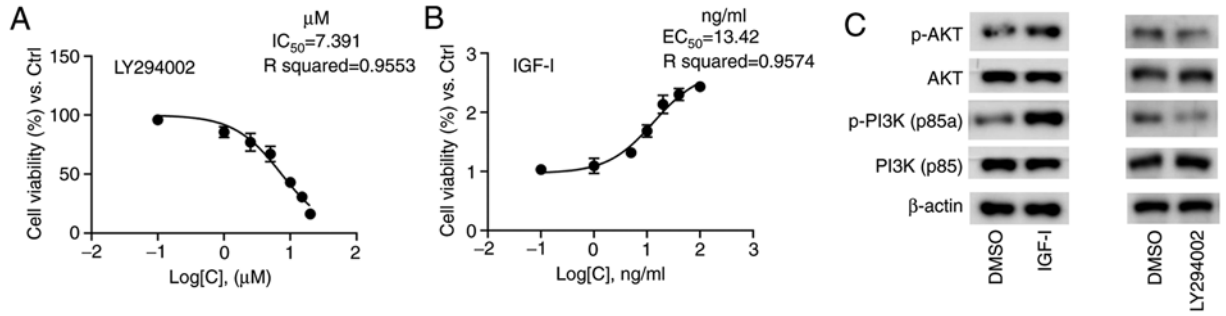

Figure 1. Sensitivity and dosage of LY294002 and IGF-I in NOZ cells. (A) The IC_{50} value of LY294002 against NOZ cells was 7.39 μM, as calculated by the inhibition curve. (B) The EC_{50} value of IGF-I against NOZ cells was 13.42 ng/ml, according to the calculation of the activation curve. (C) IGF-I notably elevated the expression levels of p-PI3K p85a and p-AKT, whereas LY294002 markedly inhibit the expression levels of p-PI3K p85a and p-AKT. The expression levels of the total PI3K and the total AKT were unaffected by IGF-I or LY294002. All of the experiments were repeated three times. IGF-I, insulin-like growth factor I; p-, phosphorylated; Ctrl, control.

at room temperature for 1 h. The incubation with primary antibody at 4˚C lasted 12 h, followed by the 2-h co-incubation with HRP-conjugated secondary antibody (1:5,000) at room temperature. The intensities of the signals were determined using a Gel Doc 2000 system (Bio-Rad Laboratories, Inc.) after being visualized with an electrochemiluminescence kit (Wuhan Boster Biological Technology Ltd.).

RNA interference. The short hairpin (sh)RNA sequences to specifically knockdown ERRα, PGC-1α and PGC-1β were 5'-GCGAGAGGAGUAUGUUCUA-3', 5'-GAUGUGAACGACUUGGAUACA-3' and 5'-UGUAGUUCUGUACAACUUCGG-3', respectively. The sequence for negative control (scrambled sequence) was 5'-TTCTCCGAACGTGTCACGT-3'. All sequences were constructed by Genomeditech Biotechnology, and were inserted into the PGMLV-SC5 lentivirus core vector (Genomeditech Biotechnology). In serum-free medium, the concentrated viruses with a MOI of 40 were then infected into the NOZ cells using ViaFect™ transfection reagent (Promega Corporation) following the manufacturer's instructions at 37˚C. The supernatant was replaced with complete culture medium after 24 h. Subsequent experimentations were performed after 120 h.

Construction of plasmids and transfection. pGL3-Basic-3X ERE-TATA-luc that contains triple the AGGTCANNN TGACCT, plasmids with WT PGC-1α [pCDNA3.1(+)-3 x Flag-C-M-PGC-1α-WT] and mutant-type (MT) PGC-1α [pCDNA3.1(+)-3 x Flag-CM-PGC-1α-2x9] were synthesized by Genomeditech Biotechnology, in accordance with the protocol described in a previous study (16). A total of 2 μg constructed plasmids were then transfected into the NOZ cells using ViaFect Transfection reagent at 37˚C. The supernatant was replaced with complete culture medium after 24 h. The expression level was analyzed via western blot analysis after 120 h. Moreover, the empty vector-infected cells (Mock-transfected) were used as the control.

Dual luciferase reporter gene assay. pGL3-Basic-3X ERE-TATA-luc was applied to detect ERRα activity. pRL-TK plasmids (25 ng; Genomeditech Biotechnology) containing PGC-1α WT or PGC-1α 2x9 (250 ng) and pGL3-Basic-3X ERE-TATA-luc (250 ng) were transfected into NOZ cells using 1.5 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. The activity of *Renilla* luciferase and firefly luciferase was detected on a luminometer using a SEAP Reporter Gene assay kit (Abcam; cat. no. ab133077). The empty vector-infected cells were used as the internal control. Finally, the results were expressed as the ratio of firefly luciferase activity/*Renilla* luciferase activity.

