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17β-ESTRADIOL REGULATES ADENOSINE TRIPHOSPHATE-BINDING CASSETTE TRANSPORTERS A1 EXPRESSION *VIA* ESTROGEN RECEPTOR A TO INCREASE MACROPHAGE CHOLESTEROL EFFLUX

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The liver is the focus of research on the effects of estrogen on cholesterol metabolism. Few studies have investigated the effects of estrogen on macrophages despite the significance of cells in atherosclerosis. The purpose of this study is to examine the effect of estrogen on macrophage cholesterol efflux. Macrophage cholesterol efflux, oil red O staining, RT-qPCR, Western blotting analyses were used to determine cholesterol metabolize and the expressions of adenosine triphosphate (ATP)-binding cassette transporter G1 (ABCG1) and ATP-binding cassette transporter A1 (ABCA1) in J774A.1 cells, and the effect of these treatments was compared to without adding 17β-estradiol (E2). Gain and loss of estrogen receptor alpha (ERα), liver X receptor α (LXRα) were conducted to study interactions between E2, ERα, LXRα and ABCA. Finally, in mice, we validate the relationship between ERα and ABCA1. E2 increases cholesterol efflux from macrophages and decreases the formation of lipid droplets and positively regulates the expression of ABCA1. This suggests that estrogen receptors (ERs) directly regulate ABCA1 translation. We suppressed ERα, which decreased the mRNA and protein expression of ABCA1. At the mRNA level, E2 treatment could partially counteract these phenomena, but not at the protein level. ABCA1 expression decreased after LXRα was inhibited. This suggests that ABCA1 translation is directly regulated by ERα. In the ovariectomized mouse model of ABCA1 protein expression was significantly reduced in the peritoneal macrophages of the ovariectomy (OVX) group. ABCA1 protein expression was greater in the E2+OVX group than in the OVX group. E2 contributes to the positive regulation of ABCA1 expression and promotes cholesterol efflux in macrophages by binding to ERα. The effect is independent of ABCA1 transcription regulation by LXRα.

Key words: *Adenosine triphosphate-binding cassette transporter A1, cholesterol efflux, estradiol, estrogen receptor alpha, liver X receptor alpha, ovariectomy, macrophage, atherosclerosis*

INTRODUCTION

It is anticipated that cardiovascular and cerebrovascular diseases will remain the leading causes of death on a global scale through 2040 (1). Atherosclerosis (AS) is the pathophysiologic process underlying cardiovascular and cerebrovascular diseases. AS macrophages are involved in the initiation, progression, and rupture of an infection (2). The core of atherosclerotic plaques is formed by lipid-laden macrophages that undergo further differentiation into foam cells during the AS process. Reverse cholesterol transport (RCT) describes how high-density lipoprotein (HDL) transports cholesterol from macrophages to the liver, where it is metabolized into bile acid (3). RCT can enhance the regression of AS plaques and reduce plaque instability. Cholesterol efflux from macrophages to HDL is the initial step of RCT; therefore, the cholesterol efflux capacity (CEC) is the key to the anti-atherosclerotic action of HDL (4). Previously, it was believed that HDL levels and AS levels were inversely related (5). However, current research suggests that HDL-mediated CEC correlates negatively with coronary artery disease risk and severity, but not with HDL or ApoA-I levels (6-9). The CEC of macrophages is predominantly determined by two factors: the ability of cells to release cholesterol and the capacity of plasma HDL to bind to cholesterol as an extracellular cholesterol receptor (10, 11).

The protective effects of estrogen on the cardiovascular system have been the subject of extensive research for a very long time. Observation of cholesterol metabolism *in vivo* by 3H cholesterol-labeled macrophages revealed that the estrogen receptor alpha (ERα) signal in hepatocytes enhanced the CEC of foam cells in female mice and facilitated cholesterol absorption by the liver (12). Population-based studies indicate that women with polycystic ovary syndrome (low estrogen levels) have a lower plasma CEC, and hormone replacement therapy can effectively raise plasma CEC in postmenopausal women (13, 14). These results indicate that estrogen can positively regulate HDL-involved macrophage CEC. Macrophages primarily transport cholesterol through cell membrane proteins - ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) (15-17). ABCA1 and ABCG1 are positively regulated by nuclear receptor transcription factors (LXRs) of the liver X receptor. Moreover, estrogen receptors (ERs)

are a member of the nuclear receptor transcription family. However, the role of ERs in the transcriptional regulation of ABCA1 and ABCG1 in macrophages remains unclear.

Estrogen primarily exerts its biological effects by interacting with ERs. ERs include two subtypes, ERα and ERβ, which are encoded by two distinct genes, ESR1 and ESR2. Following the combination of two proteins, dimers are transferred to the nucleus. They regulate target gene transcription by binding directly to estrogen response elements (EREs) or indirectly to DNA *via* other transcription factors (18, 19). Using NCBI to query the ABCA1 promoter sequence, the ABCA1 promoter contains an ERE-binding binding sequence that plays a role in ERE binding: GGTCA. Consequently, estrogen may contribute to regulating ABCA1 transcription through ERs. In this study, we examined the effect of 17β estradiol (E2) on macrophage cholesterol efflux to determine whether estrogen/ERs regulate ABCA1 expression.

MATERIALS AND METHODS

Cell culture

The mouse macrophage J774A.1 cells were obtained from Procell Biology (Wuhan, China) and cultivated in Phenol Red-Free DEME (BI, Beit-Haemek, Israel) with 10% FBS (Gibco, Carlsbad, CA, USA). Infection was prevented by adding 1% Penicillin-Streptomycin Solution (BI, Beit-Haemek, Israel) and 0.5 ug/ml Mycoplasma Removal Agent (Beyotime Biotechnology, Shanghai, China). The cells were grown in a 37ºC and 5% CO_2 incubator. Estradiol (Beyotime Biotechnology, Shanghai, China) and fulvestrant (MCE, Monmouth Junetion, New Jersey, USA) were dissolved in DMSO (Sigma, USA) and kept in a –80ºC refrigerator.

