

Caveolin-1 functions as a key regulator of 17 β -estradiol-mediated autophagy and apoptosis in BT474 breast cancer cells

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Abstract. Estradiol (E2) acts as a crucial regulator of cell growth by mediating autophagy and apoptosis in breast cancer cells. Caveolin-1 plays a key role in carcinogenesis through its diverse roles in membrane trafficking, cholesterol transport and cellular signal transduction. However, it remains unknown as to how caveolin-1 is associated with E2-mediated autophagy and apoptosis in breast cancer cells. To resolve this issue, in the present study, we used the human breast cancer cell line, BT474, in which caveolin-1 is abundantly expressed. We demonstrated that treatment with E2 increased the expression of caveolin-1, high mobility group box 1 protein (HMGB1) and autophagy-related proteins [Beclin-1, light chain (LC3)-II and Atg12/5] in a time-dependent manner and inhibited the apoptosis of BT474 cells. Following the knockdown of caveolin-1 expression using small interfering RNA (siRNA), the expression of HMGB1, LC3-II and Atg12/5 was decreased, autophagosome formation was inhibited and apoptosis was induced; however, Beclin-1 expression was not affected. Furthermore, we knocked down HMGB1 to validate the role of HMGB1 in E2/caveolin-1-regulated autophagy and apoptosis. Notably, the knockdown of HMGB1 decreased the expression of Beclin-1 and LC3-II and attenuated autophagosome formation and promoted apoptosis. Furthermore, caveolin-1 or HMGB1 knockdown markedly suppressed E2-induced cell growth. These results suggest that caveolin-1 is a positive regulator for E2-induced cell growth by promoting autophagy and inhibiting apoptosis in BT474 cells.

Introduction

Autophagy, programmed cell death type 2, serves as a catabolic process involving the degradation of intracellular proteins through autophagosomic-lysosomal mechanisms. Autophagy may be triggered by different stimuli, such as growth factor and

nutrient deprivation, hypoxia, cancer and other pathological conditions (1-4). Autophagy is usually known to be important for cell survival and demonstrates pro-tumor properties in carcinogenesis (5).

Apoptosis, programmed cell death type 1, differs from autophagy in morphological characteristics and physiological procedures. In general, apoptosis constitutes the primary type of cell death, and it plays an anti-survival role in a variety of intracellular and extracellular conditions. However, tangle-some interrelationships still exist between autophagy and apoptosis. Both synergetic and antergic effects are exerted between autophagy and apoptosis, depending on the cellular environment, oncogenic stage and stimuli (6,7). Multiple regulatory signaling pathways are involved in autophagy, such as PI3K, mTOR and p53 (8,9).

Caveolin-1, a 22-24-kDa scaffolding protein, acts as a critical structural component of caveolae, which constitute the main invaginations of the plasma membrane (10). Accumulating evidence has indicated that caveolin-1 functions as a critical regulatory factor in a diversity of cellular and extracellular processes, including endocytosis, cholesterol homeostasis and signal transduction (11-13). Furthermore, caveolin-1 plays important roles in tumorigenesis and demonstrates tumor-promoting and suppressing effects depending on the tumor cell types and subtypes (14,15). Studies have demonstrated that caveolin-1 plays key roles in the regulation of autophagy and apoptosis (16,17).

17 β -estradiol (E2) constitutes one of the 3 major estrogens (17 β -estradiol, estrone and estriol) in women and exerts genomic and non-genomic effects in a variety of cell types, such as breast cancer cells, osteoblasts, neurons and pancreatic cells (18-20). Several studies have suggested that E2 exerts protective effects by promoting autophagy and inhibiting the apoptosis of cells (21). However, E2 displays a protective role by suppressing ferrous citrate-induced autophagy in neurons (22). Therefore, the role and molecular mechanisms of action of E2 in autophagy and apoptosis remain elusive and controversial.

In this study, we demonstrate that in BT474 human breast cancer cells (ER⁺), caveolin-1 is highly expressed and serves as a crucial regulator of E2-mediated autophagy and apoptosis.

Materials and methods

Cell culture and treatment with E2. The BT474 human breast cancer cell line was purchased from the American Type Cell

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Culture (ATCC; Manassas, VA, USA) and the cells were cultured in RPMI-1640 medium (Gibco-Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-Life Technologies) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) in the presence of 5% CO₂ at 37°C.

E2 was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The cells were plated in 6-well plates at a density of 5,000 cells/well. The cells were washed and starved for 24 h in serum-free RPMI-1640 medium (Gibco-Life Technologies) after up to 60-80% confluence to eliminate the potential influence of estrogen in fetal bovine serum, and to trigger starvation-induced autophagy. The cells were then cultured in serum-free RPMI-1640 medium in the presence of 200 ng/ml E2 or 0.02% ethanol as the vehicle. The cells were exposed to E2 or the vehicle for 24, 48 and 72 h before they were harvested.