Statistical analysis. Quantitative data are presented as the mean ± SD based on triplicated experiments. An unpaired Student's t-test was used to compare the inter-group difference between two groups using GraphPad Prism 8.0 software (GraphPad Software, Inc.) for statistical analyses. Comparative data among multiple groups were analyzed using one-way ANOVA followed by Tukey's test, using SPSS 19.0 for Windows (IBM Corp.). The suppression curves for IGF-I, LY294002 and XCT-790 were plotted according to the results of seven differential concentrations. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sensitivity of NOZ cells to IGF-I and LY294002. The concentration gradients ranging from 0.1-20 μM were set to draw the inhibition curve, which demonstrated that the IC_{50} value of LY294002 was 7.39 μM in NOZ cells (Fig. 1A). Similarly, the activation curve for IGF-I was drawn to determine that the value of EC_{50} was 13.42 ng/ml (Fig. 1B). The final concentrations of 7 μM for LY294002 and 13 ng/ml for IGF-I were applied in the subsequent assays, and no obvious cytotoxicity was observed. The results of western blot analysis revealed that the protein expression levels of p-PI3K p85a and p-AKT were notably elevated in the NOZ cells cultured with IGF-I (Fig. 1C), indicating that IGF-I effectively activated the PI3K/AKT signaling pathway via PI3K/AKT phosphorylation. Conversely, LY294002 markedly reduced the expression levels of p-PI3K p85a and p-AKT (Fig. 1C), suggesting that LY294002 effectively diminished the PI3K/AKT signaling pathway via PI3K/AKT dephosphorylation.

Consistently, the proliferative capacity (Fig. 2A), colony formation ability (Fig. 2B) and the invasive capacity (Fig. 2C) of NOZ cells were significantly enhanced by IGF-I, but were significantly inhibited by LY294002.

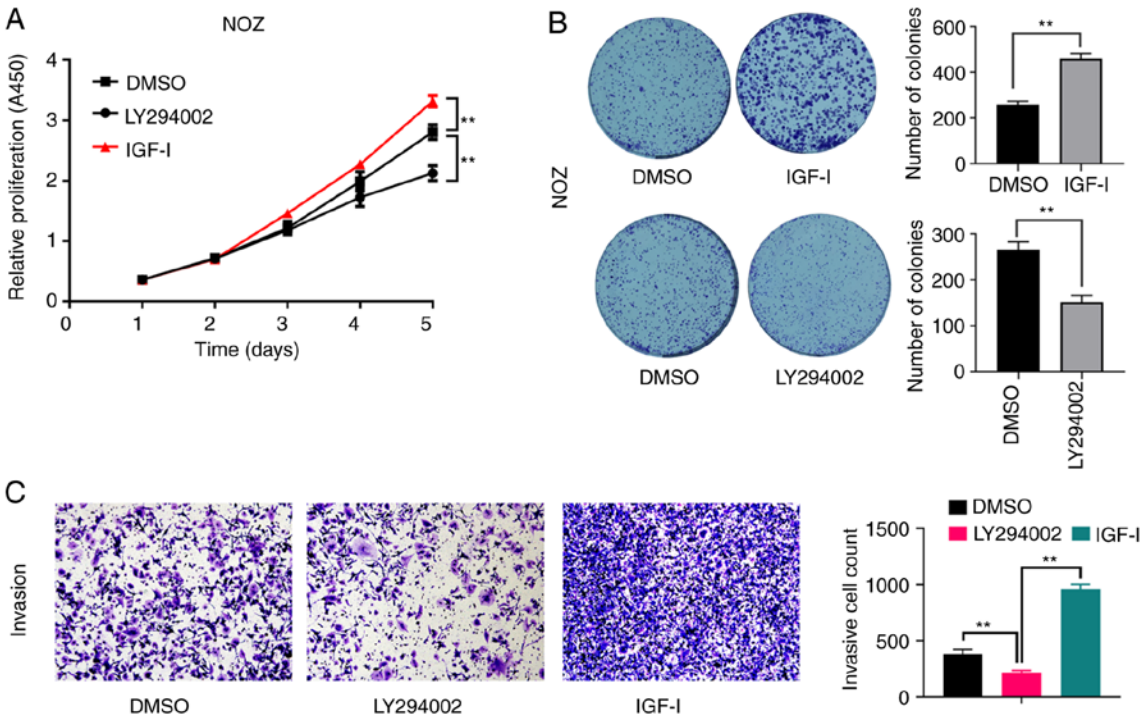

Figure 2. Biological effect of LY294002 and IGF-I on the proliferative and invasive capacity of NOZ cells. (A) A Cell Counting Kit-8 assay was performed to examine cell viability. (B) Colony formation assays. (C) Migration and invasion assays for LY294002 and IGF-I treated NOZ cells. Magnification, x20. All of the experiments were repeated three times. **P<0.01. IGF-I, insulin-like growth factor I; ERE, estrogen response element.