Oil red O staining of cells

In 6-well plates, 1x105 cells were seeded overnight per well. According subgroup, add 17β-estradiol or fulvestrant to incubate cells. After treatment, the cells were incubated without FBS for 24 hours with 50 μg/ml oxidized low-density lipoprotein (ox-LDL) (Yiyuan Biotechnology, Guangzhou, China). Oil red O staining was conducted as described in a previous study (20). The cells were fixed for 30 min in a cell fixative. The oil red O working solution (Solarbio, Beijing, China) was then added and allowed to stand at room temperature for 15 min. After washing the cells, they were observed and photographed under a microscope. Oil red O was eluted with pure isopropanol for quantitative analysis; the absorbance (OD value) was measured at 510 nm with an enzyme standard instrument.

Macrophage cholesterol efflux

The mouse macrophage cell line J774A.1 cells were plated at 5×104 cells per well, in a 24-well plate; the culture medium was DMEM without phenol red and supplemented with 0.2% BSA without fatty acid. After 24 hours of treatment per the experimental design, the medium was replaced with one containing 5 μM 22-NBD-cholesterol (Bailingwei Technology Co., Ltd., Beijing, China) and incubated for 4 hours. The cells were then washed with PBS before new media was added containing 50 μg/ml HDL (Yiyuan Biotechnology, Guangzhou, China) or apoB-depleted serum and incubated for 4 hours. After transferring the supernatant to new EP tubes, the cells were lysed with 1% Triton (Triton™ X-100) for 10 min. After collecting the lysate in an EP tube, it was centrifuged for 10 min. After collecting the lysate in an EP tube, it was centrifuged at 10000 rpm for 10 min. Lastly, supernatants and lysate were added to 96-well black plates (100 μl per well). Fluorescence (FI) was measured in a microplate reader (Gemini EM Fluorescence Microplate Reader) using 469 nm as the excitation wavelength and 537 nm as the emission wavelength. NBD-cholesterol efflux rate = the supernatants FI/(the supernatants FI+ the lysate FI) × 100%.

RNA isolation and rt-qPCR

rt-qPCR was used to analyze mRNA expression as described in a previous study (21). J774A.1 RNA was extracted using the

Table 1. Mouse primers for target gene.

Gene	**Primer**
ABCA1	Forward: 5'-AGAAGGAGGCTCGGCTGAAGG- 3' Reverse: 5'-GAGGGATGAGGCTGCTAACAAACC- 3'
ABCG1	Forward: 5'-CATGCTGCTGCCTCACCTCAC- 3' Reverse: 5'-TCTCGTCTGCCTTCATCCTTCTCC- 3'
ERα(ESR1)	Forward: 5'-GCCGCCTTCAGTGCCAACAG- 3' Reverse: 5'-GGCTCGTTCTCCAGGTAGTAGGG- 3'
LXRα(NR1H3)	Forward: 5'-AACTGAAGCGGCAAGAGGAAC- 3' Reverse: 5'-TGGCAGGACTTGAGGAGGTGAG- 3'
GAPDH	Forward: 5'-TGGTGAAGCAGGCATCTGAG- 3' Reverse: 5'-TGAAGTCGCAGGAGACAACC- 3'

Table 2. siRNA sequence for mouse target gene.

Gene	**Primer**
ERα(ESR1)	sense: 5'-GAAGGCUGCAAGGCUUUCUUUTT- 3' antisence: 5'-AAAGAAAGCCUUGCAGCCUUCTT- 3'
LXRα(NR1H3)	sense: 5'-GAAACUGAAGCGGCAAGAAGATT - 3' antisence: 5'-UCUUCUUGCCGCUUCAGUUUCTT- 3'

TRIzol reagent (Invitrogen, Carlsbad, CA, USA); 2 ug RNA was used to reverse to cDNA (Thermo Scientific, Wilmington, USA). rt-qPCR was performed with 2 µl of cDNA using the SYBR Mix (Qiagen, Hilden, Germany) on an ABI Prism 7500 real-time PCR System (Applied Biosystems). The specific primers were designed and synthesized by Shenggong Bioengineering Technology Limited (Shanghai, China). The primers used for ABCA1, ABCG1, ESR1, LXRα, and GAPDH are listed in *Table 1*. rt-qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Western blot

As described in a previous study, Western blot analysis was performed (22). The cells were collected and lysed in a RIPA buffer containing 1% cocktail protease inhibitor. Using 10% SDS-PAGE, the proteins were separated and transferred to a PVDF membrane. The PVDF membrane was blocked by a buffer containing 5% non-fat milk. The membrane was then incubated at 4ºC overnight with primary antibodies against ABCA1 (1:800 dilution, Abcam, Waltham, MA, USA), ABCG1 (1:2000 dilution, Abcam, Waltham, MA, USA), LXRα (1:1000 dilution, Abcam, Waltham, MA, USA), Erα (1:1000, Abcam, Waltham, MA, USA), and GAPDH (1:5000 dilution, CST, Cambridge, UK). The sample was then incubated at room temperature for 1.5 hours with peroxidase conjugated goat anti rabbit IgG or anti-mouse IgG labeled secondary antibodies (1:5000 dilution, BIOSS, Beijing, China). Using an ECL kit for ElectroChemi-Luminescence (ECL) detection, specific complexes were visualized (Affinity Bioreagents, Golden, CO, USA).

Si-RNA transfection

Small interfering RNA (siRNA) transfections were performed utilizing the Rect siRNA/miRNA Transfection Reagent (Changzhou Biogenerating Biotechnology Corp., Jiangsu, China). The molecule design and synthesis of all siRNAs were obtained from Sangon (Shanghai, China), including siERα, siLXRα, and sivector. siRNAs were transfected at a final concentration of 30 nM for cell transfections. The cells were harvested 48 hours after transfection, to complete the associated experiments. As a negative control, mock cells were transfected with the RFect siRNA transfection reagent. All siRNAs utilized in our study are listed in *Table 2*.

