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Estrogen promotes autophagy in the mammary epithelial cells of dairy sheep via the CXCL12/CXCR4 axis

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Abstract

Mammary gland development and lactation in dairy sheep are regulated by hormones and autophagy; however, the role of estrogen-mediated autophagy remains unclear. This study determined that estrogen enhances autophagy, promotes CXCR4 and CXCL12 gene expression, and increases the number of autolysosomes in sheep mammary epithelial cells. Co-treatment with a CXCR4 overexpression vector and the small-molecule alternative of CXCL12, NUCC-390, significantly upregulated *ATG5* and *LC3* gene expression, increased the abundance of the autophagy-associated protein ATG5 and the LC3II/I ratio, and increased the consumption of the autophagy substrate P62. These results suggest that CXCR4 and CXCL12 signaling promotes autophagy in mammary epithelial cells. Conversely, co-treatment with a CXCR4-specific blocker and estrogen inhibited autophagic changes in ATG5, P62, and LC3 levels, reducing the number of autophagosomes and autolysosomes. Overall, this study demonstrated that estrogen promotes autophagy in sheep mammary epithelial cells through the CXCL12/CXCR4 signaling axis, revealing the underlying mechanisms behind estrogen-mediated autophagy.

Lay Summary

Sheep milk is in increasing demand worldwide for its unique nutrients. Mammary physiological processes and lactation performance are regulated by hormones, and studies have shown that estrogen has the ability to regulate autophagy during mammary development, but the specific mechanism is not clear. Therefore, this study investigated the role of estrogen on autophagy in mammary epithelial cells and analyzed the regulatory mechanism of estrogen on mammary epithelial cells by screening the CXCL12 small-molecule substitute NUCC-390 and the CXCR4 specific blocker AMD3100 by virtual molecular docking technology, and the results indicated that estrogen promotes autophagy of mammary epithelial cells in sheep through the CXCL12/CXCR4 signaling axis. This study provides novel insights into the mechanism of autophagy in mammary gland development and health, and offer a molecular basis for improving lactation performance in dairy animals.

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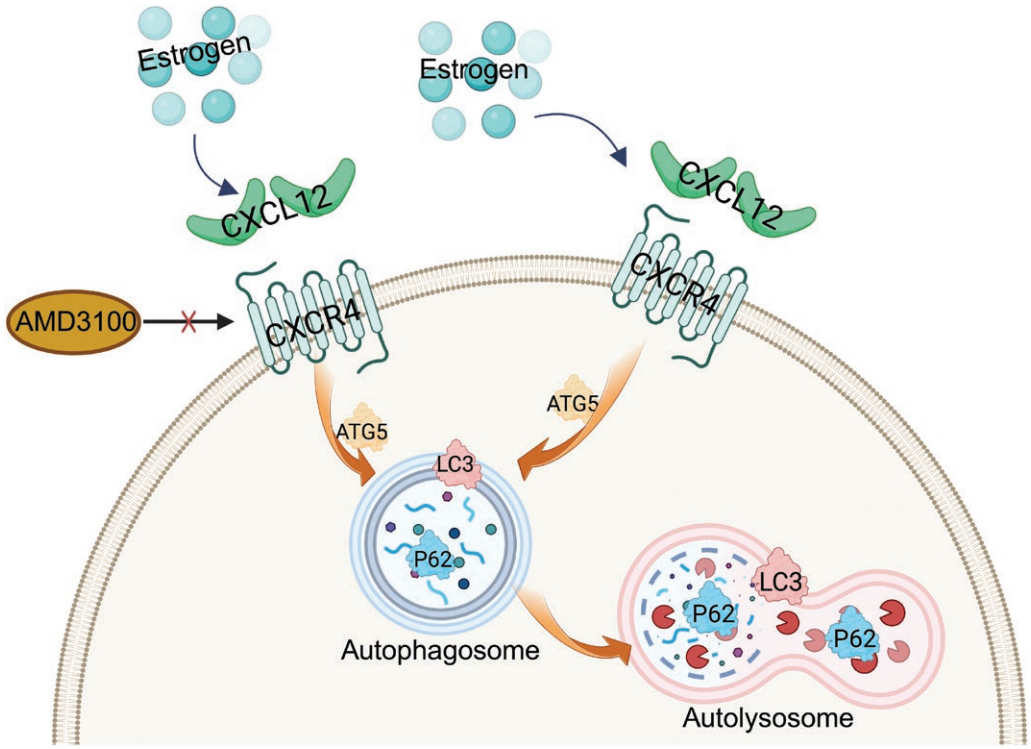
Graphical Abstract



Schematic diagram of estrogen enhances autophagy via CXCL12/CXCR4 signaling axis. Picture created in BioRender. Chu, T. (2025) https://BioRender.com/n01s394.

Key words: autophagy, CXCL12/CXCR4 signaling, estrogen

Abbreviations: CCK-8, Cell Counting Kit-8; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C chemokine receptor 4; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; E2, β-estradiol; EdU, 5-Ethynyl-2′-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; NC, negative control;SD, standard deviation; SMEC, sheep mammary epithelial cell

Introduction

Global sheep milk production is approximately 10.618 Mt (FAOSTAT, 2022), and sheep milk is in increasing demand due to its high protein, rich vitamins, low lactose and its use in a variety of functional products, including drinking milk, cheese, and nutraceutical products (Pulina et al., 2018; Nudda et al., 2020). Thus, the mammary glands of dairy sheep are of great economic importance, with their primary function involving the synthesis and secretion of milk (Altamirano et al., 2023). Mammary gland health and lactation performance are regulated by the breeding strategy employed, alongside the physiological state and number of mammary epithelial cells (**MECs**) present (Perruchot et al., 2016). In addition, lactation performance and milk composition have been linked to exogenous growth hormone, estrogen and progestagen levels (Toaff et al., 1969; Peel et al., 1983), with estrogen playing a crucial role in with mammary gland development (Gagniac et al., 2020). In particular, estrogen mediates the proliferation of MECs, activates mammary duct morphogenesis, and regulates functional differentiation and morphogenesis of the mammary gland during pregnancy (Lamote et al., 2004).

Prior studies have revealed that estrogen not only regulates mammary gland development but also balances the expression of core autophagy proteins through different transcription factors, miRNAs and histone modifications by signaling pathways downstream of the receptor (Xiang et al., 2019). Autophagy, a process involving the self-degradation and recycling of cellular components (Wollert et al., 2019), regulates the functional differentiation of MECs during mammary gland development (Elswood et al., 2021). This process is particularly important for mammary gland remodeling during lactation in dairy animals (Motyl et al., 2007). Additionally, autophagic lysosomes degrade pathogens in the mammary glands of dairy animals, thereby controlling bacterial infection (Xu et al., 2022). However, the molecular mechanism of autophagy in dairy sheep mammary glands has not yet been characterized. Autophagy is regulated by multiple proteins, biomarker proteins, autophagic flux, autophagy substrates, and other key autophagic processes (Klionsky et al., 2016). Notably, estrogen is involved in the regulation of autophagy-related genes via ligand-activated estrogen receptors, which directly bind to promoters of these genes, enhancing autophagy induction in bovine MECs (Zielniok et al., 2017). Therefore, the modulation of estrogenic actions associated with autophagy may influence mammary gland development, remodeling, and lactation. However, whether estrogen directly promotes autophagy in the MECs of dairy sheep remains unclear.

C-X-C motif chemokine ligand 12 (CXCL12) and C-X-C chemokine receptor 4 (CXCR4) are widely expressed in various cells (stromal fibroblasts, vascular endothelial cells and osteoblasts) and tissues (liver, brain, pituitary gland, kidneys, lymph nodes and colon etc.). As members of the chemokine family, they are involved in various physiological and pathological processes (Wu et al., 2023). Recent studies have suggested that estrogen is involved in the transcriptional

regulation of CXCL12/CXCR4, particularly in mammary gland (Sauve et al., 2009; Mousavi et al., 2020). Additionally, the CXCL12/CXCR4 signaling axis has been implicated in the regulation of autophagy (Yu et al., 2017; Ashley et al., 2021). However, the CXCL12/CXCR4 signaling pathway has yet to be comprehensively investigated in the context of estrogen-mediated autophagy in dairy sheep mammary epithelial cells (**SMECs**).

To investigate the molecular mechanisms by which estrogen regulates autophagy in SMECs, we studied the SMECs to reveal the relationship between estrogen, CXCR4/CXCR12 and autophagy. We found that estrogen enhanced autophagy in dairy SMECs. Moreover, we demonstrated that the activation of the CXCL12/CXCR4 axis was closely related to estrogen-induced autophagy in these SMECs. Overall, these findings provide novel insights into the mechanism of autophagy in mammary gland development and health, and offer a molecular basis for improving lactation performance in dairy animals.