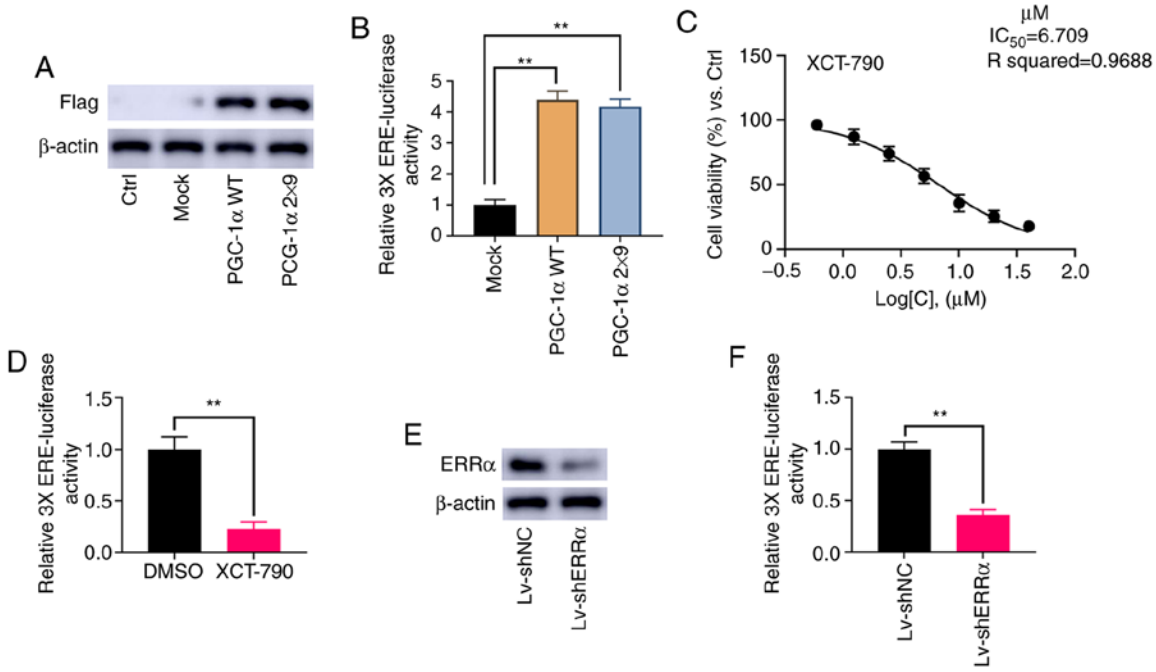

Figure 3. Detection of ERRα activation. (A) Overexpression of PGC1-α WT and PGC1-α 2x9 were validated via western blotting. (B) The activity of 3X ERE luciferase reporter was enhanced by PGC1-α WT and PGC1-α 2x9, compared with that of Mock. Empty vector-infected cells (Mock) were used as the control. (C) IC_{50} value of XCT-790 against NOZ cells was 6.709 μM, according to the calculation of the inhibition curve. (D) The activity of 3X ERE luciferase reporter was reduced by the treatment of 6 μM XCT-790. (E) Knockdown of ERRα was validated using western blotting. (F) The activity of 3X ERE luciferase reporter was diminished in the ERRα-knockdown cells. All of the experiments were repeated three times. **P<0.01. WT, wild-type; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1-1α, peroxisome proliferator-activated receptor-γ coactivator-1-α; Ctrl, control; NC, negative control; sh, short hairpin RNA.

Detection of ERRα activation. PGC-1α can activate ERRα, as well other receptors (16). To specifically and selectively activate ERRα, the current study followed the protocol described by Gaillard *et al* (16) and Chang *et al* (27), replacing both L2 and L3 motifs in WT PGC-1α with L3-09 peptides to generate PGC-1α 2x9. PGC-1α and PGC-1α 2x9

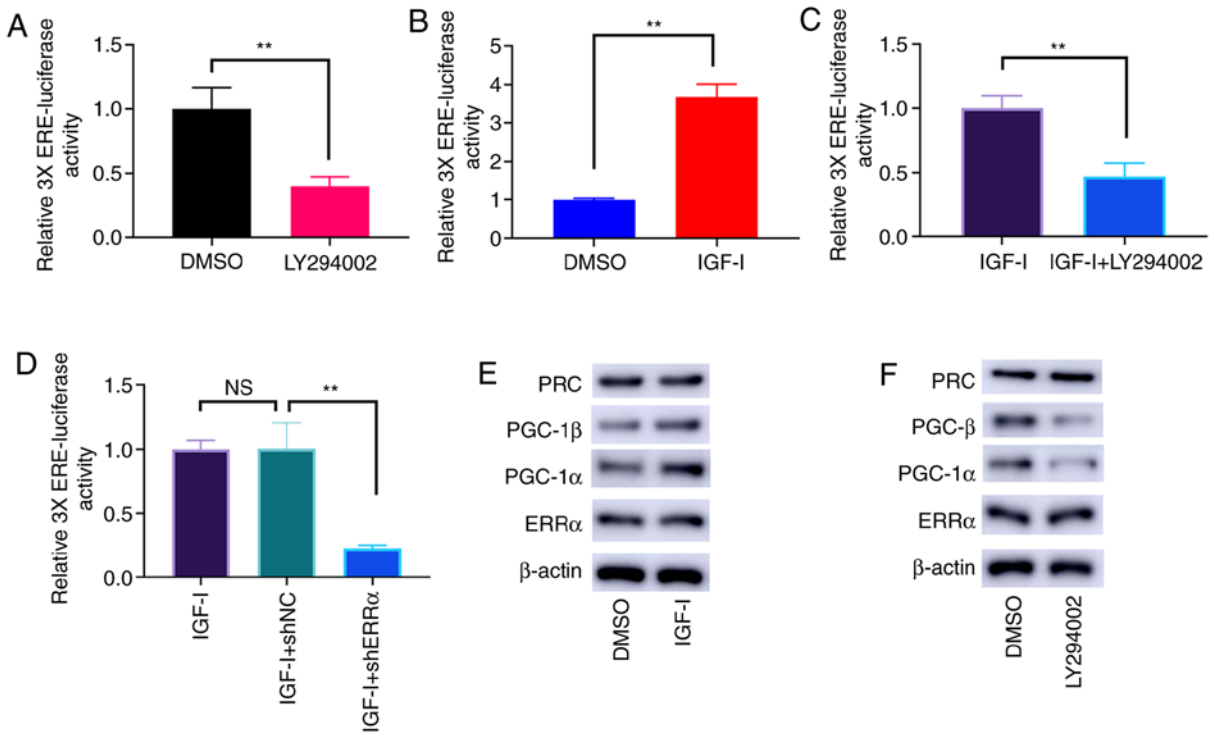

Figure 4. PI3K/AKT phosphorylation activates ERRα. (A) The inhibition of the PI3K/AKT phosphorylation by LY294002 effectively decreased the activity of ERRα. (B) PI3K/AKT phosphorylation induced by IGF-I effectively enhanced the activity of ERRα. (C) LY294002 treatment antagonized the upregulated ERRα activity induced by IGF-I. (D) The knockdown of ERRα antagonized the upregulated ERRα activity caused by IGF-I. (E) Protein expression levels of PGC-1α and PGC-1β were notably elevated by IGF-I. The expression levels of PRC and ERRα were not affected. (F) The protein expression levels of PGC-1α and PGC-1β were reduced by LY294002, while those of PRC and ERRα were not altered. All of the experiments were repeated three times. **P<0.01. IGF-I, insulin-like growth factor I; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1-1, peroxisome proliferator-activated receptor-γ coactivator-1; PRC, PGC-related coactivator; NC, negative control; sh, short hairpin RNA.