Flow cytometry

After being fixed with methanol for 5 min, the cells were permeabilized with 0.1% Tween for 20 min. To inhibit nonspecific interactions, the cells were subsequently incubated with 10% goat serum. The cells were then incubated at room temperature for 30 min with 2 µg of primary antibodies against ABCA1 (Abcam, Waltham, MA, USA) / 1×10^6 cells. Goat Anti-Mouse IgG/PE antibody (bs-0296G-PE, BIOSS, Beijing, China) was used as the secondary antibody at a 1/100 dilution for 30 min at room temperature. A negative control lacking the primary antibody was formulated and 10,000 events were held. FlowJo was used to perform the data analysis. Data analysis was conducted using FlowJo.

Animal experiments

Seven-week-old female $apoE^{-/-}$ mice were purchased from Beijing Sibeifu (the production license number of the experimental animals is SCXK (Beijing, China) 2019-0010). After a week of acclimation, the ovaries of the mice in the ovariectomized group were surgically removed, whereas the ovaries of mice in the sham-operated group were left intact. During the same 8-week period, the

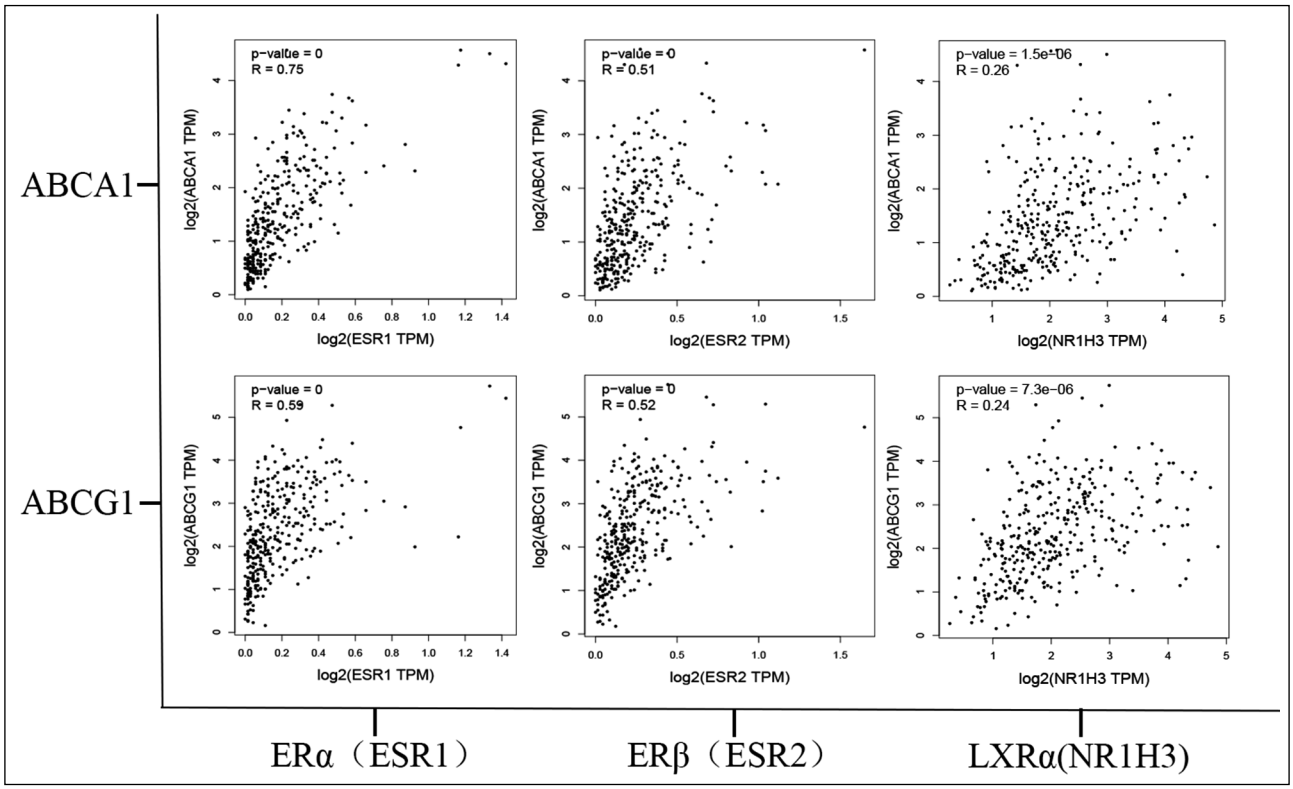

Fig. 1. The correlation between ERs and cholesterol efflux related gene expression. The GEPIA database revealed a correlation between ERs and ABCA1/G1 gene mRNA in peripheral blood samples from healthy individuals. We analyzed the correlation using Pearson's correlation analysis; R was the Pearson's correlation coefficient.

estrogen gavage group received 0.3 mg/kg/d of 17β-estradiol (Solarbio, Beijing, China), whereas the control group received the same volume of solvent. Then, each mouse was fed a Western diet containing 0.15% cholesterol and 40% fat.

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee and the Animal Ethics Committee of the First Afliated Hospital of Xinjiang Medical University in XinJang, China.

Oil Red O staining of frozen sections of aortic root

After 30 min of fixation with a tissue fixative containing 4% paraformaldehyde , the mouse heart was dehydrated and then embedded in OCT. Coronal sections were cut using the freezing microtome (8–10 μM). The angle of the cut plane was perpendicular to the tricuspid valve's orifice. The slices were stored in a refrigerator at –40ºC as a precaution. Before being stained for 15 minutes in the dark with oil red O, slices were soaked for 10 minutes in PBS solution. They were then soaked and washed 3 times with PBS for 5 minutes each. After washing, the films were sealed with glycerin, and images were captured under a microscope.

Isolation of mouse peritoneal macrophages

The abdominal cavities of mice were injected with 3 ml of sterile 3% thioglycolate. After 72 hours, the mice were euthanized and their peritoneum was lavaged with 1640 medium that had been cooled to 4ºC. We repeated the procedure once to collect all macrophages. The cell suspension was centrifuged at 1000 rpm for 8 minutes and the supernatant was discarded. After resuspending the cell pellet in 1640 medium containing 20% FBS, the cells were seeded in a cell culture dish and incubated for 2 hours. The adhesion-producing cells were macrophages.