Materials and Methods

SMECs culture and treatment

Mammary gland tissues from 3 dairy sheep were obtained from a crossbred generation of East Friesian and Hu sheep at peak lactation, and all experimental procedures were approved by the Animal Care and Use Committee of Northwest A&F University, Yangling, China. SMECs were isolated and cultured from these tissues according to the methods established by Hao et al. (2022). In brief, the mammary tissues were cut into 1 mm^3 pieces, washed in PBS containing 5% penicillin–streptomycin solution, and then spread evenly in a 3.5 cm cell culture dish. The tissues were cultured with modified medium composed of 90% DMEM/F12 basal medium (SH30023.01, Cytiva, USA), 10% fetal bovine serum, 0.1 mg/mL penicillin-streptomycin solution (P1400, Solarbio, Beijing, China), 5 μg/mL insulin (I8040, Solarbio), 10 ng/mL epidermal growth factor (PHG0313, Gibco, USA), and 1 μg/mL hydrocortisone (IH0110, Solarbio) in a cell culture incubator (Thermo Fisher, US) with 37 °C, 5% CO_2. After 7 to 8 d of tissue culture, the cells crawled out from mammary tissue, and when the cell growth reached about 80%, the MECs were isolated and purified with 0.25% trypsin-EDTA, after repeated digestion and culture 3 times, purified MECs were obtained for further experiments, and the MECs used in this study were all within 10 generations of the passaged culture. For cell treatments, β-estradiol (E2; GC11282, Globo, USA) was diluted to a working concentration of 10 mM using dimethylsulfoxide (DMSO). Then, a blank control group (BLANK) and DMSO vector controls were set up with 10, 25, 50, 75, and 100 μM estrogen-containing culture medium.

5-Ethynyl-2′-deoxyuridine (EdU) and Cell Counting Kit-8 (CCK-8) assays

To investigate the effect of estrogen on the proliferation of SMECs, EdU (C10310-1, Ribobio, Guangzhou, China) and CCK-8 assays (K009-500, ZETA life, USA) were employed as previously described (Liu et al., 2024).

Total RNA extraction and quantitative reverse-transcription PCR analysis

Total RNA was extracted from the SMECs using the Seven-Fast Total RNA Extraction Kit (SM130-02, Seven Biotech, Beijing, China) according to the manufacturer's instructions. RNA concentration and quality were then detected using a microplate spectrophotometer (Epoch, BioTek Instruments Inc., USA); RNA purity of all samples was determined to be within the range of 1.8 to 2.0 (A260/A280).

RNA was reverse transcribed into cDNA using the Takara One Step Reverse Transcription Kit (RR092A, Takara Bio Inc. Co. Ltd, Japan). Relative gene expression was quantified by real-time fluorescence quantitative PCR with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, USA) using the SYBR Green Premix Pro Taq HS qPCR Kit (A313-05, GenStar, China). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* as a reference. Primer sequences are provided in Table S1.

Total protein extraction and western blotting

Total cellular proteins were extracted from SMECs using protein lysates containing 1% protease inhibitor (T6583; Target Molecule Corp. Shanghai, China) and 1% phosphatase inhibitor (GRF102, EpiZyme, Shanghai, China). Protein concentration was determined using a BCA kit (PC0020, Solarbio). Samples were then prepared with a 5 × Protein Loading Buffer (P1040, Solarbio) and boiled at 100 °C for 10 min. Next, 10 to 30 μg of each protein sample was upsampled and separated by SDS-PAGE; proteins were then transferred to a PVDF membrane, which was blocked with 5% skim milk for 1 h and then incubated with specific primary and HRP-coupled secondary antibodies. Immunoreactive bands were visualized using chemiluminescent detection reagents, and band intensity was analyzed using ImageJ software. The primary antibodies used were as follows: ATG5 (ET1611-38, Huabio, China), β-actin (AY0573, ABways, China), p62 (AF5384, Tsingke Biotech Co., Ltd, China), LC3I/II (ET1701-65, Huabio), CXCR4 (AF5297, Tsingke Biotech Co., Ltd), mTOR (ET1608-5, Huabio), and p-mTOR (HA600094, Huabio), and the secondary antibody used in this research is HRP-labeled Goat Anti-Rabbit IgG(H + L) (A0208, Beyotime Biotechnology).

CXCR4 overexpression vector construction double

Upstream and downstream primers were designed and synthesized based on the full-length CDS sequence of the *CXCR4* gene from the NCBI database (NM_001277168.1). The primer sequences were as follows: *CXCR4*-F (5′ to 3′): ATGGAAGGGATCCGTATATTCAC, *CXCR4*-R (5′ to 3′): TTAGCTGGGAGTGAAAACTGGAAG. Firstly, PCR amplification conditions were conducted as follows: 95 °C for 5 min, 32 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. PCR amplification products were then purified before being ligated to the pMDTM19-T vector. The ligation system consisted of 5 μL of Solution I, 1 μL of the pMD19-T vector, 3 μL of the purified target gene fragment, and 1 μL of ddH_2O. The ligated product was transformed into DH5α cells, which were selectively cultivated in LB solid medium (peptone, yeast extract, NaCl, and agar powder included). After incubation for 15 h, a monoclonal population was picked and amplified in LB liquid medium before being assessed by bacteriophage PCR. After screening, the bacterial suspension was sequenced (Sangon Biotech Co., Ltd, Shanghai, China). The bacterial samples that met the requirements of the sequencing results were expanded, cultured, and used for plasmid extraction.

The plasmids and pcDNA3.1 vector were double digested with NheI and HindIII; this digestion system consisted of 2

μL of Buffer M (1×), 1 μL of HindIII, 1 μL of NheI, 8 μL of T vector containing the target fragment, and 8 μL of ddH_2O. The target DNA fragment was recovered and ligated with the pcDNA3.1 vector; the ligated product was then transformed into DH5α cells. After incubation for 15 h, a monoclonal population was picked for PCR detection, and the selected strains were further sequenced (Sangon Biotech Co., Ltd). The strains containing DNA that aligned with the established sequencing results were amplified; the extracted plasmid, named pcDNA-CXR4, was transferred into the cells for overexpression.

CXCR4 model construction and molecular docking

The SWISS-MODEL online tool (https://swissmodel.expasy.org/assess/) was used to retrieve CXCR4 templates with high homology to dairy sheep, based on the amino acid sequence of the dairy sheep *CXCR4* gene from the NCBI database (https://www.ncbi.nlm.nih.gov/). The 3-dimensional structure of dairy sheep CXCR4 was homologously modeled; the accuracy of this model was assessed using a Ramachandran plot. Virtual molecular docking was then performed using AutoDock Version4.2 software, with the dairy sheep CXCR4 protein being used as the receptor (Version 4.0); this enabled the identification of small-molecule compounds that could bind to CXCR4. The key docking sites were analyzed using PyMOL software (Version 2.6).

RNA interference

CXCR4 interfering RNAs were designed and synthesized using GEMMA software based on the *CXCR4* gene CDS sequence from the NCBI database. The resulting siRNA1, siRNA2, and control si-NC sequences are detailed in Table S2. The siRNA with optimal interference efficiency was named CXCR4-siRNA.

CXCL12 concentrations in SMEC supernatants

After treating SMECs with 50 μM estrogen for 24 h, the cell culture medium was collected. The concentration of CXCL12 in the supernatants of the medium were then detected using a sheep CXCL12 enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions (YJ951147, Yuanji Biotechnology Center, Shanghai, China).

Autophagic flux analysis

Treated cells were fixed in 4% paraformaldehyde for 10 min at 25 °C, stained with 4',6-diamidino-2-phenylindole dye for 15 min, and encapsulated with an anti-fluorescence quenching sealant (P0128S, Beyotime Biotechnology Co., Ltd, Shanghai, China). Subsequently, autophagosomes and autolysosomes were imaged under a confocal microscope (LSM980, ZEISS, Germany).

For transmission electron microscopy, SMECs were fixed with 2.5% glutaraldehyde (G1102, Servicebio, Wuhan, China) for 30 min, then with 1% osmium tetroxide for an additional 1 h. Subsequently, cells were dehydrated using acetone, permeabilized with a dehydrating agent, and embedded with Epon-812. Ultrathin sections (60 to 90 nm) were cut using an ultrathin sectioning machine (EM UC7, Leica, Germany) mounted onto a copper mesh, and stained with uranyl acetate for 10 to 15 min and lead citrate for 1 to 2 min. Finally, samples were imaged using a transmission electron microscope (**TEM**; JEM-1400FLASH, JEOL Ltd, Japan).

Statistical analysis

Student's t-test was used for comparisons between 2 groups, and one-way ANOVA was performed for comparisons among multiple groups. All data were presented as the mean ± standard deviation and analyzed using GraphPad Prism 9.0 software. Statistical significance was set at $P < 0.05$.

