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17β-Estradiol on the Expression of G-Protein Coupled Estrogen Receptor (GPER/GPR30) Mitophagy, and the PI3K/Akt Signaling Pathway in ATDC5 Chondrocytes *In Vitro*

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Background: Osteoarthritis is a progressive inflammatory joint disease resulting in damage to articular cartilage. G-protein coupled estrogen receptor (GPER/GPR30) activates cell signaling in response to 17β-estradiol, which can be blocked by the GPR30 agonist, G15, an analog of G-1. The aims of this study were to investigate the effects of 17β-estradiol on the expression of G-protein coupled estrogen receptor (GPER/GPR30) on mitophagy and the PI3K/Akt signaling pathway in ATDC5 chondrocytes *in vitro*.

Material/Methods: Cultured ATDC5 chondrocytes were treated with increasing concentrations of 17β-estradiol with and without G15, p38 inhibitor (SB203580), JNK inhibitor (SP600125), PI3K inhibitor (LY294002, S1737), and mTOR inhibitor (S1842). Expression of GPER/GPR30 and components of the PI3K/Akt pathway in cultured ATDC5 chondrocytes were detected by immunofluorescence (IF) staining, Western blot, and real-time polymerase chain reaction (RT-PCR). Transmission electron microscopy (TEM) and IF were used to detect mitophagosomes. Expression of LC-3, LAMP2, TOM20, Hsp60, p-Akt, p-mTOR, p-p38, and p-JNK was investigated by Western blot. Proliferation and viability of the ATDC5 chondrocytes were determined using BrdU and MTT assays.

Results: In 17β-estradiol-treated ATDC5 chondrocytes, increased expression of GPER/GPR30 was found, but fewer mitophagosomes were observed, and decreased numbers of TOM20-positive granules were co-localized with decreased LAMP2 and increased expression levels of TOM20, Hsp60, p-Akt, and p-mTOR, and reduced expression of LC3-II, were found. In 17β-estradiol-treated ATDC5 chondrocytes, the proliferation and viability of the 17β-estradiol-treated ATDC5 chondrocytes were significantly elevated.

Conclusions: Treatment with 17β-estradiol protected ATDC5 chondrocytes against mitophagy via the GPER/GPR30 and the PI3K/Akt signaling pathway.

MeSH Keywords: **Chondrocytes • Estradiol • Mitochondrial Degradation • Phosphatidylinositol 3-Kinases**

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Background

The steroid hormone, 17 β -estradiol, plays an important role in mammalian physiology and pathology. Estrogen receptors (ERs) and 17 β -estradiol combine to regulate many physiological functions of the reproductive, cardiovascular, nervous, muscular, skeletal, and endocrine systems [1]. Currently, three main ERs have been identified, ER α [2], ER β [3] and the G-protein coupled estrogen receptor (GPER/GPR30) [4]. The conventional effects of estrogen are mediated by classical nuclear hormone receptors (ER α and ER β), which act as ligand-activated transcription factors that usually take hours to days to mediate genomic signaling [5].

The G-protein coupled estrogen receptor (GPER), GPER/GPR30 is a member of the superfamily of 7-transmembrane GPERs, which has been recognized to mediate rapid cellular responses that occur in seconds to minutes, including the activation of ion channels, kinases, and signaling pathways [6,7]. Recent studies have identified GPER/GPR30 as a critical mediator of cell signaling in response to estrogen. However, these events cannot be explained by the actions of classical nuclear ER α or ER β alone. GPER/GPR30 is widely distributed throughout the body, with the highest expression in the lymphoid system, lungs, heart, placenta, liver, skeletal muscle, kidneys, and chondrocytes of the growth plate [8–10]. ER α and ER β are localized mainly in the cell nucleus but are also found in the mitochondria [11]. The estrogen receptor GPER/GPR30 is mainly localized in the endoplasmic reticulum but is also found in the mitochondria [12].

ER α , ER β , and GPER/GPR30 located in mitochondria are also known as mitochondrial estrogen receptors (mtERs) [12,13]. The mtERs have been detected in many tissues and cell types and may mediate the protective effects of estrogen directly on mitochondria [12,14]. GPER/GPR30 has been shown to play a role in the protective effects of estrogen on neurons, endothelial cells, and C2C12 murine skeletal myoblasts [12,15,16]. In C2C12 murine skeletal myoblasts, ER α or ER β may also act together with GPER/GPR30 to mediate the actions of estrogens. Currently, the role of GPER/GPR30 in the effects of estrogen on chondrocytes remains unclear.

A previously published study has shown that 17 β -estradiol reduces oxidative stress and inflammatory responses by activating the PI3K/Akt signal transduction pathway in the Raw 264.7 macrophage cell line [17]. 17 β -estradiol has been shown to protect osteoblasts against serum deprivation-induced apoptosis by promoting autophagy through the ER-ERK-mTOR pathway [18]. Also, 17 β -estradiol has been shown to be a potential treatment for spinal cord injury due to its ability to stimulate early astroglial responses and cytokine release, which reduce the activation of calpain, a family of Ca²⁺-activated neutral

cysteine endoproteases, and inhibition of cell apoptosis [19,20]. Recent studies have shown that estrogen has a potentially protective effect on chondrocytes and might have benefits in the treatment of osteoarthritis [21–23]. However, the molecular mechanisms of estrogen-mediated protection of chondrocytes remain unclear.

Autophagy is a process of degradation of cytoplasmic macromolecules and organelles and is a biological phenomenon found in eukaryote cells [24]. Autophagy is highly regulated to maintain the balance between the synthesis, degradation, and subsequent recycling of cell products that are essential for cell growth, survival, differentiation, development, and homeostasis. Under normal conditions, autophagy occurs at a low or basal rate in cells but can be upregulated in response to environmental stress and signaling stimuli, such as cell starvation, amino acid depletion, oxidative stress, cell proliferation, hypoxia, and by several pharmacological agents [25,26]. Furthermore, autophagy plays a significant role in many human diseases, including cancer [27], infectious diseases [28], cartilage injury and osteoarthritis [29], and pulmonary disease [30]. The regulation of the activity of autophagy is closely associated with tumor formation, progression, and response to therapy [31]. In cancer cells, chemical inhibitors of autophagy enhance apoptosis via active-site mTOR inhibitors or dual PI3K/mTOR inhibitors, which suggests that the PI3K/Akt pathway is involved in the process of autophagy [32].