Statistical analysis

A minimum of three independent replicates was carried out in each experiment. IBM SPSS Statistics 23.0 was used to conduct statistical analyses and GraphPad Prism 8 was used. Whether the quantitative data conformed to a normal distribution was tested by the Kolmogorov-Smirnov test. The normally distributed quantitative data were described as mean ±standard deviation (SD) and compared by one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnett test. The non-normally distributed quantitative data were expressed as medians and quartiles and compared using the Mann-Whitney U test. Correlation analysis was adopted using Pearson's correlation. P-value of <0.05 was regarded as statistically significant.

RESULTS

A correlation exists between ERα mRNA and ABCA1 mRNA in the peripheral blood of healthy individuals

To develop a comprehensive understanding of the relationship between ERs and cholesterol efflux-related gene mRNA expression levels, we used the online GEPiA (Gene Expression

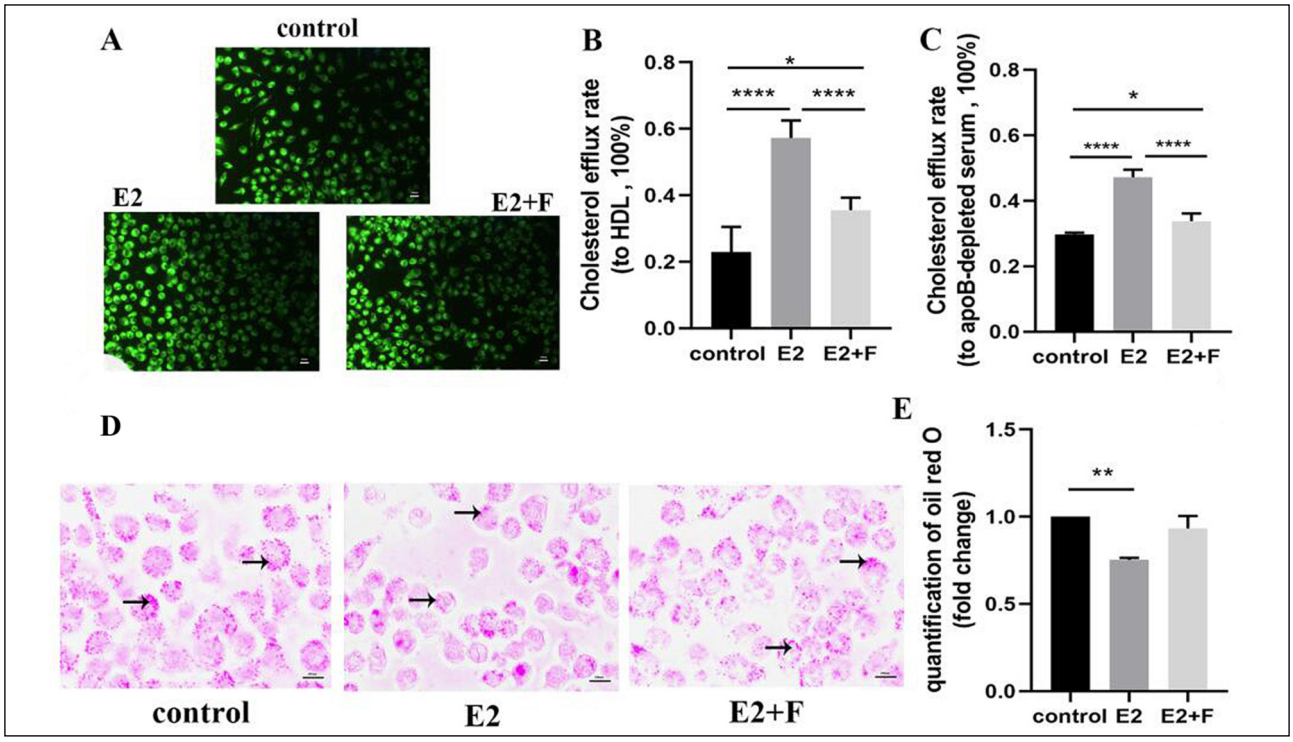

Fig. 2. Estradiol promotes cholesterol efflux from macrophages. The cells were divided into three groups: the control group, the group treated with estradiol (E2, 100 nM), and the group treated with estradiol and fulvestrant (E2+F, 1 uM). J774A.1 22-NBD-cholesterol was incubated with cells for four hours. Then, for four hours, we modified the medium to induce cholesterol efflux. (A): Fluorescence distribution of NBD in macrophages; NBD-cholesterol was responsible for the green fluorescence. (B): Comparison of the HDL-induced cholesterol efflux rates from macrophages in the three groups. (C): Comparison of the apoB-depleted serum-induced cholesterol efflux rates from macrophages in the three groups. (D): Oil-red O staining of various groups of macrophages (200× magnification). The amount of oil red O stained lipid droplets in the macrophages of the E2 group was significantly lower than that of the other two groups (see the arrow). (E): Quantification of oil red O staining. The data were analyzed using a one-way ANOVA test with multiple comparisons. *P<0.05, **P<0.01, ****P<0.0001.

Profiling Interactive Analysis) database (http://gepia.cancer-pku.cn/detail.php?clicktag=correlation) to analyze the correlation. The monocytes in circulation give rise to the macrophages in the atherosclerotic plaque. As a result, we selected a sample of peripheral blood from a healthy human. We examined LXRα, a gene known to control the transcriptional regulation of ABCA1/G1. We used the Pearson's correlation coefficient to analyze the correlations between variables. The correlation between ERα (ESR1) and ABCA1 mRNA expression was the strongest (R=0.75). The coefficient of correlation between ABCG1 and ERα was 0.59. The correlation between LXRα and ABCA1/G1 mRNA expression was less than 0.5 (*Fig. 1*).