were successfully overexpressed in NOZ cells (Fig. 3A). As shown in Fig. 3B, the relative activity of 3X ERE TATA dual luciferase reporter was significantly increased by PGC-1α and PGC-1α 2x9 (P<0.01).

As a specific inverse agonist of ERRα, XCT-790 can inhibit the activation of ERRα (28). The results demonstrated that the IC_{50} value of XCT-790 in NOZ cells was 6.71 μM (Fig. 3C), and therefore, a final concentration at 6 μM XCT-790 was applied in subsequent assays. As presented in Fig. 3D, 6 μM XCT-790 significantly inhibit the activation of 3X ERE TATA dual luciferase reporter (P<0.01). Moreover, it was found that the knockdown of ERRα significantly reduced the activation of 3X ERE TATA dual luciferase reporter (P<0.01; Fig. 3E and F). These results indicated that the relative 3X ERE TATA luciferase activity was consistent with the activity of ERRα.

PI3K/AKT phosphorylation triggers the ERRα activity. The dephosphorylation of PI3K/AKT by LY294002 led to the lower activities of ERRα (Fig. 4A). Conversely, PI3K/AKT phosphorylation induced by IGF-I enhanced the activities of ERRα (Fig. 4B), and this effect was offset by LY294002 (Fig. 4C) and ERRα knockdown (Fig. 4D). Nevertheless, the protein expression level of ERRα was not affected by PI3K/AKT phosphorylation. As potential coactivators of ERRα, PGC-1α and PGC-1β expression was notably elevated by PI3K/AKT phosphorylation (Fig. 4E). Conversely, dephosphorylation of PI3K/AKT by LY294002 reduced the protein expression levels of PGC-1α and PGC-1β (Fig. 4F). However, the protein expression level of PGC-related coactivator (PRC) was not affected by PI3K/AKT phosphorylation (Fig. 4E and F).

PGC-1α and PGC-1β mediate the activation of ERRα enhanced by IGF-I. As shown in Fig. 5A and B, PGC-1α and PGC-1β were effectively knocked down by Lv-shPGC-1α and Lv-shPGC-1β. Moreover, the loss of PGC-1α and PGC-1β antagonized the increased ERRα activity caused by IGF-I (Fig. 5C and D). Similarly, the enhanced cell viability caused by IGF-I was antagonized by the knockdown of PGC-1α and PGC-1β and the treatment of LY294002 (Fig. 5E-G). The effect of LY294002 treatment and the knockdown of PGC-1α and PGC-1β also antagonized the increased colony formation and invasive ability of NOZ cells (Fig. 6A and B). Therefore, the activation effect of PI3K/AKT on ERRα was attributable to its ability of elevating PGC-1α and PGC-1β expression.

Discussion

The vast majority of GBC cases are diagnosed at the advanced stages, and the low 5-year survival rate of patients with advanced GBC is aggravated by low sensitivity to chemoradiotherapy and targeted therapy (29). Moreover, the molecular mechanisms that underlie the onset and progression of GBC continue to defy the medical community (30). Thus, additional efforts are required to develop novel effective targeted therapies, which are considered to be the key to improve the prognosis and the quality of life of patients with GBC.