Mitochondria drive the major metabolic pathways of the cell. Mitophagy is a form of selective autophagy that is involved in the removal of dysfunctional mitochondria through degradation in the autophagy-lysosomal system [33]. Microtubule-associated protein-1 light chain-3 (LC3) is the mammalian ortholog of the yeast autophagy-associated protein, ATG8. During the process of autophagy, cytosolic LC3 (LC3-I) binds phosphatidylethanolamine to form an LC3-phosphatidylethanolamine conjugate (LC3-II), which is a good index of the process of autophagy [34]. TOM 20 is a subunit of the translocase of the outer membrane (TOM) complex that is responsible for recognizing early mitochondrial sequences and is considered to be a potential marker of mitophagy [35]. Also, some specific antioxidant enzymes that are located in the mitochondria, including heat shock protein 60 (HSP60), peroxiredoxin 3 (Prx3), and thioredoxin 2 (Trx2) protect mitochondria against reactive oxygen species (ROS)-induced damage by catalyzing the reduction of H₂O₂ to H₂O [36–38].

Recent studies have shown that 17 β -estradiol is not only associated with oxidative stress, the inflammatory response, and apoptosis but is also associated with autophagy. The neuroprotective effects of 17 β -estradiol have been shown to be related to the suppression of excessive autophagy in a rat spinal cord injury model, indicating that the protective effect of estrogen

against spinal cord injury occurs through GPER/GPR30 and not through nuclear ERs [39]. G15 is an analog of G-1 that binds to GPR30 with high affinity but has no affinity for ER α and ER β . It has been shown that when G15 was administered, the effects of GPER/GPR30 of estrogen on glomerular endothelial cells were reversed [40]. Estradiol has been shown to modify the PI3K/Akt-mTOR signaling pathway and to regulate autophagy, reducing the extent of damage following spinal cord injury [41]. LY294002 is a PI3K inhibitor, which is a morpholino-derivative of quercetin. Rapamycin is a specific mTOR antagonist. LY294002 and rapamycin have been used in several studies to provide evidence for the involvement of the PI3K-mTOR pathway in various biological systems [42,43]. SB203580 is a p38 mitogen-activated protein kinase inhibitor, and SP600125 is a broad-spectrum JNK inhibitor for JNK1, JNK2, and JNK3, which have been used to block the downstream signaling elements of p38 and JNK in the PI3K-mTOR pathway [44].

Currently, the molecular mechanisms by which estrogen protects chondrocytes and might alleviate osteoarthritis remain unclear. The aims of this study were to investigate the effects of 17 β -estradiol on the expression of GPER/GPR30 on mitophagy and the PI3K/Akt signaling pathway in ATDC5 chondrocytes *in vitro*. The findings of this study might provide insight into estradiol as a potential treatment for osteoarthritis.

Material and Methods

Reagents and antibodies

The 17 β -estradiol was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM)/F12 containing L-glutamine and HEPES, and fetal bovine serum (FBS) were obtained from HyClone (Logan, UT, USA). The selective the G-protein coupled estrogen receptor (GPER/GPR30) antagonist, G15, was purchased from Tocris Bioscience (Minneapolis, MA, USA). The p38 inhibitor (SB203580), JNK inhibitor (SP600125), PI3K inhibitor (LY294002, S1737), mTOR inhibitor (S1842) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Beyotime Biotechnology (Jiangsu, China). The GPER/GPR30 polyclonal antibody (sc-48525-R) used at a dilution of 1: 200 for Western Blot (WB) and at a dilution of 1: 50 for immunofluorescence (IF), the Lamp2 polyclonal antibody (sc-8100) (IF 1: 50), the TOM20 polyclonal antibody (sc-11415) (WB 1: 200, IF 1: 50), the LC3 monoclonal antibody (sc-376404) (WB 1: 100), and the Hsp60 polyclonal antibody (sc-1052) (WB 1: 200) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The total/phosphor-Akt polyclonal antibody and total/phosphor-p38 antibody were purchased from Abcam (Cambridge, MA, USA). The total/phosphor-mTOR antibody was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The total/phosphor-JNK antibody was purchased from

ImmunoWay (Newark, DE, USA). The β -actin monoclonal antibody (AF0003) (1: 1000) was purchased from Biosynthesis Biotechnology (Beijing, China). The IF staining kit with Alexa Fluor 555-labeled donkey anti-rabbit IgG (P0179) was purchased from Beyotime Biotechnology (Jiangsu, China). IFKine[®] green-conjugated donkey anti-goat IgG (A24231) was obtained from Abbkine Scientific Co., Ltd. (California, USA). The Cell Proliferation ELISA bromodeoxyuridine (BrdU) colorimetric kit was purchased from Roche Diagnostics (Penzberg, Germany).

Cell culture

The mouse chondroprogenitor cell line ATDC5 was purchased from Kebai (Nanjing, China). The ATDC5 cells were cultured in DMEM/F12 medium supplemented with 5% FBS. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. The cell culture medium was replaced every two days. Before the addition of 17 β -estradiol, the cells were transferred into serum-free medium in Petri dishes and incubated for 24 hours, and then treated with increasing concentrations of 17 β -estradiol (0 μ M, 10⁻³ μ M, 10⁻² μ M and 10⁻¹ μ M) with or without the addition of 15 μ M G15 or 20 μ M of LY294002, and incubated for a further 24 hours.

Real-time polymerase chain reaction (RT-PCR)

For the analysis of the expression levels of mRNA coding for GPER/GPR30, total RNA was extracted from the ATDC5 chondrocytes using TRIzol reagent (Life Technologies Co., Carlsbad, CA, USA). Real-time polymerase chain reaction (RT-PCR) was performed to detect the expression of GPER/GPR30 mRNA using TaqMan reagents (Takara, Otsu, Japan). The following specific primers were used: GPER/GPR30 forward, 5'-AACAGAGCAGCGATCTGGAC-3' and GPER/GPR30 reverse, 5'-GCAGAGTCCCTGGATGGCTT-3'.

The data were normalized based on the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which is an internal control for reverse transcription (RT) and reaction efficiency. The following specific primers were used: GAPDH forward, 5'-GGAAGGGCTCATGACCACAGT-3', and GAPDH reverse, 5'-GGAAGGCCATGCCAGTGA-3'. The reactions were amplified at 95°C for 30 s followed by 40 cycles of 95°C for 5 s followed by 60°C for 30 s. All primers and TaqMan probes specific to GPER/GPR30 and GAPDH were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA).

Immunofluorescence (IF) analysis

The ATDC5 chondrocytes were cultured in 24-well plates. After incubation, the cells were fixed with 4% paraformaldehyde buffered with 0.1 M phosphate (pH 7.3) for 30 min. The fixed

samples were then washed with phosphate-buffered saline (PBS) for 10 min. The cells were treated with 0.1% TritonX-100 for 30 min to permeabilize the cell membrane and then washed with PBS for 10 min. The samples were blocked with 5% bovine serum albumin (BSA) in TBST for 30 min and then incubated overnight at 4°C with the following specific primary antibodies: goat anti-Lamp2 (1: 50), rabbit anti-TOM20 (1: 50), and rabbit anti-GPER/GPR30 antibody (1: 50). Then, the ATDC5 chondrocytes were washed with PBS for 10 min and incubated for 30 min with the following secondary antibodies: donkey anti-goat IgG (1: 1000) and donkey anti-rabbit IgG (1: 1000). The cells were stained with DAPI for 5 min, washed with PBS for 15 min and observed with an Olympus FV1000 confocal laser-scanning microscope with the peak emission wavelengths of 518 nm (green) and 565 nm (red).