Estradiol-stimulated macrophage cholesterol efflux

Observing the effect of estrogen on cholesterol efflux from a mouse macrophage cell line (J774A.1), the cellular cholesterol efflux rate was measured using 22-NBD-cholesterol (green fluorescence). Cells were treated with estradiol in the presence or absence of fulvestrant, a nonselective estradiol receptor antagonist, for 24 hours. As previously stated, serum-depleted HDL and apoB induce cholesterol efflux. After 4 hours of 22-NBD-cholesterol treatment of the J774A.1 cell, the NBD-labeled cholesterol was evenly distributed throughout the cells (*Fig. 2A*). Irrespective of whether HDL or standard serum-induced cholesterol efflux, the estradiol group (E2) had a greater cholesterol efflux rate than the control group. To examine whether estradiol acted through the ERs, cells were treated with estradiol and fulvestrant (E2+F). Fulvestrant partially reversed a portion of this pro-cholesterol efflux effect (*Fig. 2B* and *2C*). This finding demonstrates that estradiol promotes cholesterol efflux through functional mechanisms in macrophages. We utilized 50 ug/ml ox-LDL to induce the transformation of macrophages into foam cells and analyzed the cholesteryl ester content of each cell group quantitatively using oil red O staining. The amount of oil red O stained lipid droplets in the macrophages of the E2 group was significantly lower than that of the other two groups (*Fig. 2D*). Oil-red O quantification revealed that E2 reduced lipid deposition in macrophage foam cells induced by Ox-LDL (*Fig. 2E*).

17β-estradiol positively regulated the expression of ABCA1, and fulvestrant could inhibit this effect

Cholesterol efflux from macrophages is known to be dependent on ABCA1 and G1. To determine whether the role of E2 in cholesterol efflux from macrophages is achieved by the regulation of ABCA1, J774A.1 cells in the presence or absence of 1 uM fulvestrant, were treated with varying concentrations of E2. Utilizing real-time PCR, the expression of ABCA1 and ABCG1 was determined. ABCA1 mRNA expression in cells was found to be increased by three different concentrations of E2 and down-regulated by E2 plus fulvestrant (E2+F) (*Fig. 3A*). ABCG1 mRNA expression was unaffected by E2, but it was reduced in the E2+F group compared to the E2 group (*Fig. 3B*). The final experimental concentration of E2 was set at 100 nM based on the results of mRNA expression. E2 was also observed to upregulate ABCA1 on the protein level (*Fig. 3C*). Furthermore, fulvestrant inhibited the upregulation of ABCA1 by E2 to a lesser extent than the control group. There were no observed variations in ABCG1 proteins (*Fig. 3C*). Given that ABCA1 is the cell membrane protein, we determined whether an E2-mediated increase in ABCA1 protein expression occurs at the cell surface, using flow cytometry. Flow cytometry results

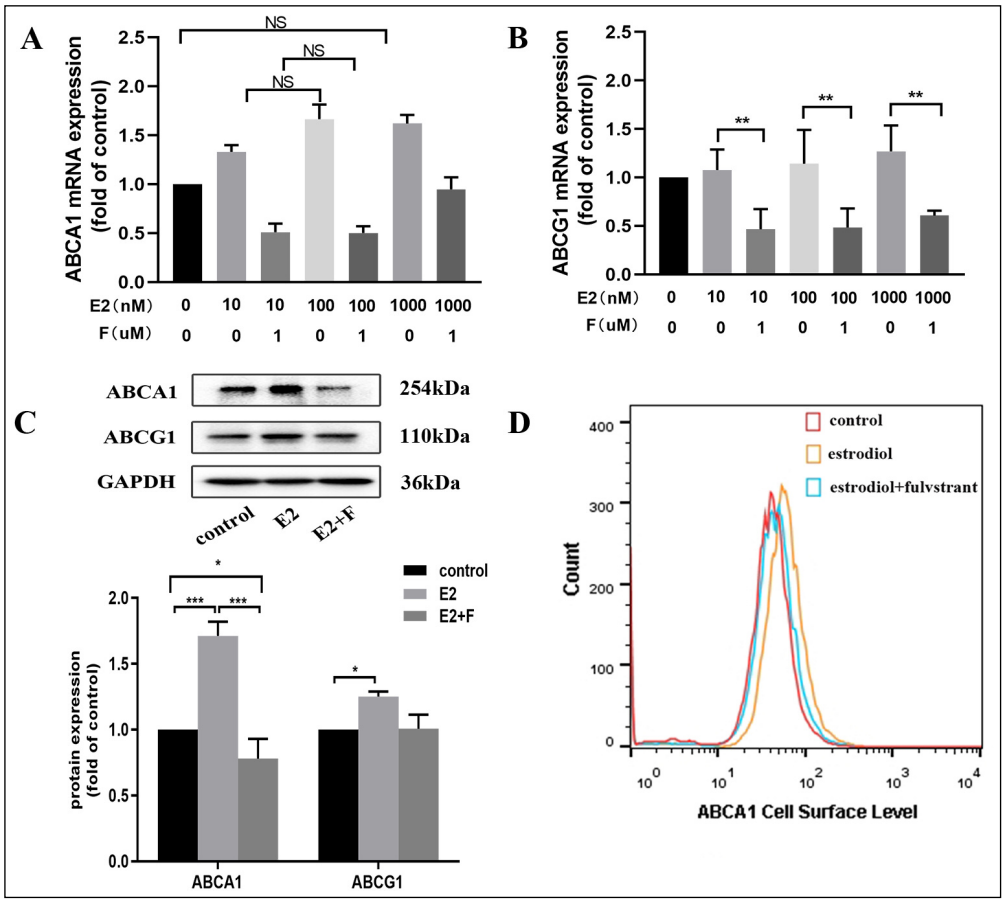

Fig. 3. The expression of ABCA1 is regulated by ERs in response to E2. RT-qPCR, Western blot, and flow cytometry were used to detect ABCA1/G1 mRNA and protein expressions after cells were treated with varying concentrations of E2 with or without fulvestrant. (A): The expressions of ABCA1 mRNA. (B): The expression of ABCG1 mRNA. (C): ABCA1/G1 protein expression. E2=100 nM, F=1 μM. (D): The expression of ABCA1 on the cell surface membrane protein. E2=100 nM, F=1 μM. The data were analyzed using a one-way ANOVA test with multiple comparisons. NS, not statistically different, **P<0.01.

indicated that estradiol can increase membrane expression of ABCA1. Likewise, fulvestrant could counteract the effect of E2 (*Fig. 3D*).