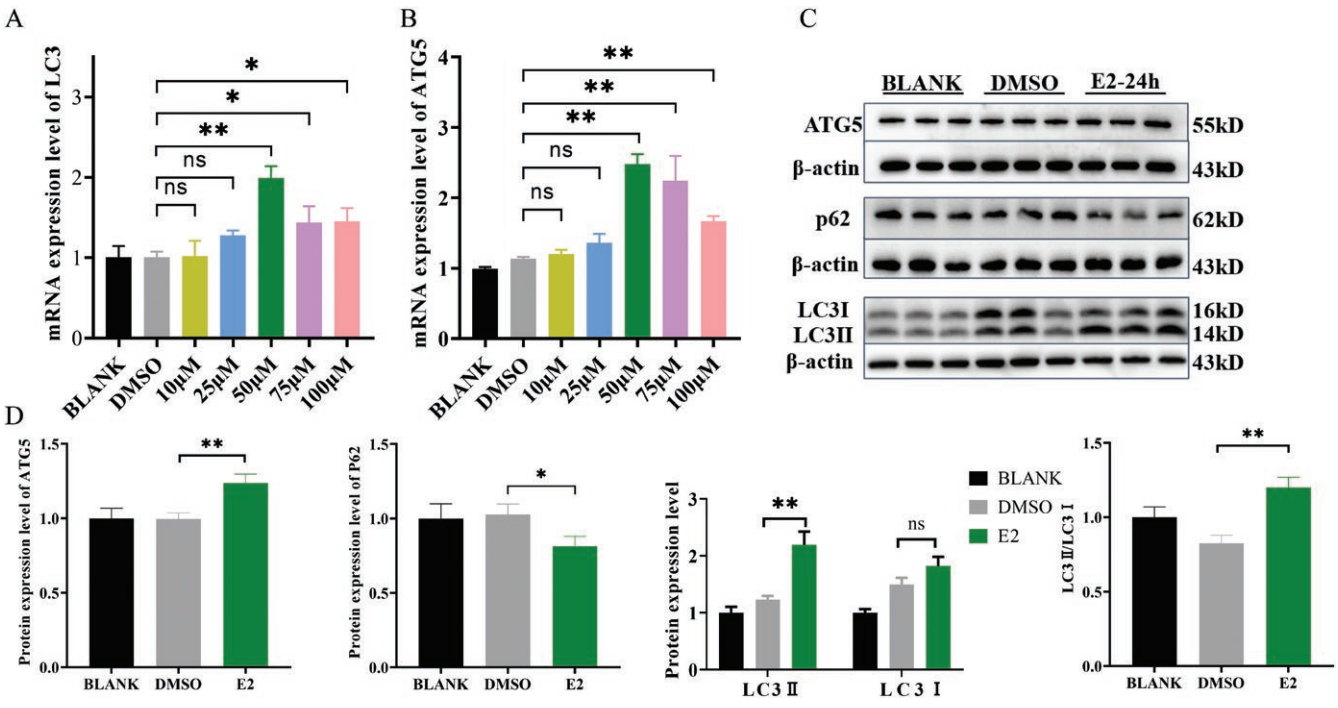


Figure 1. Estrogen enhanced autophagy flux in SMECs. (A and B) The relative mRNA levels of *LC3* and *ATG5* in SMECs treated with different concentrations of estrogen. (C and D) The relative protein levels of p62, LC3, and ATG5 in SMECs treated with different concentrations of estrogen. * $P < 0.05$, ** $P < 0.01$.

Results

Estrogen enhances autophagy in SMECs

In this study, we evaluated the proliferation of SMECs treated with different concentrations of estrogen. EdU staining revealed that SMECs proliferation decreased with increasing E2 concentrations, with a minimum concentration of 75 μM E2 significantly reducing SMECs proliferation (Fig. S1). Subsequent qRT-PCR analysis of autophagy-related gene and protein levels in estrogen-treated SMECs revealed that 50 μM estrogen treatment significantly increased the mRNA expression levels of key autophagy genes *ATG5* and *LC3* (Fig. 1A and B). In contrast, compared with 50 um group, the 75 μM and 100 μM estrogen-treated groups showed a decreased trend in these mRNA expression levels ($0.05 < P < 0.10$). Meanwhile, western blot analysis indicated a significant increase in ATG5 protein abundance ($P < 0.01$), a significant decrease in P62 protein abundance ($P < 0.05$), and a significant increase in LC3II protein abundance and the LC3II/I ratio in SMECs treated with 50 μM estrogen, while LC3I protein abundance didn't show significant difference ($P < 0.01$, Figure 1C and D).

Laser confocal microscopy analysis revealed a significant increase in the number of autophagosomes (green fluorescent protein) and autolysosomes (monomeric red fluorescent protein) in SMECs treated with 50 μM estrogen; notably, the number of fully fused autolysosomes (red) was increased ($P < 0.01$, Fig. 2A to C). These results were supported by TEM analysis, which similarly indicated an increased number of autolysosomes in SMECs treated with 50 μM estrogen (Fig. 2D and E). Overall, these findings suggest that 50 μM estrogen treatment enhances autophagy in SMECs.

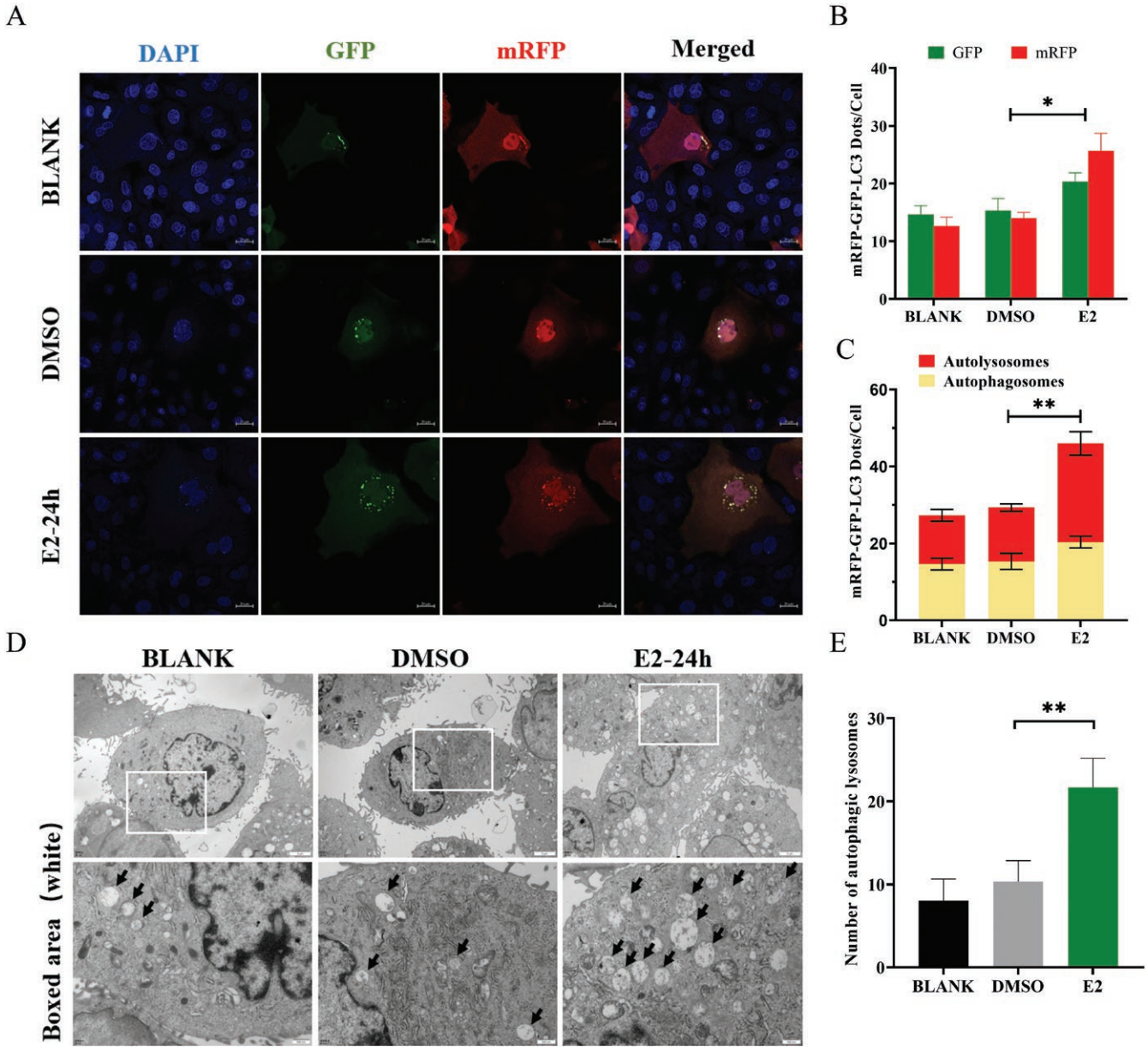


Figure 2. Effects of estrogen on the autolysosomes and autophagosomes in SMECs. (A) Representative images of autophagosomes (GFP) and autolysosomes (mRFP) observed under confocal microscopy. Scale bar = 20 μm; SMECs were transfected with the recombinant adenovirus mRFP-GFP-LC3 for 48 h, and/or treated with estrogen. (B and C) Quantitative statistics of autophagosomes and autolysosomes observed by laser confocal microscopy. (D) Representative images of autolysosomes observed under TEM. Scale bar = 2 μm or 500 nm. (E) Quantitative statistics of autolysosomes observed under TEM. * $P < 0.05$, ** $P < 0.01$.