Protein preparation and Western blot analysis

The cultured ATDC5 chondrocytes were washed with PBS, and the total protein was harvested in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China) supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Beijing, Jiangsu, China) and phosphatase inhibitors (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The protein concentration was determined with a Bicinchoninic Acid (BCA) Protein Assay Kit, according to the manufacturer's instructions (Beyotime, China). The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using a transfer buffer at 70 V for 1.5 hours. To block nonspecific binding, the membranes were incubated in dried skimmed milk powder for 120 min at room temperature and were washed three times with Tris-buffered saline (TBS) containing Tween 20 (TBST) for 30 min. The membranes were incubated overnight at 4°C with the primary antibodies and then washed with TBST for 30 min and incubated with horseradish peroxidase (HRP)-conjugated anti-species secondary antibody (Beyotime, China) for 2 hours at room temperature. After the membranes were washed three times with TBST for 30 min, the proteins were visualized with the BeyoECL plus kit (Beyotime, China).

Transmission electron microscopy (TEM) analysis

The cells were trypsinized and collected by centrifugation at 1,000 \times g for 5 min. The cells were fixed in 2.5% phosphate-buffered glutaraldehyde and post-fixed in 1% osmium tetroxide in water. After being subjected to gradient acetone dehydration, the cells were embedded and sectioned. The samples were double-stained with uranyl acetate and lead citrate, and the ultrastructure of the cells was observed with a JEM-1200EX transmission electron microscope (TEM) (Tokyo, Japan).

Cell proliferation assay

ATDC5 chondrocytes were seeded in 96-well plates (2 \times 10³ cells/well) and cultured for 24 hours. The cells were serum-deprived for 24 hours and then treated with 17 β -estradiol (0 or 10⁻¹ μ M), with or without G15 and PI3Ki for a further 24 hours. Then, the BrdU solution (1 μ M) was added, and the cells were incubated for 2.5 hours. BrdU incorporation into the DNA was measured using the Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics).

Cell viability

ATDC5 chondrocytes were seeded in 96-well plates (2 \times 10³ cells/well) and cultured for 24 hours. After incubation in serum-free DMEM/F12 medium supplemented with 17 β -estradiol (10⁻¹ μ M) at 37°C for 24 hours, 10 μ l of MTT reagent (5 mg/ml) was added, and the cells were incubated at 37°C for 4 hours. Then, the culture medium was removed, and formazan crystals were dissolved with 100 μ l of dimethyl sulfoxide (DMSO). All experiments were performed in triplicate. The optical density (OD) was measured using a Spectra Max Plus 384 Microplate Reader (Molecular Devices, Ismaning, Germany) at 570 nm (reference wavelength).

Statistical analysis

The data were presented as the mean \pm standard deviations (SD). Normality was assessed with the Shapiro-Wilk test, and the homoscedastic sequence of random variables was assessed using Bartlett's method (also known as the method of averaged periodograms). The data that passed the homogeneity test were analyzed with the Student's t-test or one-way analyses of variance (ANOVA), followed by the least significant difference (LSD) test. The statistical analysis was performed using SPSS version 15.0 (SPSS, Inc., IL, USA). $P < 0.05$ was considered statistically significant.

Results

G-protein coupled estrogen receptor (GPER/GPR30) expression in ATDC5 chondrocytes was stimulated by 17 β -estradiol

Positive expression of GPER/GPR30 in the ATDC5 cells was detected by real-time polymerase chain reaction (RT-PCR) (Figure 1A), Western blot (Figure 1B, 1C), and immunofluorescence (IF) staining (Figure 1D).

In this study, serum-starved ATDC5 chondrocytes were treated with different concentrations of 17 β -estradiol (0 μ M, 10⁻³ μ M, 10⁻² μ M and 10⁻¹ μ M, with or without 15 μ M G15) for 24