The expression of ABCA1 was upregulated by 17β-estradiol via ERα and independent of LXRα

ER antagonists prevented E2 from promoting cholesterol efflux and ABCA1 expression, which suggests that E2 regulates ABCA1 through ERs. After considering the highest correlation between ERα and ABCA1 in human peripheral blood, we hypothesized that after binding to ERα, estradiol could participate in the transcriptional regulation of ABCA1 and play a role in the transcriptional activity of nuclear receptors. Small interfering RNAs (siERα and siLXRα) were utilized to suppress the expressions of ERα and LXRα. We designed three siRNAs for ERα and LXRα and used rt-qPCR to validate one siRNA and the optimal interference conditions. In addition, correlations were calculated between ABCA1 and ERα/LXRα mRNA expression. Similar to the results of correlation analysis in human peripheral blood, the expressions of ABCA1 mRNA was highly correlated with the expression of ERα mRNA (correlation coefficient, r=0.9451) (*Fig. 4A*). Significant correlation exists between the expressions of ABCA1 mRNA and LXRα mRNA (correlation coefficient, r=0.9487) (*Fig. 4B*). Both mRNA and protein levels of ABCA1 were reduced after ERα knockdown. E2 treatment could only partially counteract these phenomena at the mRNA level, but not at the protein level (*Fig. 4C* and *4D*). To exclude the possibility of an indirect effect of LXRα, its expression was inhibited with siLXRα. Not surprisingly, the mRNA and protein levels of ABCA1 were diminished. On the basis of LXRα inhibition, it was determined that the expression of ABCA1 was augmented by E2 addition (*Fig. 4C* and *4D*). In addition to LXRα, the E2-ERα pathway plays an important role in the regulation of macrophage ABCA1 expression.

17β-estradiol can regulate the expression of ABCA1 in macrophages in vivo

ApoE$^{-/-}$ mice were divided into three groups according to the experimental design: sham operation (sham), ovariectomy (OVX), and ovariectomy plus E2 gavage (OVX+E2). Each group included 8 mice (n=8). After the abdominal localization of mice under anesthesia, a skin incision was made. The incision was then closed with sutures in the sham group. In the OVX group, the ovary and uterine cornu were removed as a marker following ovarian exploration. The OVX+E2 group received E2 on a daily basis after ovarian resection. Olive oil was administered by gavage to both the placebo and OVX groups simultaneously. The three mice groups were fed a high-fat diet. The fact that the average body weight of mice in the OVX and OVX+E2 groups was greater than that of mice in the sham group was statistically significant. However, there was no significant

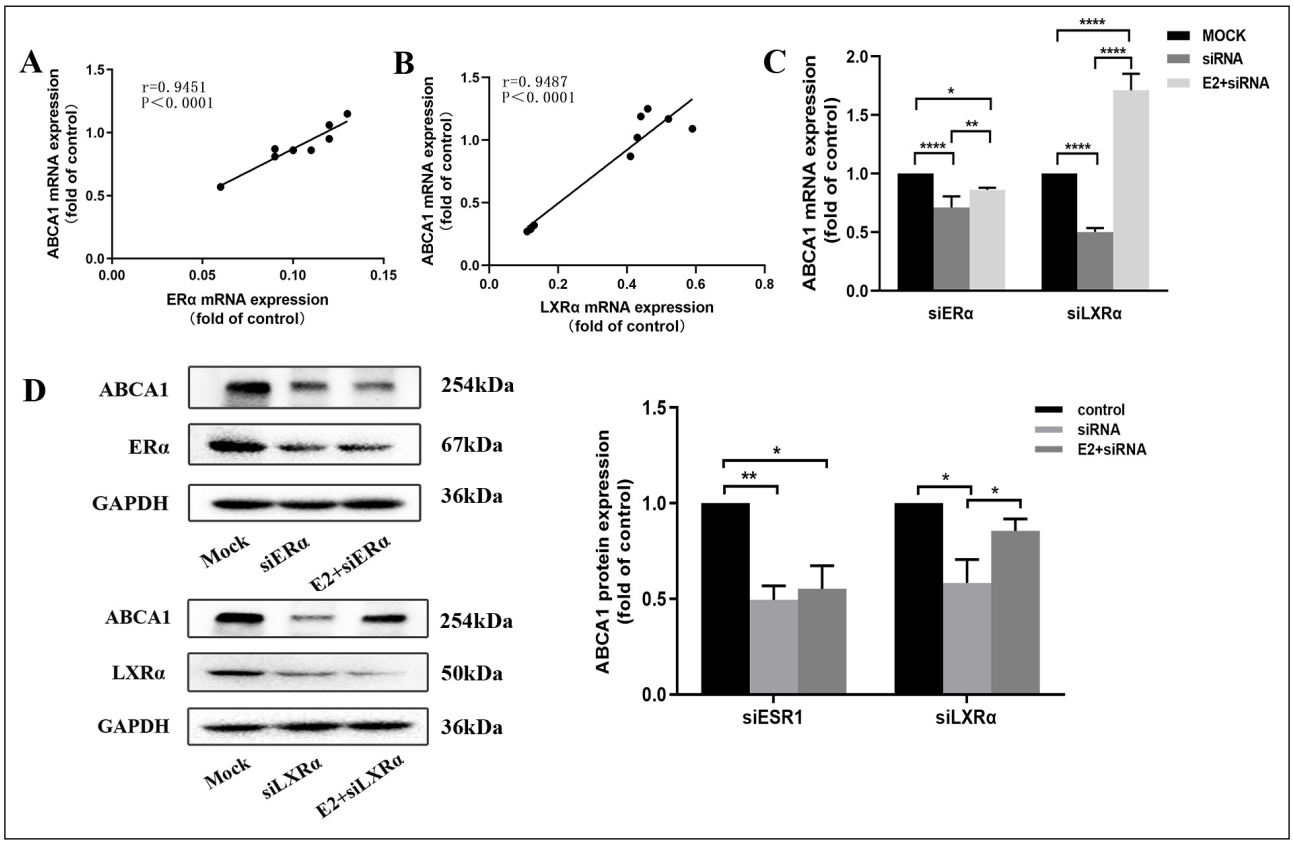

Fig. 4. Estradiol upregulates ABCA1 expression independent of LXRα *via* ERα. Small interfering RNA was used to knock down 100 copies of ERα or LXRα to detect the expression of ABCA1. As a control (mock), the same transfection reagent was used to treat cells. Concentration of E2 was 100 nM. (A): Correlation analysis between ERα and ABCA1 mRNA (Pearson's correlation coefficient). (B): Correlation analysis between LXRα and ABCA1 mRNA (Pearson's correlation coefficient). (C): Levels of ABCA1 mRNA expression following siRNA knockdown, in the presence or absence of E2. (D): Levels of ABCA1 protein expression following siRNA knockdown, in the presence or absence of E2. Pearson's correlation coefficient was used for the correlation analysis. The data were analyzed using a one-way ANOVA test with multiple comparisons. *P<0.05, **P<0.01, ****P<0.0001.