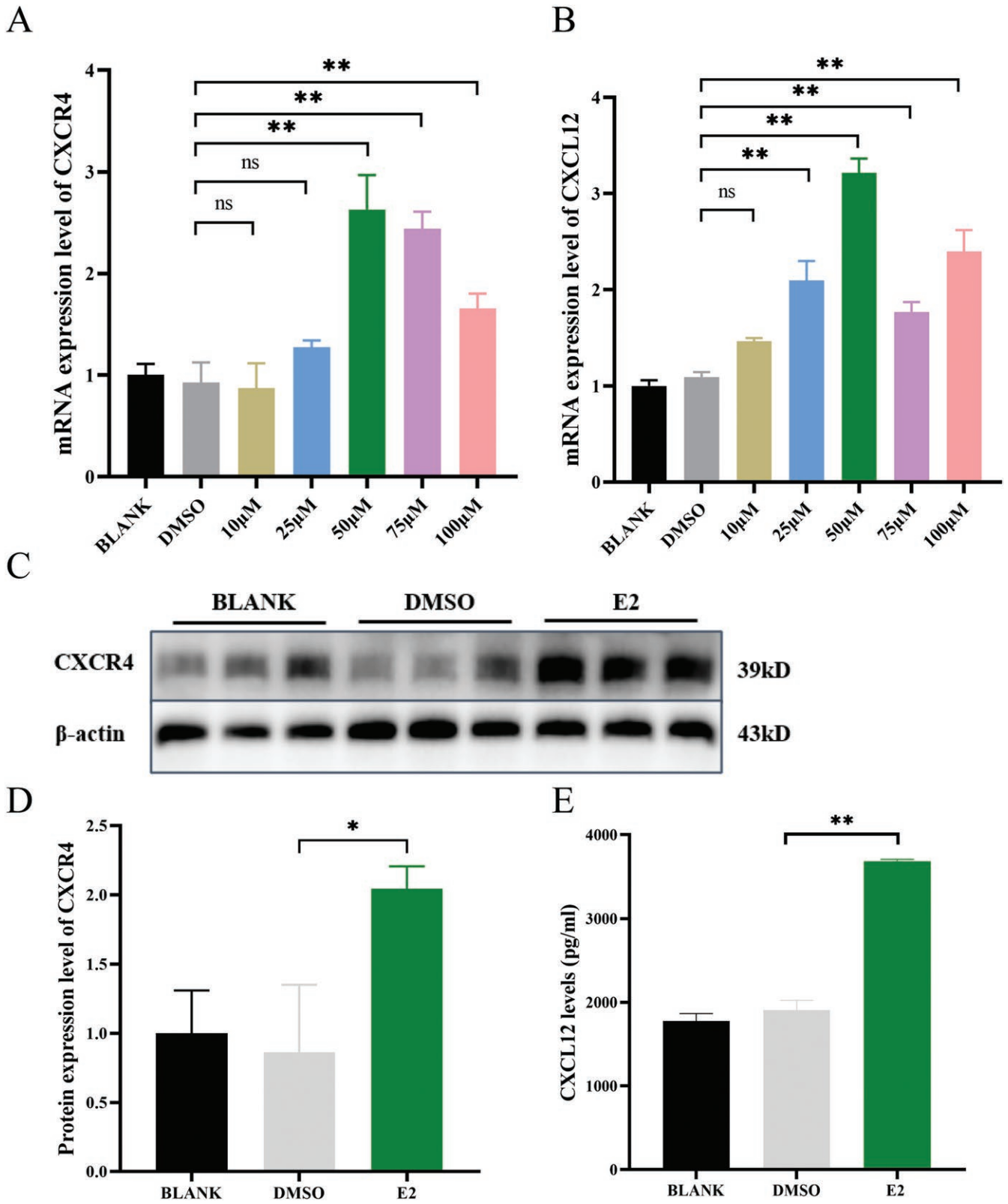


Figure 3. Estrogen enhanced the expression of CXCR4 and CXCL12. (A) The mRNA expression level of *CXCR4* in SMECs treated with different concentrations of estrogen. (B) The mRNA expression level of *CXCR12* in SMECs treated with different concentrations of estrogen for 24 h. (C and D) The abundance of CXCR4 protein in SMECs treated with 50 μM estrogen for 24 h. (E) The concentration of CXCL12 in cell culture medium. * $P < 0.05$, ** $P < 0.01$.

Estrogen upregulates CXCR4 and CXCL12 expression in SMECs

To investigate whether estrogen affects CXCL12 and CXCR4 expression levels, we performed qRT-PCR, western blotting, and ELISA analyses. qRT-PCR results demonstrated that 50, 75, and 100 μM estrogen treatment groups exhibited a significant increase in CXCR4 and CXCL12 mRNA expression levels compared to those observed in the negative

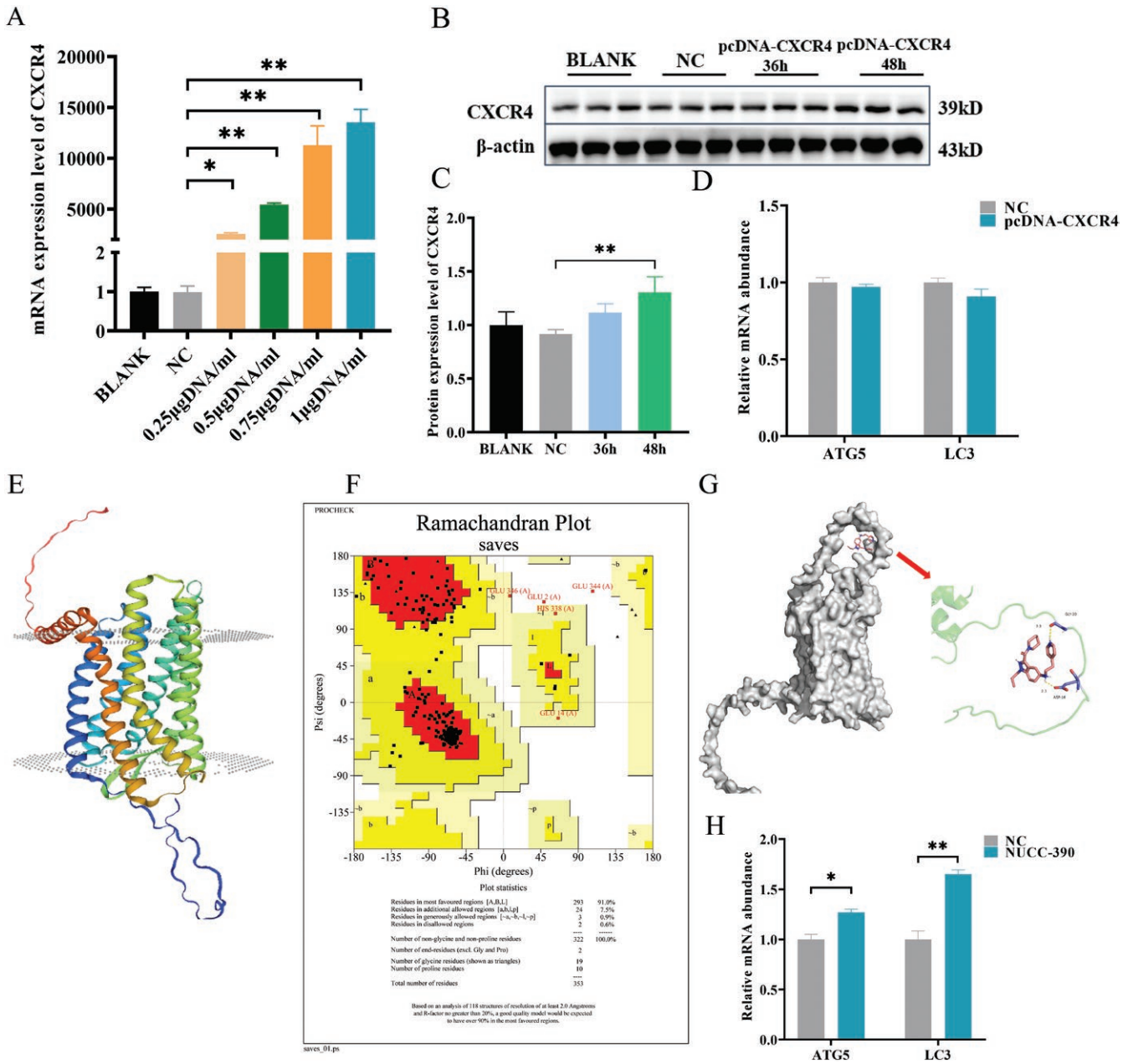


Figure 4. CXCR4 overexpression promoted autophagy flux in SMECs. (A) The transfection efficiency of SMECs treated with pcDNA-CXCR4. (B and C) The protein expression levels of CXCR4 in SMECs transfected with pcDNA-CXCR4 at different time. (D) The mRNA expression levels of *ATG5* and *LC3* in SMECs transfected with pcDNA-CXCR4 for 48 h. (E and F) The homologous modeling and credibility detection of CXCR4. (G) The binding site of NUCC-390 and CXCR4 protein. (H) The mRNA expression levels of *ATG5* and *LC3* in SMECs treated with NUCC-390 for 24 h. $n = 3$, * $P < 0.05$, ** $P < 0.01$.

control (**NC**) group ($P < 0.01$, Fig. 3A and B). Meanwhile, western blot analysis indicated that SMECs treated with 50 μM estrogen displayed significantly increased CXCR4 protein levels ($P < 0.01$, Fig. 3C and D). Finally, ELISA analysis revealed elevated CXCL12 content in the culture medium of estrogen-treated SMECs ($P < 0.01$, Fig. 3E). These results demonstrated that 50 μM estrogen promotes autophagy and enhances CXCR4/CXCL12 expression in SMECs.