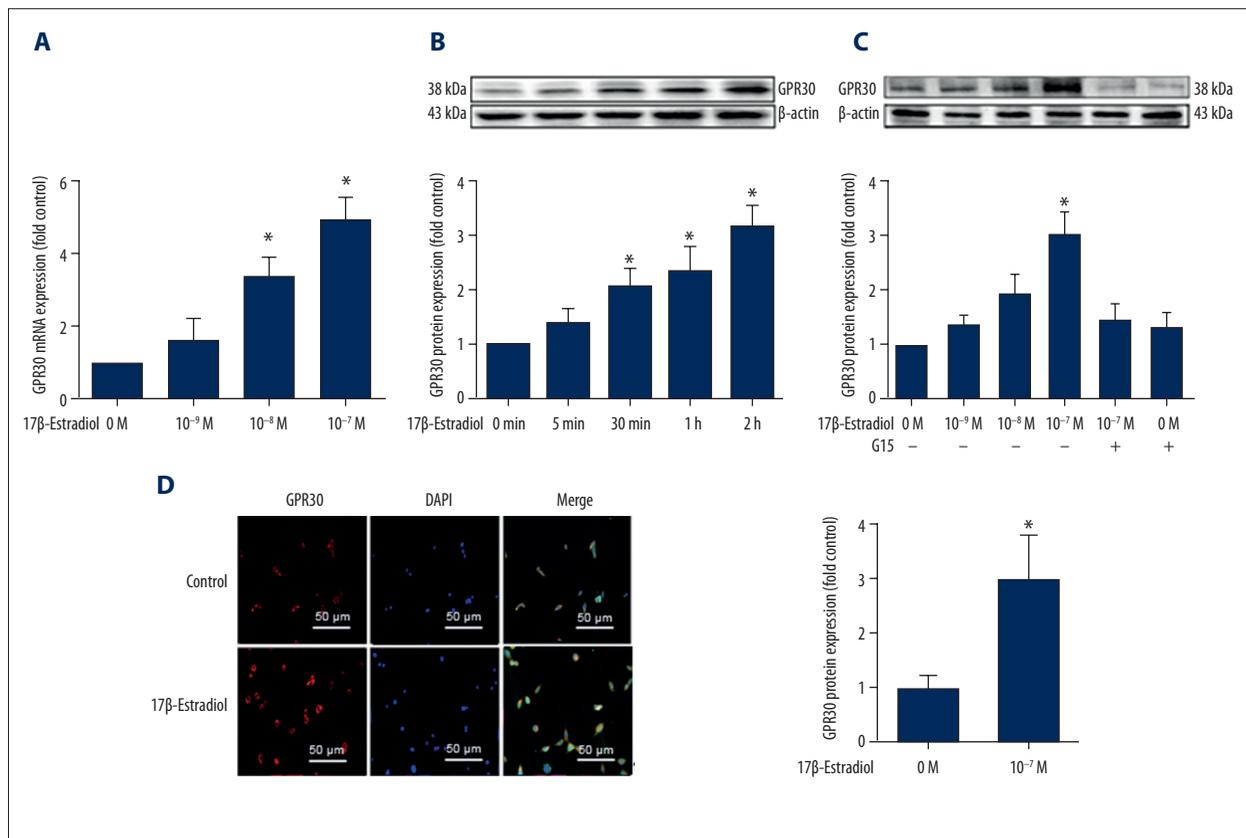


Figure 1. Expression of the G-protein coupled estrogen receptor (GPER/GPR30) stimulated by 17 β -estradiol in serum-starved ATDC5 chondrocytes. **(A)** Expression of GPER/GPR30 mRNA in serum-starved ATDC5 chondrocytes treated with increasing concentrations of 17 β -estradiol (0 μ M, 10⁻³ μ M, 10⁻² μ M, and 10⁻¹ μ M), as assessed with quantitative real-time polymerase chain reaction (RT-PCR). **(B)** Expression of GPER/GPR30 protein in ATDC5 chondrocytes treated with 17 β -estradiol (0 μ M, 10⁻³ μ M, 10⁻² μ M, or 10⁻¹ μ M) for 0, 5, 30, 60, or 120 min, as assessed with Western blot. **(C)** Expressions of GPER/GPR30 protein in serum-starved ATDC5 chondrocytes treated with increasing concentrations of 17 β -estradiol (0 μ M, 10⁻³ μ M, 10⁻² μ M, and 10⁻¹ μ M) with or without 15 μ M of G15, as assessed by Western blot. **(D)** Confocal immunofluorescence (IF) staining of ATDC5 chondrocytes for GPER/GPR30 (red). The cell nuclei are stained with 4', 6-diamidino-2-phenylindole (DAPI) (blue). The intensity of the GPER/GPR30 staining is increased in the serum-starved ATDC5 cells that were cultured in serum-free medium with 17 β -estradiol ($P < 0.05$). The figure data represent experiments performed in triplicate. The asterisks indicate significant differences compared with the control (* $P < 0.05$).

hours. G15 is a high-affinity and selective GPER antagonist. The data from this study indicated that 17 β -estradiol could increase GPER/GPR30 mRNA and protein levels in a dose-dependent manner up to the highest dose of 10⁻¹ μ M ($P < 0.05$) (Figure 1A, 1C). Also, the level of expression of GPER/GPR30 protein increased in a time-dependent manner.

Western blot analysis showed that estradiol significantly increased the protein expression over time (as assessed at 0 min, 5 min, 30 min, 1 hour, and 2 hours) (Figure 1B). Western blot showed that the GPER protein was expressed at a higher level in the serum-deprived 17 β -estradiol-treated ATDC5 chondrocytes compared with cells that were treated with 17 β -estradiol plus the GPER/GPR30-specific antagonist G15 (15 μ M) (Figure 1C). Also, immunofluorescence (IF) staining showed that, compared with the control group, 17 β -estradiol increased

the expression of GPER/GPR30 in the cell membrane and the cytoplasm ($P < 0.05$) (Figure 1D).

Inhibition of mitophagy in ATDC5 cells was involved in the function of 17 β -estradiol via GPER/GPR30

Mitophagy was visualized based on mitophagosomes or autophagosomes seen on transmission electron microscopy (TEM) and the co-localization of mitochondria with mitophagic proteins and lysosomes on IF staining. In this study, the prominent features of the mitophagosomes and autophagosomes were analyzed by TEM (Figure 2). Compared with the control group, fewer mitophagosomes were observed in the 17 β -estradiol-treated ATDC5 cells, which indicated that 17 β -estradiol inhibited mitophagy in the ATDC5 cells. Also, a significantly increased number of double membrane vacuoles were seen in

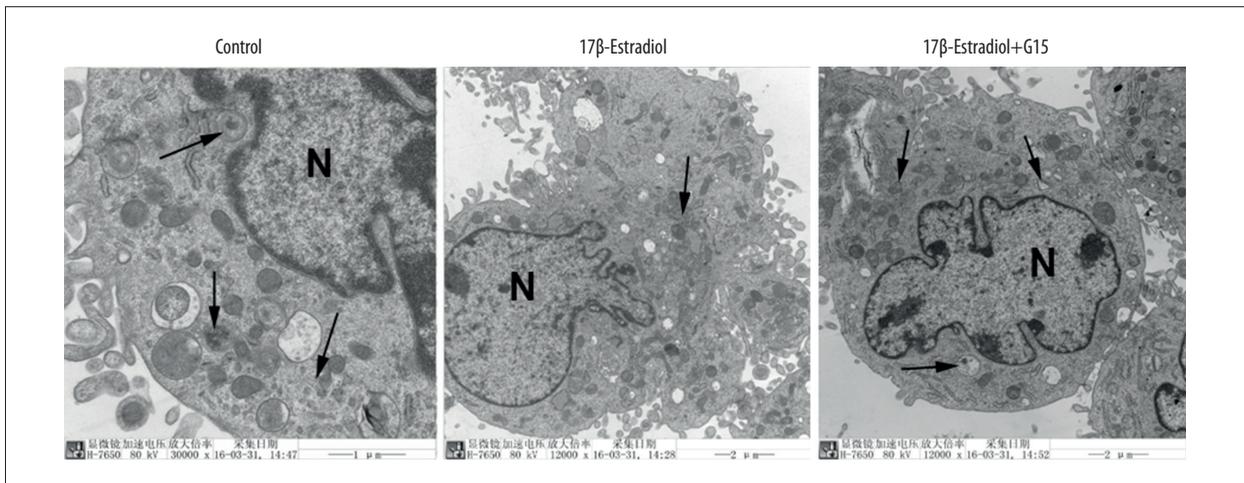


Figure 2. Transmission electron microscopy (TEM) showing mitophagosomes in the ATDC5 cells. Double membrane vacuoles (mitophagosomes) in the ATDC5 cells that were treated with 17 β -estradiol (0 μ M and 10⁻¹ μ M) with or without 15 μ M of G15 seen by transmission electron microscopy (TEM). N represents the nucleus. The vacuoles are indicated with arrows. Black bar=5 μ m.