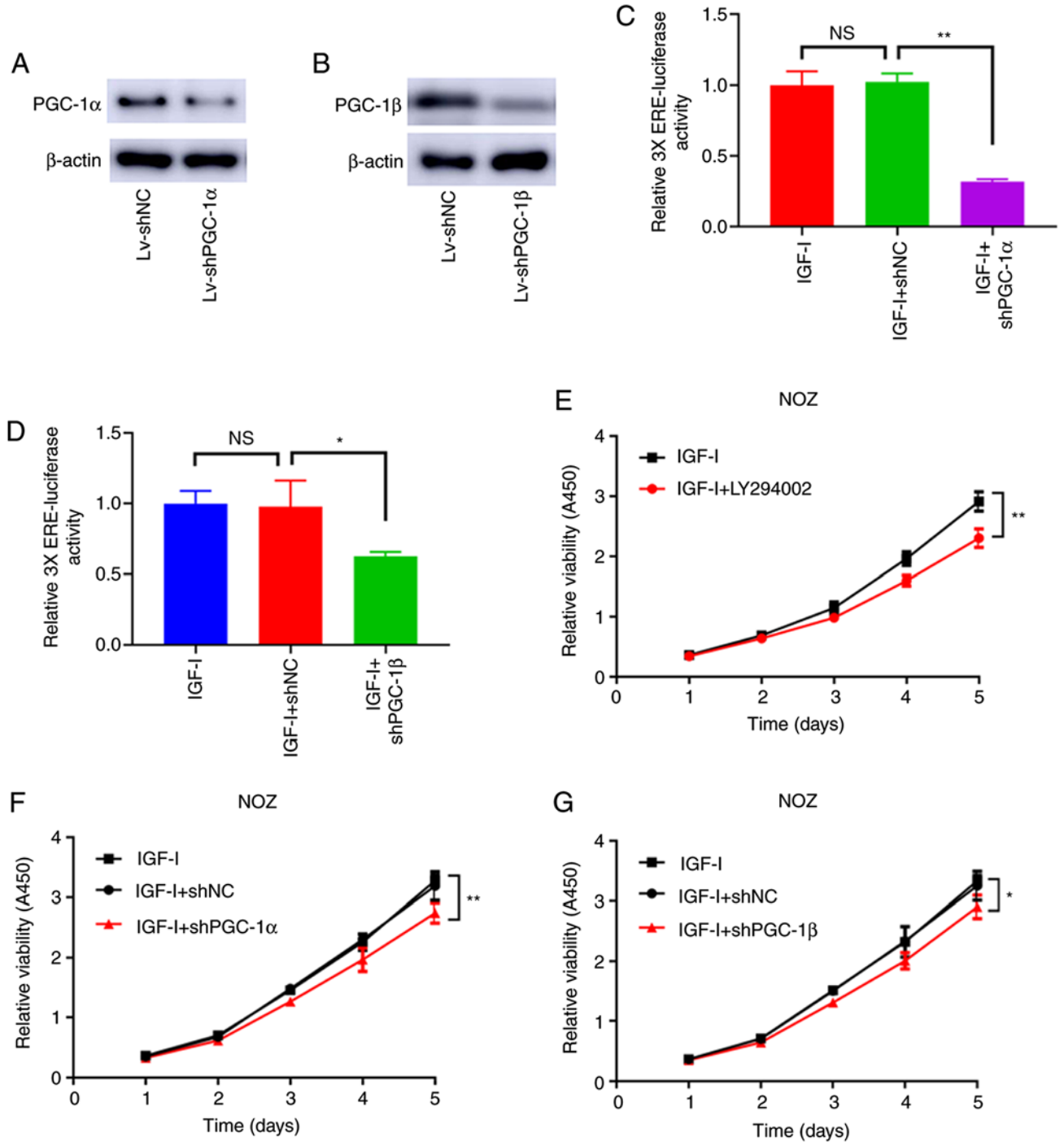

Figure 5. Knockdown of PGC-1α and PGC-1β attenuates the effect of IGF-I on ERRα. (A) Lv-shPGC-1α and (B) Lv-shPGC-1β effectively knocked down PGC-1α and PGC-1β, respectively. Knockdown of (C) PGC-1α and (D) PGC-1β attenuated the upregulated activity of ERRα caused by IGF-I. (E) LY294002 treatment and knockdown of (F) PGC-1α and (G) PGC-1β reduced NOZ cell viability that was enhanced by IGF-I. All of the experiments were conducted in triplicate. *P<0.05, **P<0.01. IGF-I, insulin-like growth factor I; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1-1, peroxisome proliferator-activated receptor-γ coactivator-1; NC, negative control; sh, short hairpin RNA; NS, not significant.

Our previous study reported that ERRα enhanced the transcription of Nectin-4, thereby triggering the PI3K/AKT signaling pathway to promote the growth and metastasis of GBC (25). As an orphan nuclear receptor in the nucleus, ERRα bears structural resemblance to ERα. Nevertheless, ERRα cannot be activated by estrogen (11). The majority of the genes under the regulation of ERRα are distinct from those mediated by ERα. The PGC-1 family serves as co-activators to activate ERRα, which, once activated, can regulate the expression levels of genes that are involved in the tricarboxylic acid cycle, lipid metabolism and oxidative phosphorylation (27). Accumulating evidence has shown that ERRα may be involved in a wide variety of cancer types (31). Therefore, in-depth examination into the molecular mechanisms that affect the activity of ERRα could shed light on ERRα targets. For example, in a recent study, Yang *et al* (15) revealed that F-box and leucine-rich repeat protein 10 increased ERRα enrichment at the promoter region of its