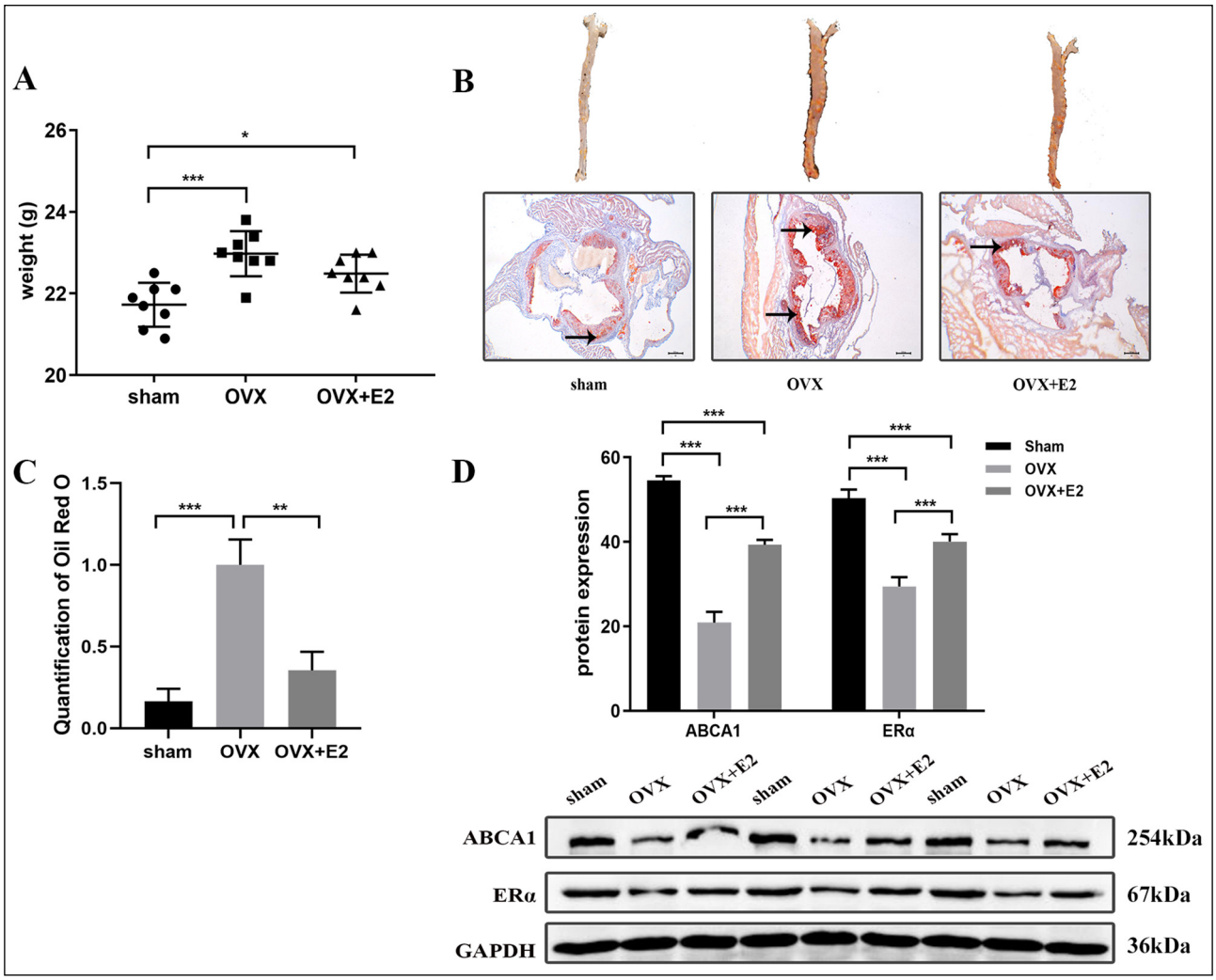

Fig. 5. E2 may regulate the expression of ABCA1 in mouse macrophages. The ApoE$^{-/-}$ mice were separated into three treatment groups: the sham operation group (sham), the ovariectomized group (OVX), and the estradiol gavage group after ovariectomy (OVX+E2). For eight weeks, the three groups of mice were fed a high-fat diet. (A): Weight comparison of mice among the three groups. (B): Aortic oil red O staining. The area stained with oil red O in the figure is the lipid deposition area in the aortic root. Lipid droplets stained with oil red O is designated by the arrow. (C): Quantitative analysis of oil red O. (D): The protein expression of ABCA1 and ERα in mouse peritoneal macrophages. The data were analyzed using a test of one-way ANOVA with multiple comparisons.*P<0.05, **P<0.01, ***P<0.001.

weight difference between the OVX and OVX+E2 groups (*Fig. 5A*). Oil red O staining of the aortic roots of the same mice revealed that the OVX group had a larger plaque area than the sham and OVX+E2 groups (*Fig. 5B* and *5C*). Macrophages were removed from the peritoneum of mice and their protein expression levels were analyzed. It was found that the OVX group had significantly lower levels of ABCA1 protein expression compared to both the sham group and the OVX+E2 group. Furthermore, protein expression of ABCA1 was lower in the OVX+E2 group, than in the sham group. Lowest ER expression was observed in the OVX group, followed by the sham group, and then the OVX+E2 group (*Fig. 5D*).