CXCL12/CXCR4 signaling promotes SMECs autophagy

The pcDNA-CXCR4 vector constructed in this study was transferred into SMECs to detect its efficiency. Notably, the mRNA expression of *CXCR4* was elevated in SMECs transfected with pcDNA-CXCR4, indicating that the vector exceptional overexpression efficiency (Fig. 4A). Excessive pcDNA-CXCR4 transfection impaired cell proliferation (Fig. S2); thus, a pcDNA-CXCR4 concentration of 0.5 μg/mL was selected for subsequent experiments. Compared to SMECs transfected with pcDNA3.1 (empty vector, NC group), those transfected with 0.5 μg/mL pcDNA-CXCR4 exhibited a significant increase in CXCR4 protein levels ($P < 0.01$, Fig. 4B and C); however, the mRNA expression levels of *ATG5* and *LC3* did not show significant differences (Fig. 4D).

To target CXCR4 activation, we aimed to identify a small-molecule substitute of CXCL12. Specifically, we

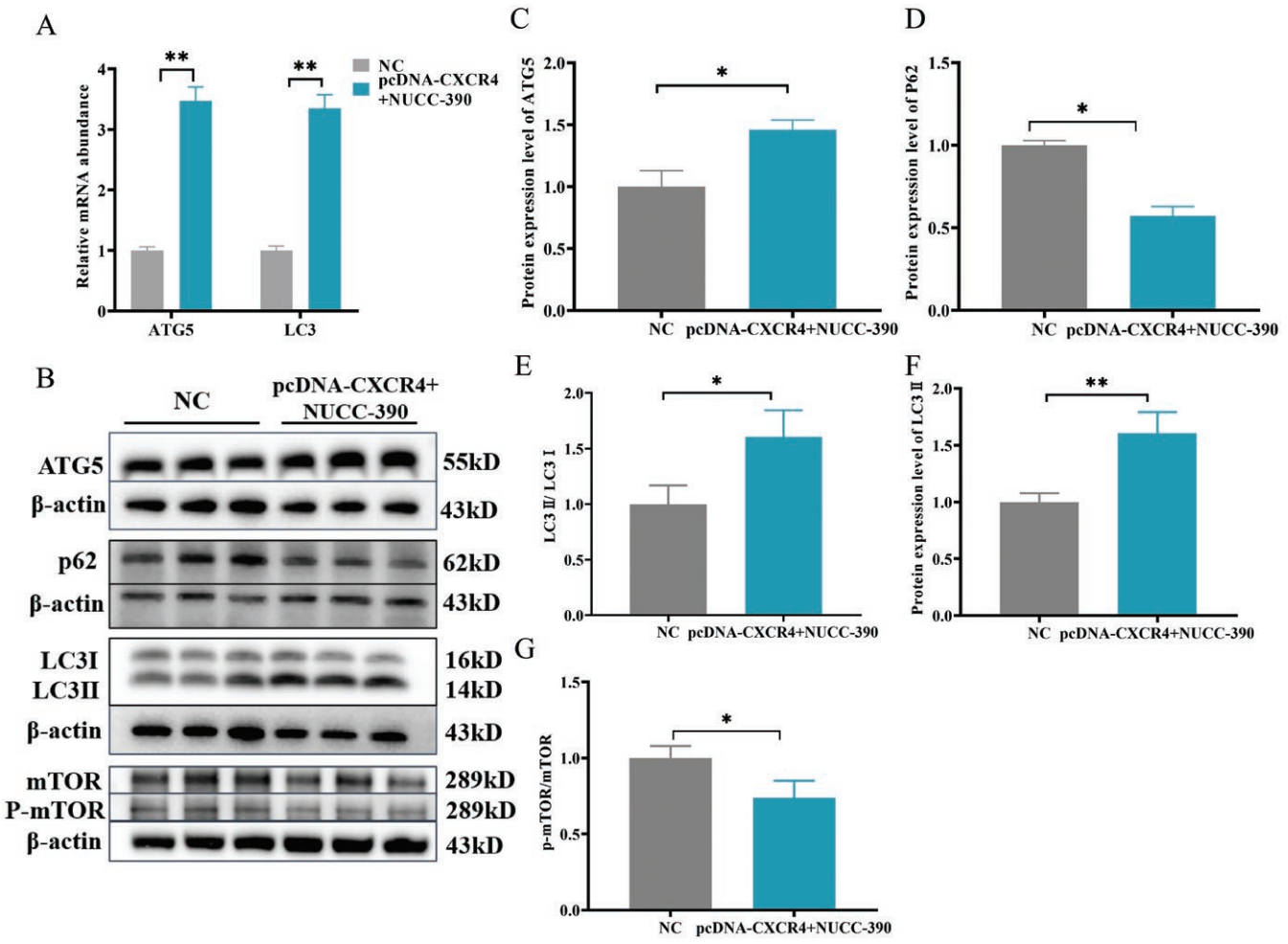


Figure 5. Enhancing CXCL12/CXCR4 signal transduction promoted autophagy flux in SMECs. (A) The mRNA expression levels of *ATG5* and *LC3* in SMECs treated with pcDNA-CXCR4 and NUCC-390. (B to E) The abundances of ATG5, P62, LC3, and mTOR proteins in SMECs treated with pcDNA-CXCR4 and NUCC-390. $n = 3$, * $P < 0.05$, ** $P < 0.01$.

modeled sheep CXCR4 protein using the 3D structure of human CXCR4 protein as a template; we evaluated the constructed model using a Ramachandran plot, which showed that 99.4% of the amino acid residues were located in reasonable positions, indicating successful protein structure prediction (Fig. 4E and F). Next, we employed AutoDock software for molecular docking; this analysis identified a small-molecule compound, NUCC-390, that specifically binds to this CXCR4 protein model. The lowest binding free energy of NUCC-390 docked to sheep CXCR4 was -7.6 kcal/mol, which was lower than that of the conventional molecular binding free energy (-1.2 kcal/mol). Additionally, visualization of the molecular docking results with PyMOL software revealed stable binding of NUCC-390 to the amino acid residues of CXCR4 with 3 hydrogen bonds (Fig. 4G). Treatment of SMECs with NUCC-390 significantly increased the mRNA expression levels of *ATG5 and LC3* ($P < 0.01$, Fig. 4H), indicating that NUCC-390 effectively promotes SMECs autophagy.

To investigate the regulatory mechanisms of the CXCL12/CXCR4 signaling axis on autophagy in SMECs, we treated SMECs with pcDNA-CXCR4 followed by NUCC-390 (5 μM) as the instruction of NUC390 reagent. This treatment group exhibited a significant increase in the expression levels of key autophagy genes *ATG5* and *LC3* compared to those observed in the NC group (DMSO + pcDNA3.1, empty vector) ($P < 0.01$, Fig. 5A). Moreover, ATG5 protein levels increased (Fig. 5B and C), P62 consumption increased ($P < 0.05$, Fig. 5B and D), and the LC3II/I ratio and LC3II protein abundance increased in the treatment group (Fig. 5B, E, and F). Further tests revealed that pcDNA-CXCR4 + NUCC-390 (5 μM) treatment decreased the p-mTOR/mTOR ratio ($P < 0.05$, Fig. 5B and G), suggesting that CXCL12/CXCR4 may promote autophagy by inhibiting the mTOR signaling pathway.

Additionally, confocal microscopy revealed an increased number of autophagosomes and autolysosomes in SMECs treated with pcDNA-CXCR4 + NUCC-390. Additionally, the number of fully fused autolysosomes (red) was increased ($P < 0.01$, Fig. 6A to C). TEM analysis also showed an increased number of autolysosomes in the pcDNA-CXCR4 + NUCC-390 treatment group compared to that in the NC group (DMSO + pcDNA3.1, empty vector) ($P < 0.01$, Fig. 6D and E). In conclusion, these results suggest that activation of the CXCL12/CXCR4 signaling axis promotes estrogen-associated autophagy in SMECs.