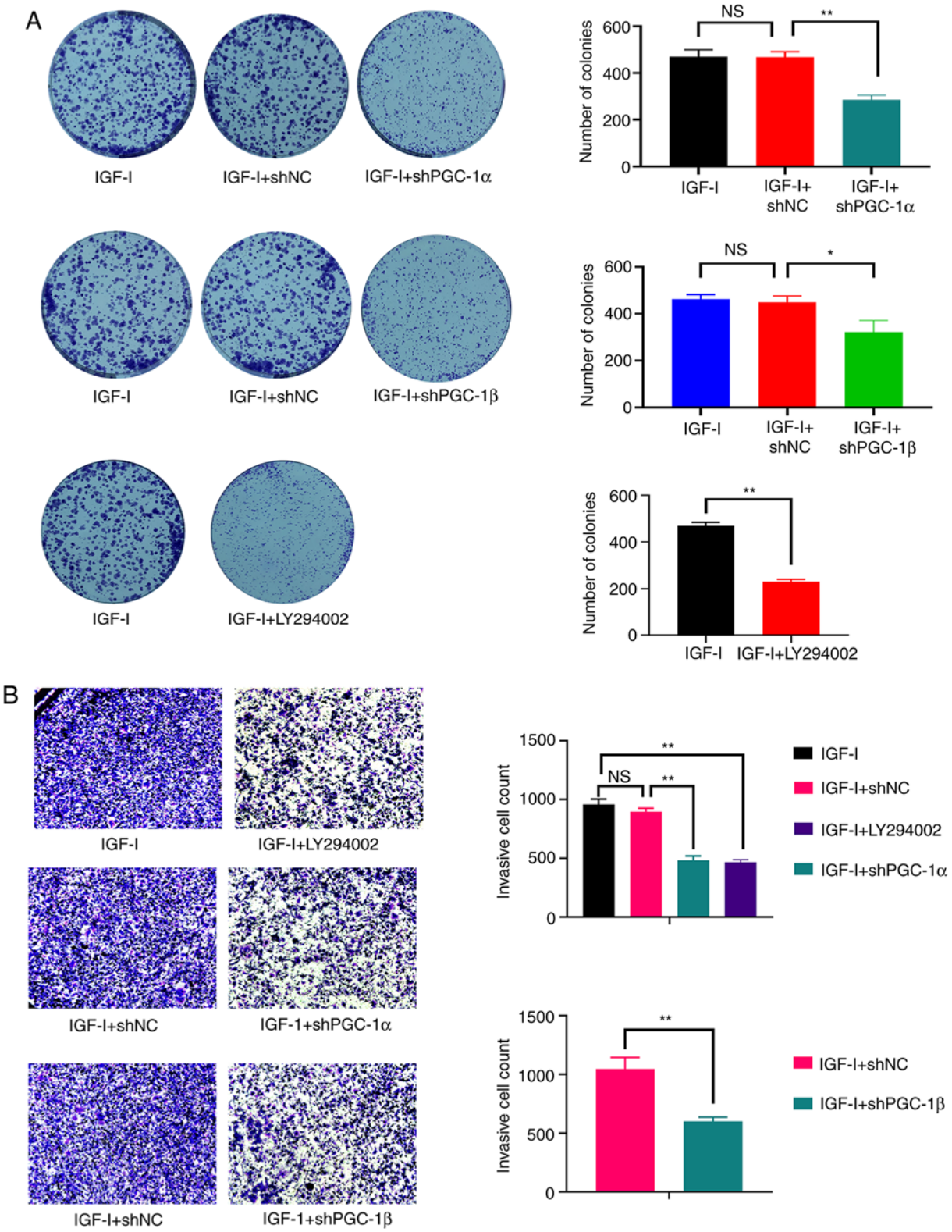

Figure 6. LY294002 treatment and the knockdown of PGC-1α and PGC-1β antagonize the effect of IGF-I on NOZ cell colony formation and invasion. (A) The cell colony formation ability elevated by IGF-I was significantly decreased by LY294002 and the knockdown of PGC-1α and PGC-1β. (B) The elevated invasive capacity of NOZ cells by IGF-I was antagonized by LY294002 and the knockdown of PGC-1α and PGC-1β. Magnification, x20. All of the experiments were conducted in triplicate. *P<0.05, **P<0.01. IGF-I, insulin-like growth factor I; PGC1-1, peroxisome proliferator-activated receptor-γ coactivator-1; NC, negative control; sh, short hairpin RNA; NS, not significant.

target genes by promoting the mono-ubiquitylation of ERRα. However, additional, novel pathogenesis mechanisms are yet to be elucidated.

The primary aim of the present study was to validate whether PI3K/AKT phosphorylation affects and regulates ERRα activity in GBC cells to form a positive feedback loop.

To that end, IGF-I and LY294002 were used to enhance and inhibit PI3K/AKT phosphorylation in NOZ cells, respectively. The present results demonstrated that the bioactivity of ERRα was upregulated and downregulated, respectively, and hence a positive feedback loop of ERRα/PI3K/AKT could be established.

The genes in the PI3K/AKT pathway show the highest frequency of aberrant expression in human cancer (17,32). The activated PI3K/AKT pathway functions to enhance the transformation, proliferation and invasion of cancerous cells. Moreover, the aberrant overexpression or activation of PI3K/AKT has been reported in various malignancies, including GBC, and is associated with an improved proliferative capacity and invasive potential of cancerous cells (17). Therefore, the PI3K/AKT signaling pathway is an ideal target to provide a promising approach for the prevention and clinical therapy of cancer cases. The PI3K/AKT signaling pathway exerts an anti-apoptotic effect mainly by influencing a variety of downstream effector molecules, such as CREB regulated transcription coactivator 1, ribosomal protein S6 kinase B1, S6 Rb and eukaryotic translation initiation factor 4E (17,32). At present, the PI3K/AKT signaling pathway and its related genes can be suppressed by applying gene intervention methods or via the treatment of small-molecule compound drugs. Blocking the activation of a variety of downstream anti-apoptotic effector molecules and promoting cell apoptosis are regarded as effective means to treat cancer (33). In the present study, it was found that PI3K/AKT phosphorylation activated ERRα, but does not promote the amplification of ERRα, which indicated that the activity of ERRα depends on the binding state rather than the total amount. The abundant factors in the ERRα/PI3K/AKT circuit are regarded as potential targets for the targeted therapy of GBC. Therefore, a novel combination therapy using the antagonist of ERRα and the inhibitors of PI3K/AKT signaling has a promising prospect to improve the prognosis of patients with GBC.