DISCUSSION

The most important risk factor for AS is local inflammation, which is triggered when cholesterol in the bloodstream attaches to the arterial wall to form lipid plaques. Animal experiments show that factors that can regulate lipid and inflammatory metabolism in the body can also regulate the process of atherosclerosis (23, 24). The accumulation of cholesterol in the form of lipid droplets and the subsequent formation of foam cells follows the phagocytosis of cholesterol-rich low-density lipoproteins (LDL) by macrophages. Macrophage-derived foam cells are the primary constituents of AS plaques. The comprehensive management of atherosclerosis can be accomplished through the regulation of macrophage-mediated inflammation and the pro-atherosclerotic pathway (25). The cholesterol efflux can reduce lipid droplets in cells and exert an anti-inflammatory effect, thereby delaying the development of AS (26). The membrane protein ABCA1 is essential for the cholesterol efflux from intracellular compartments. ABCA1 gene promoter region contains multiple transcription factor binding sites, including the ERE binding sequence (GGTCA). Previous research had focused on the mechanism by which estrogen regulates liver lipid transport and metabolism (27, 28). Our findings suggest that after binding to the ER in macrophages, estradiol can positively regulate ABCA1

expression and promote cholesterol efflux. ABCA1 can be regulated by E/ERα independently of LXRα.

Regarding the protective effects of estrogen in cardiovascular disease, the low cardiovascular risk of premenopausal women has received a lot of attention. According to studies, estrogen regulates the expression of HMG-CoA reductase and LDLR in liver cells to lower cholesterol levels in the liver and plasma (29-31). In addition, estrogen acts *via* ERα in a concentration-dependent manner, periodically inducing HDL structure and function remodeling and promoting cholesterol efflux from the liver, to reduce the risk of cardiovascular disease in reproductive-aged female mice (32). In our study, estradiol promoted cholesterol efflux and decreased intracellular lipid droplets after binding to estrogen receptors in macrophages. This demonstrates that the effect of estrogen on CEC cannot be explained solely by HDL; it is also linked to ATP-binding cassette (ABC) transporters. Whether HDL or apoB depleted serum is used as cholesterol efflux inducer, the cholesterol efflux rate in the E2 group is higher than that in the control group, and estrogen receptor antagonists can reverse the effect of estradiol on promoting cholesterol efflux. This indicates that this phenomenon is caused by the interaction between estrogen and estrogen receptor. To confirm this, we measured the expression of ABCA1 and ABCG1 in estrogen-treated J774A.1 cells. The results suggest that estradiol increased the expression of ABCA1 in macrophages, including membrane proteins. This is consistent with the findings of cell-based research (33, 34). Animal studies conducted *in vivo* revealed that myeloid-specific ER-deficient mice have reduced peritoneal macrophage ABCA1 protein expression, thereby accelerating atherosclerosis (35).

The estrogen/ERs complex can directly regulate transcription factors by binding to ERE sequences in the promoters of target genes. The sequence analysis uncovered estrogen/ERS-binding sites in the ABCA1 promoter (ERE). We confirmed that the mRNA and protein levels of ABCA1 were significantly decreased in J774A.1 cells transfected with ESR1 siRNA, and that this effect was independent of the transcriptional regulation effect of LXR on ABCA1. Knocking down LXR using siRNA. The expression of ABCA1 decreased after knocking down LXRα. After adding E2, the expression of ABCA1 increased. Additionally, at the mRNA level, ABCA1 is associated with ER, respectively, LXRα has a good positive correlation. LXRα is known to regulate the transcription of ABCA1 (36). This outcome contradicts what Wang *et al.* proposed (33). As per their findings, estradiol positively regulates LXRα *via* ERβ and indirectly regulates ABCA1 and ABCG mRNA expression in smooth muscle cells (33). ERα and ERβ have distinct tissue and cell distributions, affinities for their regulated ligands, and gene expression patterns that do not overlap (37). Consequently, the relative importance of estrogen receptors in distinct cell types may account for this disparity. Additionally, there are interactions between members of the nuclear receptor superfamily. For instance, in THP-1 macrophage-derived foam cells, the nuclear receptor PPARγ was elevated. The PPARγ-LXRα-ABCA1 axis regulates ABCA1 expression and cholesterol efflux (38). High estrogen levels in the liver of mice can enhance the binding of the estrogen-ER complex to DNA and enhance the transcriptional activity of LXRα (32). Our findings indicate that ER antagonists can significantly reduce ABCG1 expression, but the difference is not statistically significant compared to the control group. According to Rai *et al.*, estrogen increased the ABCA1 gene in the liver even in mice lacking ERα, indicating that estrogen-mediated regulation of liver ABCA1 is independent of Erα; other estrogen receptors, such as ERβ, can temporarily replace Erα's function (39).

Based on cholesterol-lowering therapy, increasing ABCA1 and ABCG1 to promote cellular cholesterol efflux is an effective strategy for reducing cardiovascular risk (36). LXR agonists can however induce SREBP-1c, resulting in elevated serum triglyceride levels and liver steatosis (40, 41). In addition to not increasing serum lipids, estrogen can promote RCT by positively regulating lipid homeostasis. It also explains why young women have a lower incidence of cardiovascular disease compared to similarly aged men. However, the risk is identical for men and postmenopausal women (due to the low estrogen state). Post-transcriptional regulation is essential for the abundance of ABCA1 proteins in addition to regulating the transcription level. Recent research has demonstrated that lncRNA MeXis can induce expression of LXRs sequences in macrophages, thereby enhancing the LXR-induced expression of ABCA1 (42). Although ERs are transcription factors, we were unable to demonstrate direct ER binding to the ABCA1 promoter or estrogen's transcriptional regulation of ABCA1 *via* ERα. Future research could use a luciferase reporter assay to confirm the direct binding of ERα and ABCA1 promoters and their participation in transcriptional activation.

The results of this study reveal that estradiol can increase ABCA1 expression *via* the ER, promote cholesterol efflux in macrophages, and reduce lipids within foam cells. Estrogen plays a complex role in the cardiovascular system. It remains debatable whether postmenopausal women benefit from hormone replacement therapy (43). Research on estrogen is advantageous for the diagnosis and treatment of AS in females. 'Precision medicine' should also account for gender differences in the diagnosis and treatment of cardiovascular diseases.

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