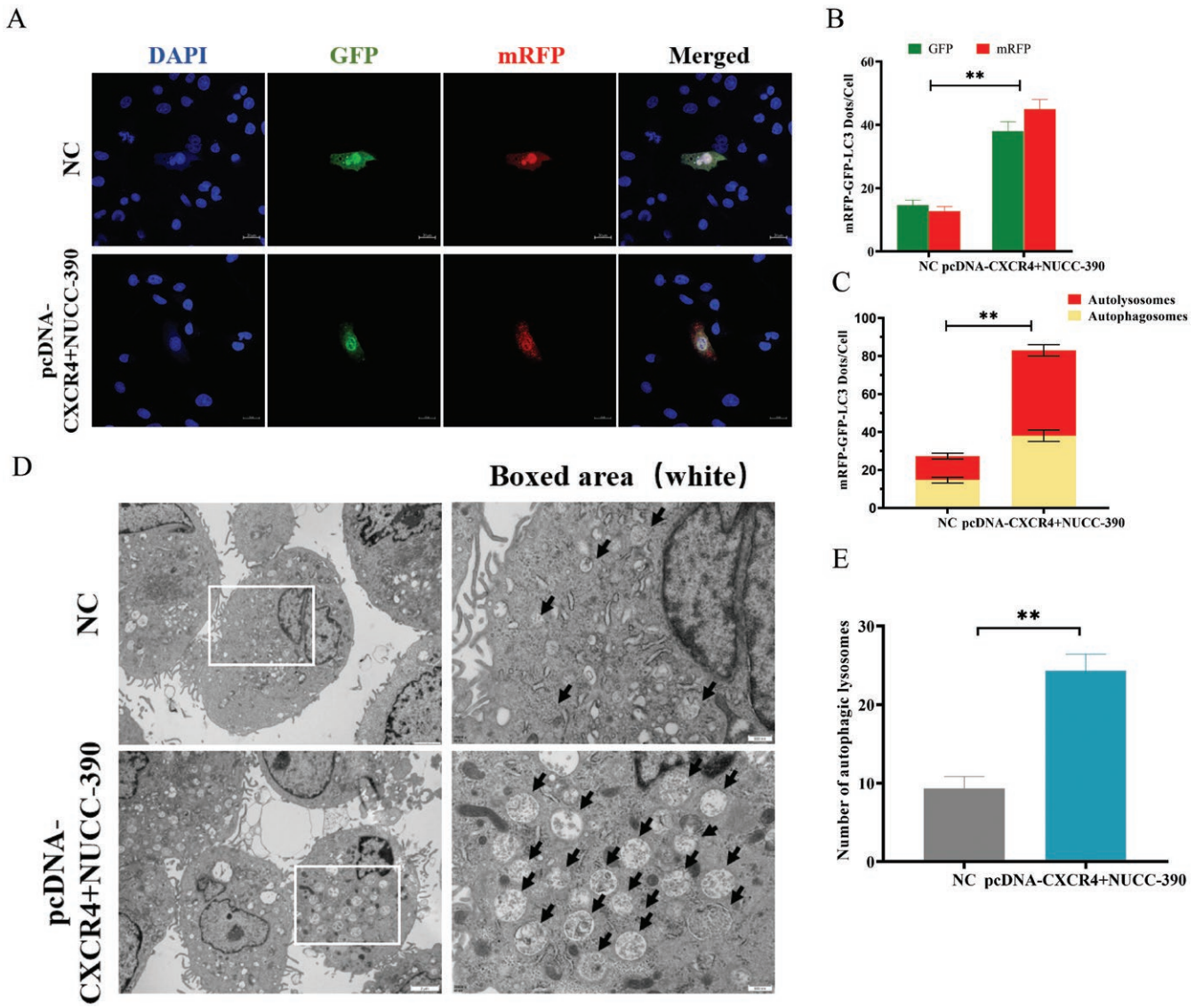


Figure 6. Effects of enhanced CXCL12/CXCR4 signaling on autophagosomes and autolysosomes in SMECs. (A) Representative images of autophagosomes (GFP) and autolysosomes (mRFP) observed under confocal microscopy. Scale bar = 20 μm; SMECs were transfected with pcDNACXCR4 + NUCC-390(5μM). (B and C) Quantitative statistics of autophagosomes and autolysosomes observed by confocal microscopy. D. Representative images of autolysosomes observed under TEM. Scale bar = 2 μm or 500 nm; SMECs were transfected with pcDNACXCR4 + NUCC-390(5μM). (E) Quantitative statistics of autolysosome observed under TEM. $n = 3$,* $P < 0.05$,** $P < 0.01$.

Blocking CXCL12/CXCR4 signaling inhibits estrogen-associated SMECs autophagy

To explore the regulatory mechanisms of autophagy in estrogen-treated SMECs, we performed siRNA interference to inhibit the CXCL12/CXCR4 signaling axis. Both si-RNA1 and si-RNA2 reduced the mRNA expression level of *CXCR4*, with si-RNA1 achieving an interference efficiency of approximately 70%; therefore, si-RNA1 (CXCR4-siRNA) was used for subsequent experiments ($P < 0.01$, Fig. 7A). Notably, CXCR4-siRNA transfection also reduced CXCR4 protein expression in SMECs ($P < 0.05$, Fig. 7B). Interestingly, CXCR4-siRNA did not significantly inhibit the estrogen-induced mRNA expression of autophagy genes *ATG5* and *LC3* in SMECs (Fig. 7C and D), and failed to significantly reduce *CXCR4* mRNA expression in the presence of estrogen (Fig. 7E).

Next, we identified AMD3100 as a CXCR4-specific blocker AMD3100 that could interfere with the binding of CXCL12 to CXCR4 in virtual molecular docking simulations. Thus, we co-treated SMECs with estrogen and 100 nM AMD3100 according to the instructions of the AMD3100 reagent. Our results demonstrated that AMD3100 could significantly inhibit the estrogen-associated increase in autophagy flux in SMECs, with a particular reduction in *LC3* and *ATG5* mRNA expression ($P < 0.05$, Fig. 8A and B); notably, ATG5 and LC3II protein levels were also decreased, while P62 protein levels increased in the estrogen + AMD3100 treatment group (Fig. 8C to G). The AMD3100 treatment group also exhibited a significant reduction in the number of autophagosomes and autolysosomes compared to the those in the estrogen treatment group (Fig. 9A to C). Taken together, these results demonstrate that blockade of the