The present study demonstrated that PGC-1α and PGC-1β were downstream targets of the PI3K/AKT signaling pathway, and that the PGC-1 family acted as the nuclear transcription co-activator that mediates multiple cellular pathways, among which the regulation of metabolism (34) and tissue-specific functions (13,35-37) are most prominent. The PGC-1 family consists of PGC-1α, PGC-1β and PRC (13). The PGC-1 family serves a critical role in the regulation of mitochondrial biogenesis and bioenergetics. Furthermore, PGC-1 co-activators are essential to sustain tumor survival and growth (38). PGC-1α activity is regulated by a number of post-translational modifications, such as methylation, phosphorylation and acetylation (39). PGC-1α and PGC-1β bind to multiple nuclear transcription factors or hormone receptors, including ER, ERR and thyroid hormone receptor. The presence of PGC-1α and PGC-1β is required for the activity of ERRα (36). In NOZ cells, the phosphorylated PI3K/AKT function could elevate the activity of PGC-1α and PGC-1β, and thereby enhance ERRα activity.

In summary, the present study reported the sensitivity and dosage of LY294002 and IGF-I in inhibiting and activating the PI3K/AKT signaling pathway in NOZ cells, respectively. The experimental results of dual luciferase reporter gene assay indicated that ERRα was positively regulated by PI3K/AKT phosphorylation. Furthermore, PGC-1α and PGC-1β were shown to mediate the activation of ERRα stimulated by PI3K/AKT phosphorylation. Thus, the combined inhibition of multiple targets in the positive feedback loop of ERRα/PI3K/AKT may present significant potential to provide promising anti-cancer solutions.

Acknowledgements

The authors would like to thank Dr Chingyi Chang at Duke University for providing guidance in designing 3X ERE-TATA-luc and PGC-1α-2x9.

Funding

This work was supported by the following Funds: Natural Science Foundation of Jiangsu Province (grant no. BK20181129), The Science Foundation of Health Commission of Wuxi (grant no. Q201714) and The Project of Public Health Research Center at Jiangnan University (grant no. JUPH201829).

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

LW, MY and HJ designed the study, analyzed the data, performed the experiments and wrote the manuscript. LW and HJ performed the critical revision of the manuscript and supervised the study. All authors read and approved the final manuscript. LW and HJ confirm the authenticity of all the raw data.

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.

References

1. Ouyang G, Liu Q, Wu Y, Liu Z, Lu W, Li S, Pan G and Chen X: The global, regional, and national burden of gallbladder and biliary tract cancer and its attributable risk factors in 195 countries and territories, 1990 to 2017: A systematic analysis for the Global Burden of Disease Study 2017. Cancer 127: 2238-2250, 2021.
2. Baiu I and Visser B: Gallbladder cancer. JAMA 320: 1294, 2018.
3. Zhang X, Kong Z, Xu X, Yun X, Chao J, Ding D, Li T, Gao Y, Guan N, Zhu C and Qin X: ARRB1 drives gallbladder cancer progression by facilitating TAK1/MAPK signaling activation. J Cancer 12: 1926-1935, 2021.
4. Sharma A, Sharma KL, Gupta A, Yadav A and Kumar A: Gallbladder cancer epidemiology, pathogenesis and molecular genetics: Recent update. World J Gastroenterol 23: 3978-3998, 2017.