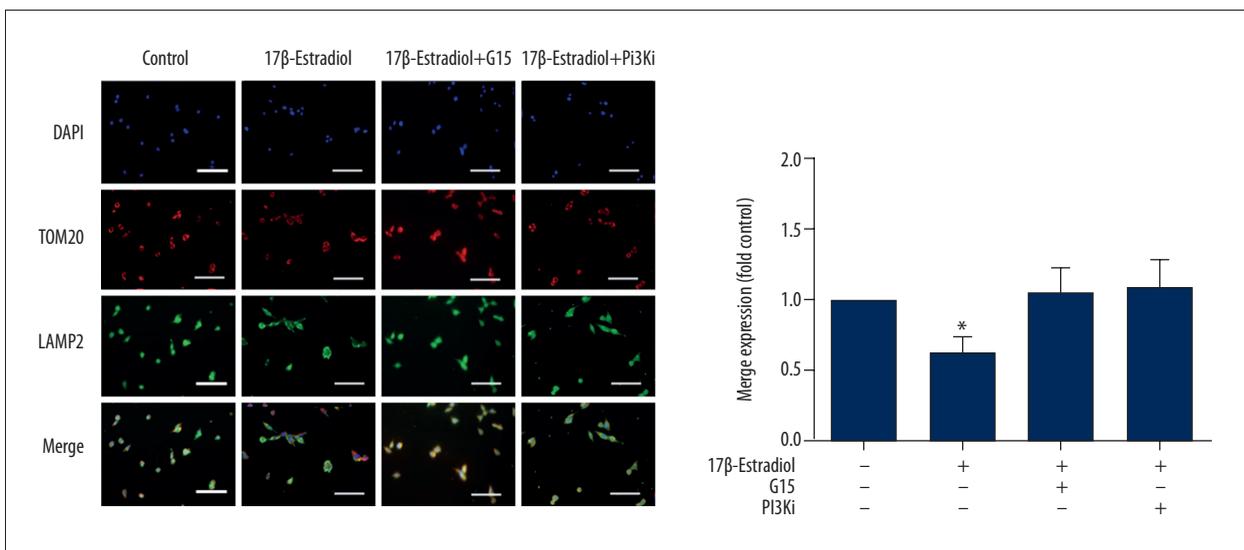


Figure 3. Confocal immunofluorescence (IF) staining for TOM20 and Lamp2 of ATDC5 cells. Confocal immunofluorescence staining for TOM20 (red) and Lamp2 (green). The cell nuclei are stained blue with 4,6-diamidino-2-phenylindole (DAPI). Serum-free ATDC5 cells were treated with 17 β -estradiol (0 μ M and 10⁻¹ μ M) with or without 15 μ M of G15 and 20 μ M of PI3K inhibitor. The staining intensity is lower in the serum-free 17 β -estradiol-treated ATDC5 cell group compared with the other groups ($P < 0.05$). White bar=50 μ m.

the ATDC5 cells that were treated with 17 β -estradiol and G15 (which inhibits binding to estrogen receptors) compared with the cells that were treated with 17 β -estradiol alone (Figure 2), which indicated that GPER was involved in mitophagy.

The co-localization of TOM20 with Lamp2 was measured with IF staining. The results indicated that decreased numbers of TOM20-positive granules were co-localized with the decreased Lamp2 in the ATDC5 cells that were exposed to 17 β -estradiol, which indicates that the number of mitophagosomes or

autophagosomes decreased (Figure 3). However, these effects of 17 β -estradiol were abolished by the presence of the inhibitors G15 or LY294002 (Figure 3).

17 β -Estradiol inhibited mitophagy by regulating the phosphorylation of LC3, TOM20, and Hsp60 in ATDC5 cells

To understand the effect of 17 β -estradiol on mitophagy protein activity, the expression of LC3 (LC3-I and LC3-II), which are typical markers of completed autophagy, TOM20, and Hsp60