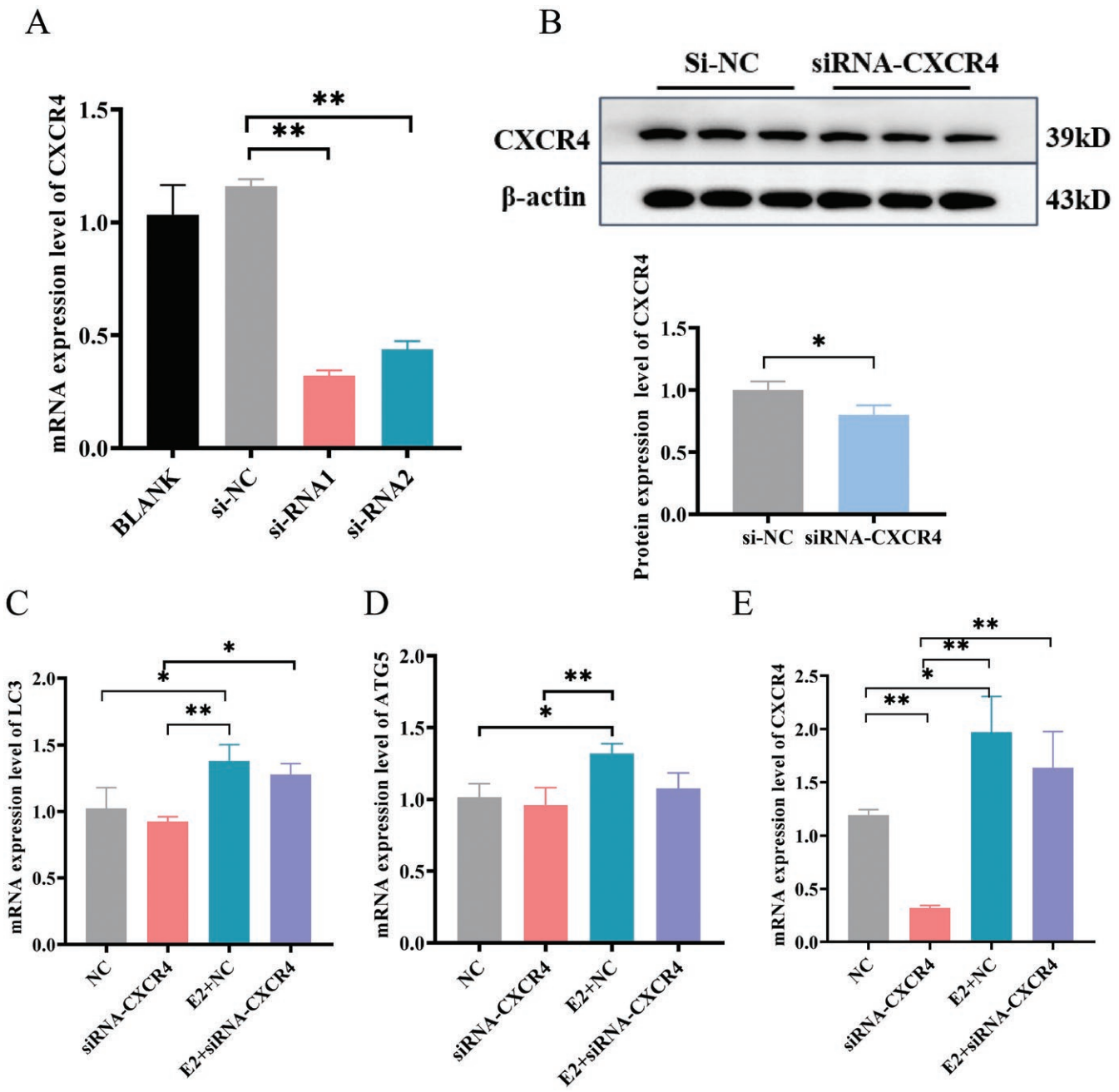


Figure 7. Effects of CXCR4-siRNA on autophagy flux in SMECs. (A) The interference efficiency of siRNA in MECs. (B) The abundances of CXCR4 protein in SMECs transfected with CXCR4-siRNA. (C and D) The mRNA expression levels of *LC3* and *ATG5* in SMECs were transfected with CXCR4-siRNA, and/or treated with estrogen. (E) The mRNA expression levels of CXCR4 in SMECs were transfected with CXCR4-siRNA, and/or treated with estrogen. $n = 3$, * $P < 0.05$, ** $P < 0.01$.

CXCL12/CXCR4 signaling axis inhibits estrogen-associated autophagy in MECs.

Discussion

In this study, we demonstrated that estrogen upregulates autophagy and elevates CXCL12/CXCR4 expression in SMECs. Notably, we also revealed that estrogen promotes autophagy in SMECs through the activation of the CXCL12/CXCR4 signaling axis, elucidating the physiological mechanisms by which estrogen regulates autophagy in the mammary gland.

Estrogen plays an important role in physiological processes in the mammary gland, regulating development and inducing changes in the immune microenvironment, either directly through specific receptors or indirectly via growth factors (Janowski et al., 2022, Zhang et al., 2019; Tower et al., 2022). Previous studies have reported that estrogen increases the formation of the membrane-bound form of LC3 (LC3-II) and the expression of LC3-I in bovine MECs (Sobolewska et al., 2011). Consistent with these findings, our study demonstrated that 50 μM estrogen elevated the expression levels of LC3 and ATG5 in SMECs. LC3-II and p62 are well-established

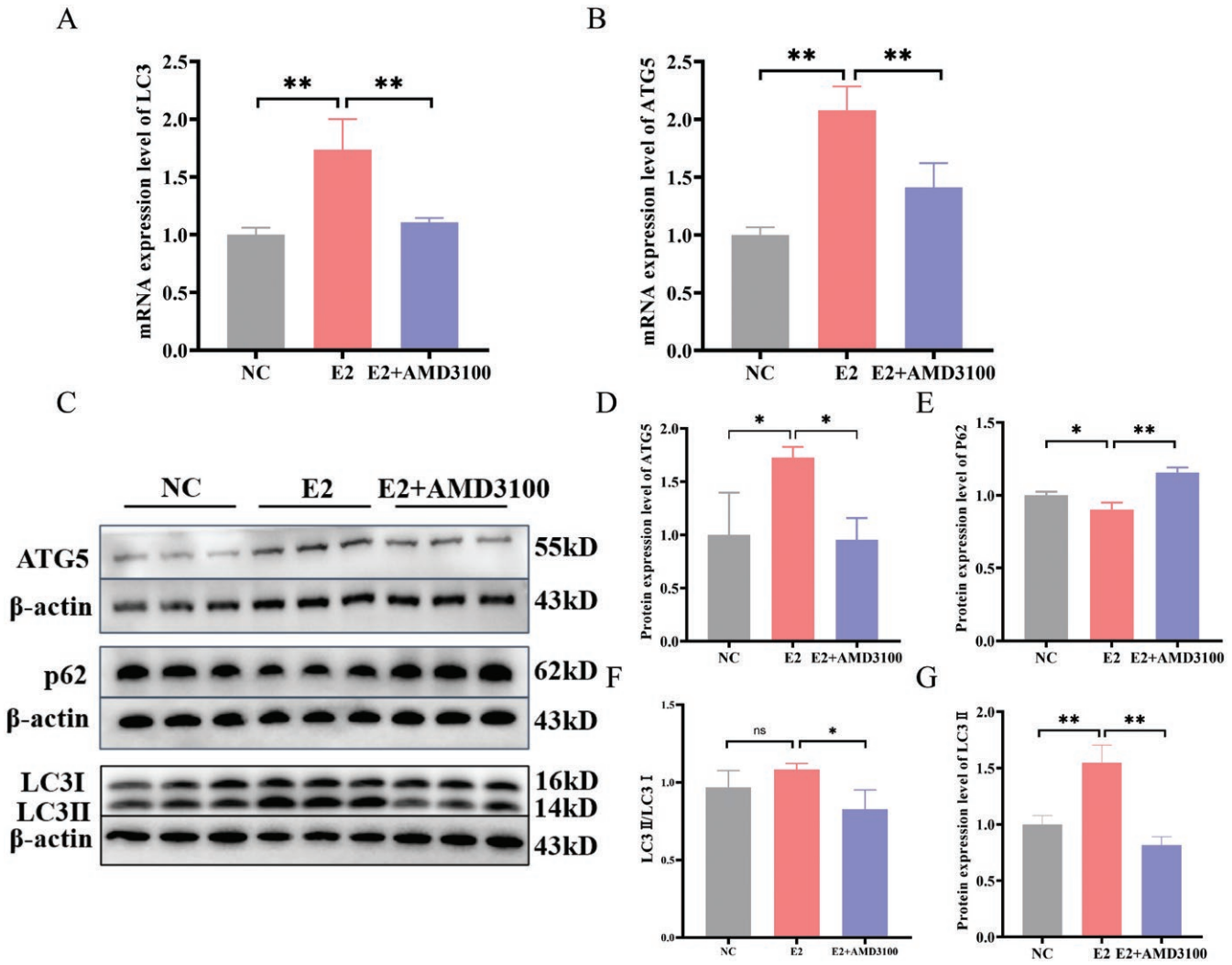


Figure 8. Blocking CXCL12/CXCR4 signaling inhibits estrogen-promoted autophagy flux in SMECs. (A and B) The mRNA expression levels of *LC3* and *ATG5* in SMECs were transfected with AMD3100, and/or treated with estrogen. (D to H) The abundances of LC3, ATG5, and p62 proteins in SMECs were transfected with AMD3100, and/or treated with estrogen.

markers of autophagic activity. Specifically, LC3-II is correlated with the number of autophagosomes, while p62 serves as an autophagic substrate, binding to ubiquitylated proteins and LC3-II to induce autophagic degradation (Mizushima et al., 2007; Gao et al., 2021). Meanwhile, ATG5 is an important molecule involved in the induction of autophagy (Arakawa et al., 2017). In summary, these findings demonstrate that estrogen promotes autophagy in SMECs.

Autophagy primarily involves the following steps: upon receiving autophagy-inducing signals, cells form phagophore membranes that selectively or non-selectively phagocytose cytoplasmic substances, forming autophagosomes; these autophagosomes dock and fuse with lysosomes to form autolysosomes, which degrade the encapsulated contents to meet cellular metabolic demands and facilitate the renewal of mitochondria and endoplasmic reticulum (Klionsky et al., 2000; Sun et al., 2021). In the present study, the numbers of autophagosomes and autolysosomes were found to be significantly higher in estrogen-treated SMECs, indicating enhanced autophagy. Additionally, the number of full fused autolysosomes exceeded that of unfused autophagosomes, an established characteristic of autophagy (Nakamura et al., 2017).

CXCL12, a chemokine belonging to the C-X-C subfamily, exerts its function by binding to CXCR4 (Luo et al., 2016). Estrogen activates the CXCR4/CXCL12 pathway in mammary cells; meanwhile, CXCR4 signaling promotes estrogen transcriptional activation (Sauve et al., 2009). In the present study, we determined that 50 μM estrogen promotes CXCL12/CXCR4 expression, suggesting that activation of the CXCL12/CXCR4 axis in SMECs may be linked to estrogen. Similarly, prior studies have shown that estrogen regulates endometrial stromal cell physiology through CXCL12/CXCR4-dependent autophagy (Mei et al., 2015).