5. Hu YP, Jin YP, Wu XS, Yang Y, Li YS, Li HF, Xiang SS, Song XL, Jiang L, Zhang YJ, *et al*: LncRNA-HGBC stabilized by HuR promotes gallbladder cancer progression by regulating miR-502-3p/SET/AKT axis. Mol Cancer 18: 167, 2019.
6. Kim M, Kim H, Han Y, Sohn H, Kang JS, Kwon W and Jang JY: Prognostic value of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) in gallbladder cancer; 65 IU/ml of CA 19-9 is the new cut-off value for prognosis. Cancers (Basel) 13: 1089, 2021.
7. Li M, Liu F, Zhang F, Zhou W, Jiang X, Yang Y, Qu K, Wang Y, Ma Q, Wang T, *et al*: Genomic ERBB2/ERBB3 mutations promote PD-L1-mediated immune escape in gallbladder cancer: A whole-exome sequencing analysis. Gut 68: 1024-1033, 2019.
8. Chen X, Wu X, Wu H, Gu Y, Shao Y, Shao Q, Zhu F, Li X, Qian X, Hu J, *et al*: Camrelizumab plus gemcitabine and oxaliplatin (GEMOX) in patients with advanced biliary tract cancer: A single-arm, open-label, phase II trial. J Immunother Cancer 8: e001240, 2020.
9. Giguère V, Yang N, Segui P and Evans RM: Identification of a new class of steroid hormone receptors. Nature 331: 91-94, 1988.
10. Deblois G and Giguère V: Functional and physiological genomics of estrogen-related receptors (ERRs) in health and disease. Biochim Biophys Acta 1812: 1032-1040, 2011.
11. Kim SY, Yang CS, Lee HM, Kim JK, Kim YS, Kim YR, Kim JS, Kim TS, Yuk JM, Dufour CR, *et al*: ESRRA (estrogen-related receptor α) is a key coordinator of transcriptional and post-translational activation of autophagy to promote innate host defense. Autophagy 14: 152-168, 2018.
12. Deblois G, Hall JA, Perry MC, Laganière J, Ghahremani M, Park M, Hallett M and Giguère V: Genome-wide identification of direct target genes implicates estrogen-related receptor alpha as a determinant of breast cancer heterogeneity. Cancer Res 69: 6149-6157, 2009.
13. Luo C, Widlund HR and Puigserver P: PGC-1 coactivators: Shepherding the mitochondrial biogenesis of tumors. Trends Cancer 2: 619-631, 2016.
14. Huss JM, Kopp RP and Kelly DP: Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. J Biol Chem 277: 40265-40274, 2002.
15. Yang Y, Li S, Li B, Li Y, Xia K, Aman S, Yang Y, Ahmad B, Zhao B and Wu H: FBXL10 promotes ERRα protein stability and proliferation of breast cancer cells by enhancing the mono-ubiquitylation of ERRα. Cancer Lett 502: 108-119, 2021.
16. Gaillard S, Grasfeder LL, Haeffele CL, Lobenhofer EK, Chu TM, Wolfinger R, Kazmin D, Koves TR, Muoio DM, Chang CY, *et al*: Receptor-selective coactivators as tools to define the biology of specific receptor-coactivator pairs. Mol Cell 24: 797-803, 2006.
17. Mayer IA and Arteaga CL: The PI3K/AKT pathway as a target for cancer treatment. Annu Rev Med 67: 11-28, 2016.
18. Yang F, Xie HY, Yang LF, Zhang L, Zhang FL, Liu HY, Li DQ and Shao ZM: Stabilization of MORC2 by estrogen and antiestrogens through GPER1-PRKACA-CMA pathway contributes to estrogen-induced proliferation and endocrine resistance of breast cancer cells. Autophagy 16: 1061-1076, 2020.
19. Xia P, Gütl D, Zheden V and Heisenberg CP: Lateral inhibition in cell specification mediated by mechanical signals modulating TAZ activity. Cell 176: 1379-1392.e14, 2019.
20. Lu Y, Tao F, Zhou MT and Tang KF: The signaling pathways that mediate the anti-cancer effects of caloric restriction. Pharmacol Res 141: 512-520, 2019.
21. Girnita L, Worrall C, Takahashi S, Seregard S and Girnita A: Something old, something new and something borrowed: Emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation. Cell Mol Life Sci 71: 2403-2427, 2014.
22. LeRoith D, Werner H, Beitner-Johnson D and Roberts CT Jr: Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16: 143-163, 1995.
23. Myers MG Jr, Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B and White MF: IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc Natl Acad Sci USA 89: 10350-10354, 1992.
24. Vanhaesebroeck B and Alessi DR: The PI3K-PDK1 connection: More than just a road to PKB. Biochem J 346: 561-576, 2000.
25. Wang L, Yang M, Guo X, Yang Z, Liu S, Ji Y and Jin H: Estrogen-related receptor-α promotes gallbladder cancer development by enhancing the transcription of Nectin-4. Cancer Sci 111: 1514-1527, 2020.
26. Zhang Y, Liu S, Wang L, Wu Y, Hao J, Wang Z, Lu W, Wang XA, Zhang F, Cao Y, *et al*: A novel PI3K/AKT signaling axis mediates Nectin-4-induced gallbladder cancer cell proliferation, metastasis and tumor growth. Cancer Lett 375: 179-189, 2016.
27. Chang CY, Kazmin D, Jasper JS, Kunder R, Zuercher WJ and McDonnell DP: The metabolic regulator ERRα, a downstream target of HER2/IGF-1R, as a therapeutic target in breast cancer. Cancer Cell 20: 500-510, 2011.
28. Theodoris CV, Zhou P, Liu L, Zhang Y, Nishino T, Huang Y, Kostina A, Ranade SS, Gifford CA, Uspenskiy V, *et al*: Network-based screen in iPSC-derived cells reveals therapeutic candidate for heart valve disease. Science 371: eabd0724, 2021.
29. Ramaswamy A, Ostwal V, Sharma A, Bhargava P, Srinivas S, Goel M, Patkar S, Mandavkar S, Jadhav P, Parulekar M, *et al*: Efficacy of capecitabine plus irinotecan vs irinotecan monotherapy as second-line treatment in patients with advanced gallbladder cancer: A multicenter phase 2 randomized clinical trial (GB-SELECT). JAMA Oncol 7: 436-439, 2021.
30. Nepal C, Zhu B, O'Rourke CJ, Bhatt DK, Lee D, Song L, Wang D, Van Dyke A, Choo-Wosoba H, Liu Z, *et al*: Integrative molecular characterization of gallbladder cancer reveals microenvironment-associated subtypes. J Hepatol 74: 1132-1144, 2021.
31. Ranhotra HS: Estrogen-related receptor alpha and cancer: Axis of evil. J Recept Signal Transduct Res 35: 505-508, 2015.
32. Mercurio L, Albanesi C and Madonna S: Recent updates on the involvement of PI3K/AKT/mTOR molecular cascade in the pathogenesis of hyperproliferative skin disorders. Front Med (Lausanne) 8: 665647, 2021.
33. Song M, Bode AM, Dong Z and Lee MH: AKT as a therapeutic target for cancer. Cancer Res 79: 1019-1031, 2019.
34. Villena JA: New insights into PGC-1 coactivators: Redefining their role in the regulation of mitochondrial function and beyond. FEBS J 282: 647-672, 2015.
35. Lin J, Handschin C and Spiegelman BM: Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab 1: 361-370, 2005.
36. Patten IS and Arany Z: PGC-1 coactivators in the cardiovascular system. Trends Endocrinol Metab 23: 90-97, 2012.
37. Li S, Liu C, Li N, Hao T, Han T, Hill DE, Vidal M and Lin JD: Genome-wide coactivation analysis of PGC-1alpha identifies BAF60a as a regulator of hepatic lipid metabolism. Cell Metab 8: 105-117, 2008.
38. Vernier M and Giguère V: Aging, senescence and mitochondria: The PGC-1/ERR axis. J Mol Endocrinol 66: R1-R14, 2021.
39. Chambers JM and Wingert RA: PGC-1α in disease: Recent renal insights into a versatile metabolic regulator. Cells 9: 2234, 2020.