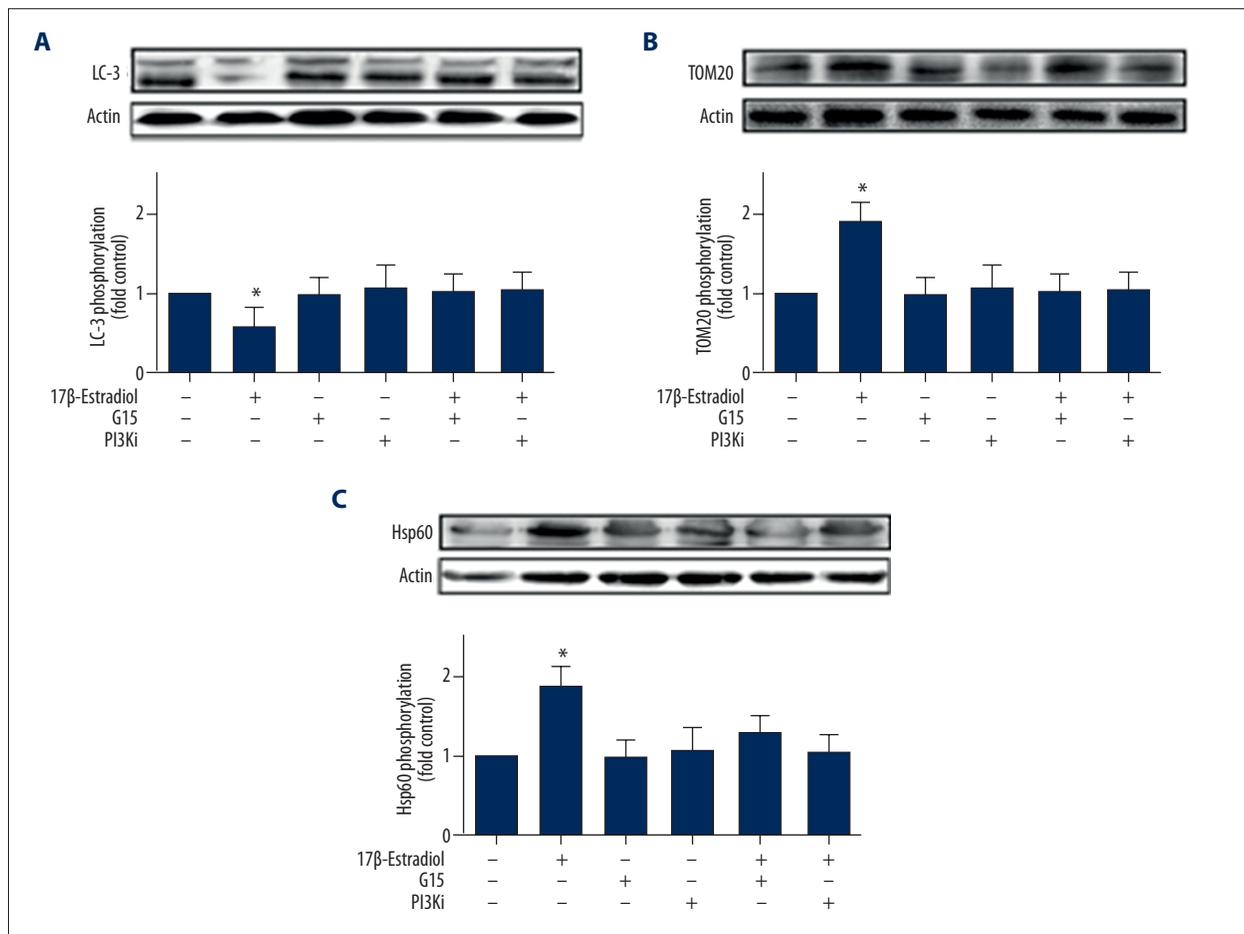


Figure 4. Western blot showing expression of the LC3, TOM20, and Hsp60 proteins in ATDC5 cells treated with 17β-estradiol with or without G15 or the PI3K inhibitor. **(A)** Western blot shows expression of the LC3 protein in ATDC5 chondrocytes that were treated with 17β-estradiol (0 μM and 10⁻¹ μM) with or without 15 μM of G15 and 20 μM of PI3K inhibitor. **(B)** Western blot shows expression of the TOM20 protein in ATDC5 chondrocytes that were treated with 17β-estradiol (0 μM and 10⁻¹ μM) with or without 15 μM of G15 and 20 μM of PI3K inhibitor. **(C)** Western blot shows expression of the Hsp60 protein in ATDC5 chondrocytes that were treated with 17β-estradiol (0 μM and 10⁻¹ μM) with or without 15 μM of G15 and 20 μM of PI3K inhibitor. The figure data represent experiments performed in triplicate. The asterisks indicate significant differences compared with the control (* P<0.05).

proteins were measured by Western blot. The expression level of LC3-II was significantly decreased in the 17β-estradiol-treated ATDC5 cells compared with the control group, and this effect was abolished in the cells that were treated with 17β-estradiol and G15 or LY294002 (Figure 4). The levels of TOM20 and Hsp60 in the group of ATDC5 cells that were exposed to 17β-estradiol were greater compared with those in the control group. Also, these effects were diminished by G15 or LY294002 treatment (Figure 4).

17β-Estradiol protected ATDC5 chondrocytes through the GPER/GPR30/PI3K/Akt pathway

The GPER/GPR30/PI3K/Akt pathway plays important roles in cell proliferation, apoptosis, and autophagy. Akt phosphorylation

has been widely used as an indicator of PI3K/Akt pathway activation. In this study, Western blot showed increased phospho-Akt protein levels in the 17β-estradiol-treated ATDC5 cells, while the level of total Akt expression remained comparable. The opposite results were observed when the cells were treated with G15 (15 μM) or the PI3K inhibitor, LY294002 (20 μM) (Figure 5A), which indicated that 17β-estradiol (10⁻¹ μM) activated the PI3K/Akt pathway via GPER/GPR30 in the ATDC5 cells. Also, the inhibition of phospho-mTOR (p-mTOR) was selected as an indicator of autophagy pathway activation. The data showed that 17β-estradiol (10⁻¹ μM) increased the p-mTOR protein levels in the ATDC5 cells, and the opposite results were found following treatment of the cells with G15 or the mTOR inhibitor, rapamycin (10⁻¹ μM) (Figure 5B), which indicated that 17β-estradiol (10⁻¹ μM) increased the