To elucidate the physiological regulatory mechanisms of CXCL12/CXCR4 signaling and autophagy in MECs, we constructed a CXCR4 overexpression vector (pcDNA-CXCR4). However, transfection of SMECs with pcDNA-CXCR4 did not enhance the expression levels of the autophagy-related genes *ATG5* and *LC3*, likely due to the need for CXCL12 to bind to its receptor CXCR4. Recent studies suggest that CXCL12 can also bind to CXCR7(Romain et al., 2014). Therefore, using the 3-dimensional structure of CXCR4, we identified a small-molecule substitute of CXCL12, NUCC-390, which specifically binds

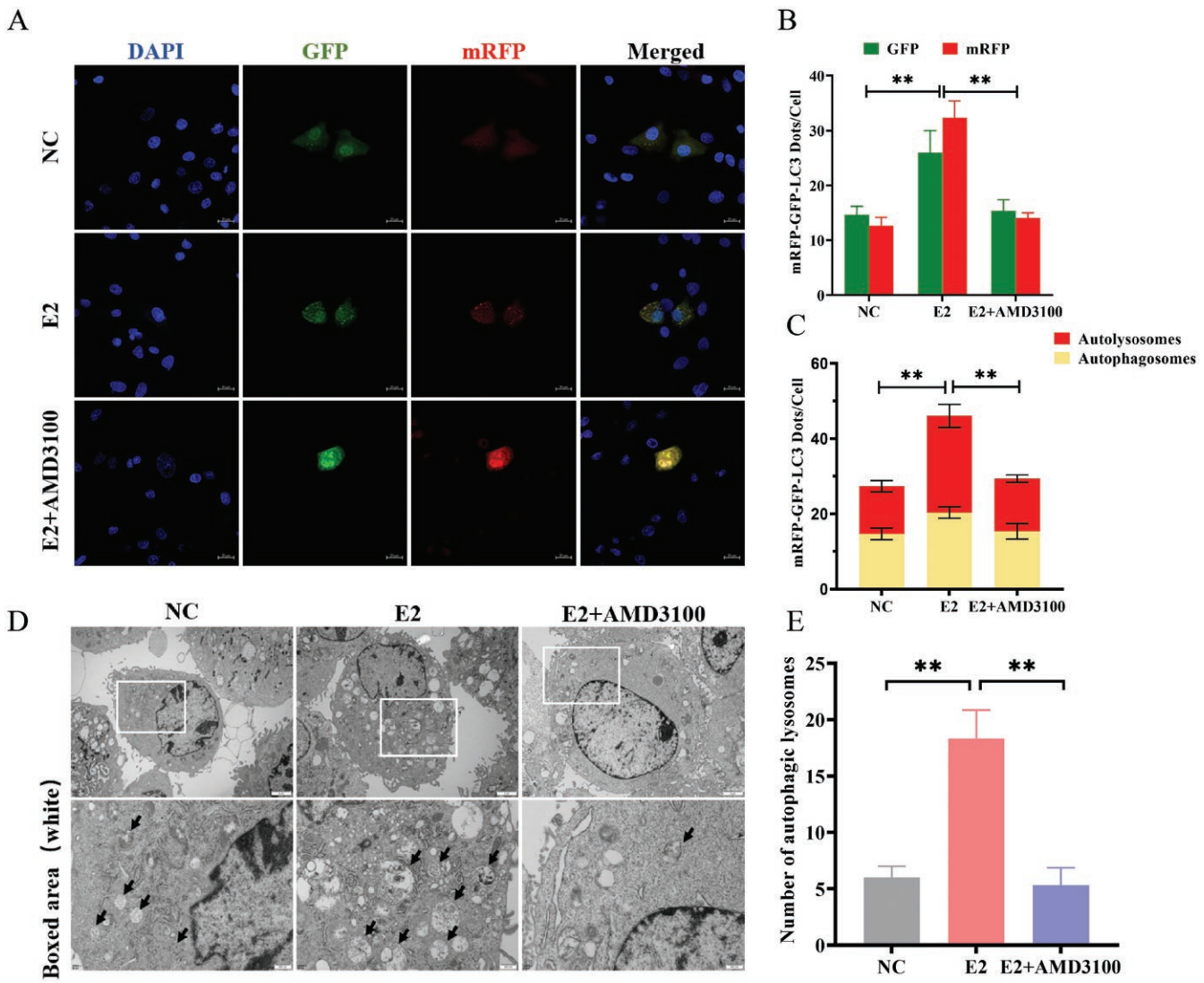


Figure 9. Effects of blocking CXCL12/CXCR4 signaling on autophagosomes and autolysosomes in SMECs. (A) Representative images of autophagosomes (GFP) and autolysosomes (mRFP) observed under confocal microscopy. Scale bar = 20 μm; MECs were transfected with estrogen + ADM3100. (B and C) Quantitative statistics of autophagosomes and autolysosomes observed by laser confocal microscopy. D. Representative images of autolysosomes observed under TEM. Scale bar = 2 μm or 500 nm; SMECs were transfected with estrogen + ADM3100. E. Quantitative statistics of autolysosomes observed under TEM. $n = 3$, * $P < 0.05$, ** $P < 0.01$.

to CXCR4. Moreover, prior study has demonstrated that NUCC-390 can activate the CXCR4 receptor (Negro et al., 2019). Notably, our study also revealed that treatment with pcDNA-CXCR4 and NUCC-390 significantly increased autophagy levels in SMECs, demonstrating that CXCR4 specifically binds to CXCL12 to activate its functional role, and thus positively regulates autophagy via the CXCR4/CXCL12 signaling axis.

Several studies have explored the mechanism by which CXCR12/CXCR4 regulates autophagy as a target for disease prevention and cancer therapy (Yin et al., 2018; De Sanctis et al., 2023; Zhang et al., 2023). Notably, our study revealed that the mTOR signaling pathway was inhibited by pcDNA-CXCR4 + NUCC-390 treatment. The mTOR signaling pathway is a crucial autophagy regulatory pathway, with its inhibition promoting the formation of the autophagy initiation complex, thereby promoting cellular autophagy (Jung et al., 2010; Deleyto et al., 2021). Ultimately, this suggests that the CXCL12/CXCR4 signaling axis may regulate autophagy via the mTOR signaling pathway.

Estrogen enhances cellular autophagy and protects organs through various pathways. In neuronal cells, estrogen enhances autophagy and promotes the degradation of β-amyloid, thereby providing neurological protection in patients with Alzheimer's disease (Cui et al., 2022). In human neuroblastoma cells, overexpression of the estrogen receptor promotes the expression of autophagy-related genes *LC3* and *ATG7*, leading to an increase in the number of autophagosomes (Wei et al., 2019). To further confirm the role of CXCL12/CXCR4 in estrogen-promoted autophagy, we performed mRNA interference assays. Although siRNA-CXCR4 treatment significantly reduced CXCR4 expression levels, it failed to reduce the upregulated autophagy induced by estrogen. This may be due to the stronger effect of estrogen on *CXCR4* expression compared to siRNA-CXCR4 interference.

Using virtual molecular docking technology, we identified AMD3100, a small-molecule compound that possesses a stronger binding ability to CXCR4 than CXCL12. Thus, AMD3100 competitively binds to CXCR4 and is used as a target for the treatment of CXCR4-related cancer, inflammation, and acquired immune deficiency syndromes (De Clercq, 2003). In the present study, co-treatment of SMECs with AMD3100 and estrogen significantly inhibited estrogen-associated autophagy. These results suggest that estrogen-associated promotion of autophagy in SMECs is mediated by activation of the CXCL12/CXCR4 signaling axis.

In conclusion, our study demonstrated that 50 μM estrogen treatment can enhance autophagy in SMECs. Similarly, the CXCL12 substitute NUCC-390 activated the CXCR4 receptor to promote autophagy. Meanwhile, the CXCR4-specific blocker AMD3100 inhibited estrogen-associated promotion of autophagy. Thus, our study revealed that estrogen enhances autophagy in SMECs through the CXCR12/CXCR4 signaling axis. These findings provide a theoretical foundation for understanding the regulation of autophagy by this signaling axis. However, our study has certain limitations. In particular, we only demonstrated the regulatory mechanisms of estrogen, the CXCR12/CXCR4 signaling axis, and autophagy in vitro. Therefore, future research should focus on understanding how estrogen and autophagy regulate physiological processes during different lactation periods and how these processes impact the lactation performance of dairy sheep; ultimately, these studies may provide new perspectives for improving the economic value of dairy animals.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Disclosures

None of the authors have any conflict of interest to declare.

Author Contributions

Tingting Chu (Conceptualization, Data curation, Writing—original draft), Jiashun Tong (Funding acquisition, Investigation), Zhongshi Zhu (Formal analysis, Methodology), Lei Sun (Validation, Visualization), Jiuzeng Cui (Resources, Software), Yue Jiang (Methodology, Supervision), Jiaxin Liu (Formal analysis, Software), Ahmad Naseer (Project administration, Validation), Lei Zhang (Software, Supervision), and yuxuan song (Conceptualization, Funding acquisition, Project administration, Writing—review & editing)

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