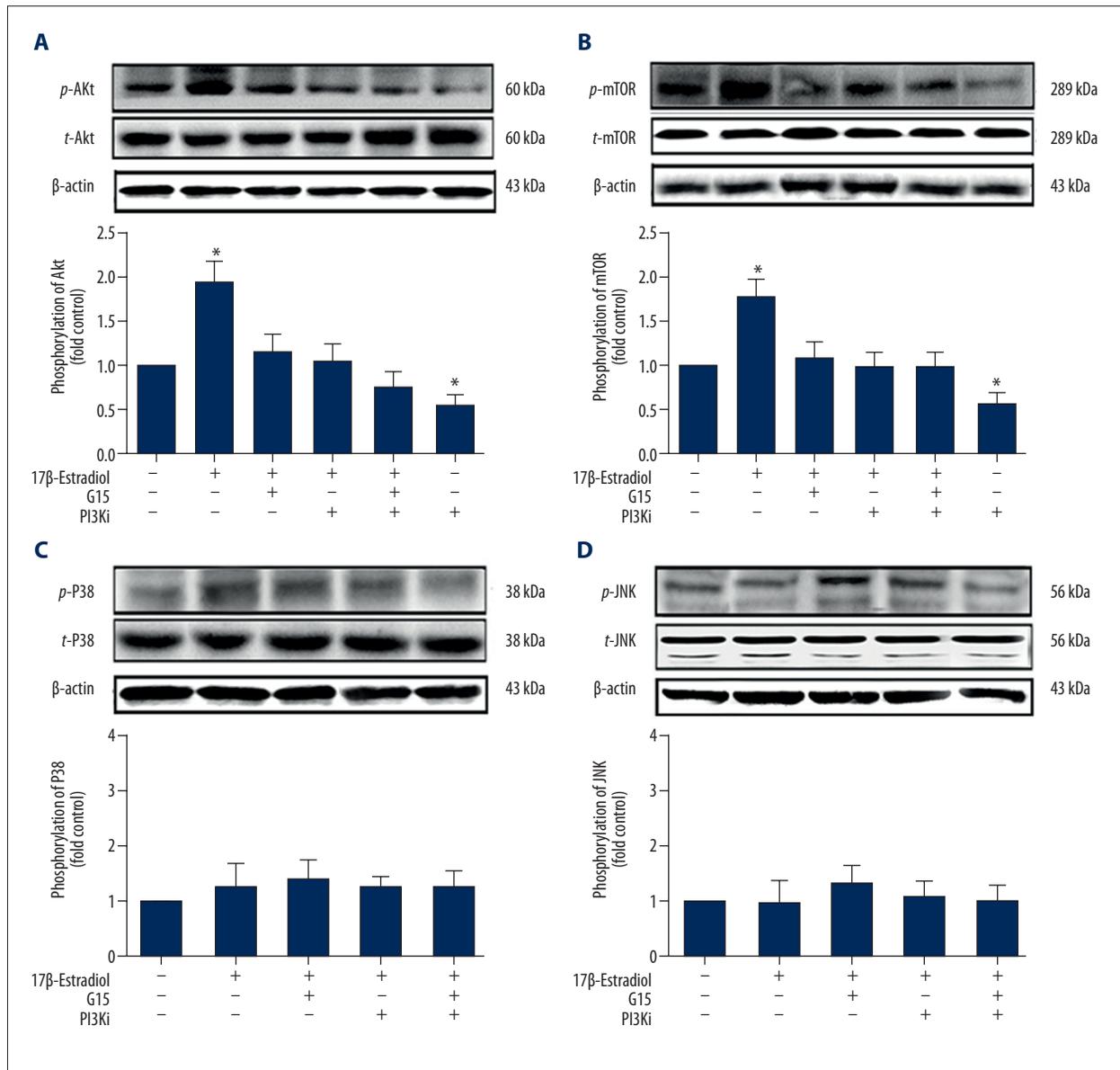


Figure 5. Western blot showing expression of p-Akt, p-mTOR, p-p38, and p-JNK in ATDC5 cells treated with 17 β -estradiol with or without G15, the PI3K inhibitor, the mTOR inhibitor, the p38i inhibitor, and the JNK inhibitor. **(A)** Western blot shows the expression of p-Akt in ATDC5 cells treated with 17 β -estradiol (0 μ M or 10⁻¹ μ M), with or without G15, the PI3K inhibitor, the mTOR inhibitor, the p38i inhibitor, and the JNK inhibitor. **(B)** Western blot shows the expression of p-mTOR in ATDC5 cells treated with 17 β -estradiol (0 μ M or 10⁻¹ μ M), with or without G15, the PI3K inhibitor, the mTOR inhibitor, the p38i inhibitor, and the JNK inhibitor. **(C)** Western blot shows the expression of p-p38 in ATDC5 cells treated with 17 β -estradiol (0 μ M or 10⁻¹ μ M), with or without G15, the PI3K inhibitor, the mTOR inhibitor, the p38i inhibitor, and the JNK inhibitor. **(D)** Western blot shows the expression of p-JNK in ATDC5 cells treated with 17 β -estradiol (0 μ M or 10⁻¹ μ M), with or without G15, the PI3K inhibitor, the mTOR inhibitor, the p38i inhibitor, and the JNK inhibitor. The figure data represent experiments performed in triplicate. The asterisks indicate significant differences compared with the control (* $P < 0.05$).

phosphorylation of mTOR via GPER/GPR30 in the ATDC5 cells and inhibited mitophagy.

Also, the effect of 17 β -estradiol on the ATDC5 cells was abolished by the GPER/GPR30 inhibitor G15 (Figures 2–6), the

PI3K inhibitor LY294002 (Figures 3–6), and by the mTOR inhibitor (Figure 5B), which suggests that 17 β -estradiol inhibited mitophagy through the PI3K/Akt pathway via GPER/GPR30.

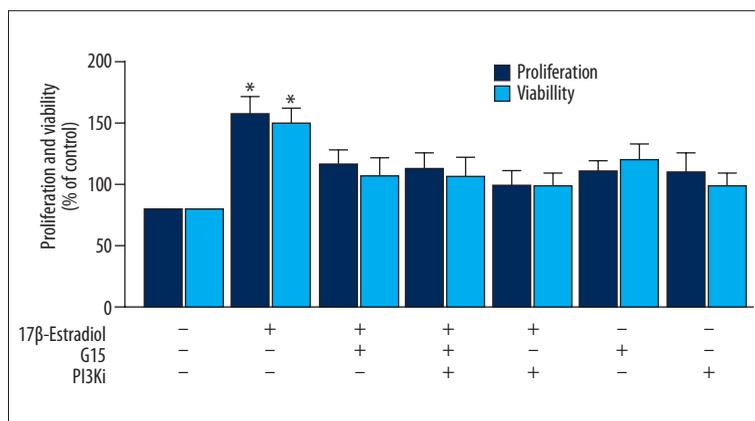


Figure 6. BrdU and MTT assays for proliferation and viability of ATDC5 chondrocytes that were treated with 17 β -estradiol with or without G15 and PI3K inhibitor. Analysis of the proliferation and viability of ATDC5 chondrocytes that were treated with 17 β -estradiol (0 μ M and 10⁻¹ μ M) with or without G15 and PI3K inhibitor as assessed with the BrdU assays and the MTT assay. The figure data represent experiments performed in triplicate. The asterisks indicate significant differences compared with the control (* $P < 0.05$).

Mitogen-activated protein kinases (MAPKs), including those of the JNK and p38 signaling pathways, have also been linked to the regulation of autophagy [45]. To further determine whether estradiol could protect ATDC5 chondrocytes through the GPER/GPR30/JNK or GPER/GPR30/p38 pathways, serum-free ATDC5 cells were treated with 10⁻¹ μ M of 17 β -estradiol with or without the JNK inhibitor, SP600125 (10 μ M), the p38 inhibitor, SB203580 (10 μ M), G15 (15 μ M), or with the combination of inhibitors for 24 hours. The data indicated no changes in p38 or JNK phosphorylation in the 17 β -estradiol-treated ATDC5 cells, and no reversal of effects was observed with G15, the p38 inhibitor, or the JNK inhibitor (Figure 5C, 5D).

Finally, a BrdU assay and an MTT assay showed that the proliferation and viability of the 17 β -estradiol-treated ATDC5 chondrocytes were increased by 1.5-fold compared with the control group ($P < 0.05$), and the effects were reduced by treatment with G15 and the PI3K inhibitor, LY294002 (Figure 6). These findings indicated that 17 β -estradiol promoted the proliferation and viability of the ATDC5 chondrocytes under serum-starved conditions through the GPER/GPR30/PI3K/Akt pathway.

Discussion

Osteoarthritis is a degenerative joint disease that is characterized by articular cartilage damage. Chondrocytes are the main cell type that form the articular cartilage, and the gradual loss of these cells is the main cause of osteoarthritis [46]. There has been increasing published evidence that estrogen can protect chondrocytes and alleviate osteoarthritis *in vivo* and *in vitro* [21–23]. In this study, the proliferation and viability of 17 β -estradiol-treated ATDC5 chondrocytes were significantly increased compared with the control group, which confirmed the protective effect of 17 β -estradiol on chondrocytes. However, the molecular mechanism of the estrogen-mediated protection of chondrocytes from damage remains unclear.

In the present study, fewer mitophagosomes were observed using transmission electron microscopy (TEM), and decreased numbers of TOM20-positive granules were co-localized with decreased Lamp2 shown by immunofluorescence (IF) analysis in the 17 β -estradiol-treated ATDC5 cells, which showed that mitophagy was suppressed by 17 β -estradiol. These findings are consistent with those of a recent study that showed that the protective effect of estradiol was due to the inhibition of autophagy in neurocytes [41]. There is increasing evidence to indicate that mitochondria are essential for cell survival and cell death because they can be considered to be the metabolic ‘powerhouse’ of the cell [47].

Mitochondria are essential for the intermediary metabolism of protein, carbohydrate, and fat. Mitochondria also play a key role in adenosine triphosphate (ATP) production and provide more than 90% of the energy of the cell. It has been established that the activity of mitochondria modulate cell survival, cycle differentiation, cell proliferation, apoptosis, and the generation of reactive oxygen species (ROS) [48,49]. Oxidative stress can lead to mitochondrial damage, and if that damage surpasses the membrane potential across the inner mitochondrial membrane, the entire population of dysfunctional mitochondria is removed via the autophagy pathway, a process known as mitophagy.

Autophagy has been proposed to be a ‘double-edged sword’; while mitophagy can protect cells from apoptosis by removing mitochondria that have been damaged by oxidative stress, excessive mitophagy can reduce essential cellular components through the degradation of the bulk cytoplasm and thus cause cell death [50]. In ATDC5 cells *in vitro*, excess autophagy can be induced by glucocorticoids (GCs), which results in a significant reduction in cell viability [51]. Previous studies have shown that autophagy could reduce the growth rate of growing cells through the elimination of growth-promoting molecules and organelles, such as the protein p62 (sequestosome-1). When p62 is depleted or knocked out, mTOR activation fails, which leads to an impairment of cell growth [52]. In response to excessive mitophagy, mitochondria employ other mechanisms to repair or remove damaged cell components, such as products arising

during cycles of fusion and fission, and act as an organelle quality control mechanism known as the mitochondrial unfolded protein response (UPR^{mt}). These mechanisms ensure that the mitochondria maintain their normal functions [53]. The cell can activate the UPR^{mt} pathway to protect the mitochondria from dysfunction by increasing mitochondrial chaperone levels, including Hsp60 and Hsp70, and increasing the expression of proteases [54,55]. TOM20 is a protein marker in the mitochondrial outer membrane. Serum starvation of HeLa cells can induce mitophagy along with an increased expression of LC3-II and can decrease the expression of TOM20 [56]. Similarly, in this study, the expression level of the protein LC3-II was reduced, while the expressions of the mitochondrial outer membrane protein TOM20 and Hsp60 were increased. Therefore, it can be concluded that 17 β -estradiol might protect ATDC5 chondrocytes by inhibiting mitophagy and may help to repair the mitochondrial damage.

In this study, mitophagy was suppressed by 17 β -estradiol in the ATDC5 cells via the G-protein coupled estrogen receptor (GPER/GPR30) and the PI3K/Akt-mTOR signaling pathway, which suggests that the PI3K/Akt-mTOR signaling pathway might be a target for osteoarthritis therapy. Previous studies have shown that 17 β -estradiol can bind to GPER/GPR30 receptors that are expressed in cardiomyocytes and cancer cells to affect their physiological functions [57,58]. In this study, GPER/GPR30 was expressed in ATDC5 chondrocytes, and treatment with 17 β -estradiol at increasing doses (0 μ M, 10⁻³ μ M, 10⁻² μ M, and 10⁻¹ μ M) increased the GPER/GPR30 protein levels in a dose-dependent manner, with strong increase at the concentration of 10⁻¹ μ M, which indicates that 17 β -estradiol might act on GPER/GPR30 to exert its physiological regulatory function in chondrocytes.

Also, in this study, Western blot analysis also showed a time-dependent induction of the levels of the GPER/GPR30 protein. Immunofluorescence (IF) staining was performed and showed that GPER/GPR30 was present in the regions outside the nucleus in ATDC5 chondrocytes, which implies that the GPER/GPR30 receptor may not be an intra-nuclear receptor and that 17 β -estradiol may act on ATDC5 chondrocytes through GPER/GPR30 in the membrane, cytoplasm and mitochondrial. More studies are needed to elucidate the distribution of GPER/GPR30 in ATDC5 chondrocytes, to clarify the mechanism of action of GPER/GPR30 in ATDC5 cells. The effects of 17 β -estradiol were abolished by the GPER/GPR30 antagonist G15, which caused an increase in the expression of LC3-II and reductions in the expressions of TOM20 and Hsp60. Therefore, 17 β -estradiol may inhibit mitophagy via GPER/GPR30. The role of ER α and ER β in mediating 17 β -estradiol actions on ATDC5 chondrocytes remain to be studied, to further evaluate the possible interactions among ER α , ER β , and GPER/GPR30 in 17 β -estradiol actions on chondrocytes.

The PI3K/Akt signaling pathway is involved in almost every aspect of physiological and pathological cell functions, including

growth, tumorigenesis, apoptosis, and autophagy [59,60]. In this study, the phospho-Akt protein level was increased in the ATDC5 chondrocytes that were cultured with 17 β -estradiol for 24 hours, while the expression levels of the total Akt protein remained unchanged. It has previously been reported that mTOR, which is positively regulated by PI3K, is a negative regulator of autophagy. In the present study, the expression level of p-mTOR was significantly increased in the 17 β -estradiol-treated ATDC5 cells compared with the control cells. In addition to incubation with 17 β -estradiol, ATDC5 cells were incubated with an inhibitor of GPER/GPR30 (G15), an inhibitor of PI3Ki or an inhibitor of mTOR. The results showed that the inhibition of GPER, PI3K or mTOR abolished the effect of 17 β -estradiol on mitophagy, which indicated that 17 β -estradiol inhibited mitophagy through the PI3K/Akt-mTOR signaling pathway via GPER/GPR30. Similar findings have also been in previous studies. For example, 17 β -estradiol has been shown to suppress lipopolysaccharide-induced acute lung injury through PI3K/Akt signaling [61]. Moreover, 17 β -estradiol decreases the rate of apoptosis in nucleus pulposus cells in rats via the PI3K/Akt/caspase-3 pathway [62]. Furthermore, PI3K/Akt signaling is inversely associated with mitochondrial oxidative stress, and the activation of PI3K/Akt signaling reduces ROS production [63,64]. Other MAPK signaling pathways, including the JNK and p38 pathways, might be linked to the regulation of autophagy. To verify whether 17 β -estradiol also modulated mitophagy through JNK and p38, in this study, cells were pretreated with a p38i and a JNKi for 30 min. The P-p38 and P-JNK levels exhibited non significant differences between the experimental groups. These results indicated that 17 β -estradiol did not regulate mitophagy through the JNK or p38 signaling pathways.

Future studies will focus on the function of estradiol in chondrocytes and the relevant molecular mechanisms in animal models and clinical trials with the goals of identifying the clinical effects of estradiol on chondrocytes and potential therapeutic targets for osteoarthritis.

Conclusions

The findings of the present study have shown that 17 β -estradiol protected ATDC5 chondrocytes cultured *in vitro* from mitophagy by activating the PI3K/Akt-mTOR signaling pathway via the G-protein coupled estrogen receptor (GPER/GPR30). The findings of this study also suggest that estradiol might be a promising treatment for osteoarthritis and that the PI3K/Akt-mTOR signaling pathway might be a potential target for future therapy for patients with osteoarthritis.

Conflicts of interest

None.

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