Antibodies. The antibodies used were as follows: rabbit anti-caveolin 1, rabbit anti-high mobility group box 1 protein (HMGB1), rabbit anti-light chain (LC)3, rabbit anti-Becn1-1, rabbit anti-Atg12 and rabbit anti-cleaved-caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Small interfering RNA (siRNA) and transfection. The human caveolin-1, human HMGB1 and negative control siRNA were designed and constructed by Guangzhou Ribio Biotech Co., Ltd. (Guangzhou, China). The sequences of caveolin-1 and HMGB1 siRNA were as follows: caveolin-1, 5'-GCAUCAACUUGCAGAAAGAdTdT-3' and 3'-dTdTTCGUAGUUGAACGUCUUCU-5'; HMGB1, 5'-GGAGGAAGAUGAAGAAGAUdTdT-3' and 3'-dTdTCCUCCUUCUACUUCUUCUA-5'. Twenty-four hours prior to transfection with siRNA, the medium was replaced with penicillin/streptomycin-free RPMI-1640 complete medium. The BT474 cells were then transfected with siRNA using Lipofectamine™ 2000 (Invitrogen-Life Technologies, Carlsbad, CA, USA) in OPTI-MEM (Gibco-Life Technologies) for 6 h, and then the medium was replaced with antibiotics-free RPMI-1640 complete medium. After being transfected for 48 h, the cells were then exposed to E2 for a further 24 h.

Western blot analysis. The BT474 cells were planted in 6-well plates until they were grown to 60-80% confluence and they were treated with different stimuli for the indicated periods of time. The extraction of protein was performed using an assay kit (Cell Signaling Technology), and the concentration of total protein was examined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). For western blot analysis, SDS-PAGE (Beyotime Institute of Biotechnology) was used to separate equal amounts (30 µg) of total protein and then the protein was transferred onto PVDF membranes (Millipore, Billerica, MA, USA) using transfer buffer (200 mM glycine, 40 mM Tris and 20% methanol) at 240 mA for 30-90 min, depending on the molecular weight of the detected proteins. The membranes were blocked with 5% non-fat milk (Cell Signaling Technology) in 0.1% TBST (Cell Signaling Technology) for 1 h, at 37°C and then incubated with the primary antibody overnight at 4°C. After washing in 0.1% TBST 3 times, 5 min each, the

membranes were incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) at a dilution 1:3,000 for 1 h, 37°C. The membranes were then washed in 0.1% TBST 3 times, 5 min each. The ECL Chemiluminescent Substrate Reagent kit (Cell Signaling Technology) was added to the membranes and the quantification of the band intensity of the blots was measured using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). The internal standard was GAPDH.

Immunofluorescence. The BT474 cells were seeded at sterile glass slides in 6-well plates. After they reached 50-60% confluence, the cells were treated with different stimuli as indicated. The medium was then discarded, and the cells were washed with cold PBS (Cell Signaling Technology) 3 times. The cells were fixed with 100% cold methanol at -20°C for 15 min. Following washing 3 times again in cold PBS, the slides were blocked with 1% bovine serum albumin in 0.1% PBST (Cell Signaling Technology) for 1 h at room temperature. The slides were incubated with primary antibody overnight at 4°C. FITC-conjugated goat-anti-rabbit IgG (Cell Signaling Technology) was used to detect the primary antibody for 1 h at room temperature in the dark. Subsequently, DAPI (0.3 µmol/l) was used to label the nuclei for 2 min at room temperature in the dark. The slides were then washed and images were acquired using an Olympus IX71 epifluorescence microscope (Olympus, Tokyo, Japan).

Monodansylcadaverine (MDC) staining. MDC (Sigma-Aldrich), an autofluorescent dye, is used to detect autophagy. Briefly, the BT474 cells were incubated with 50 µmol/l MDC for 15 min at 37°C to monitor autophagosomes. The cells were then washed with PBS 3 times immediately followed by image acquisition. The MDC-positive cells were observed under a fluorescence microscope (Olympus).

Flow cytometric analysis for apoptosis. For the apoptosis assay, an Annexin V-FITV/PI apoptosis kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) was used according to the manufacturer's instructions. Briefly, the cells (1-5x10⁵) were isolated using EDTA-free Trypsin (Beyotime Institute of Biotechnology) and collected. The cells were then centrifuged at 5,000 x g for 5 min, and they were washed 3 times with cold PBS. The cells were resuspended in 500 µl binding buffer containing 5 µl Annexin V-FITC and 5 µl propidium iodide (PI). The apoptotic cells were detected by flow cytometry using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

CCK-8 cell viability assay. The BT474 cells (5x10³ cells/well) were plated in 96-well plates and cultured for the indicated periods of time. Cell viability was measured using the CCK-8 kit (Dōjindo Laboratories, Kumamoto, Japan). Briefly, the medium was removed followed by the addition of 100 µl CCK-8 in a dilution of 1:9 to each well. The cells were then incubated at 37°C for 0.5-2 h, avoiding light. The absorbance of the optical densities was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm.

Statistical analysis. Results obtained from 3 repeat experiments are expressed as the means ± standard deviation (SD), and analyzed using SPSS 11.0 software (SPSS, Inc., Chicago,

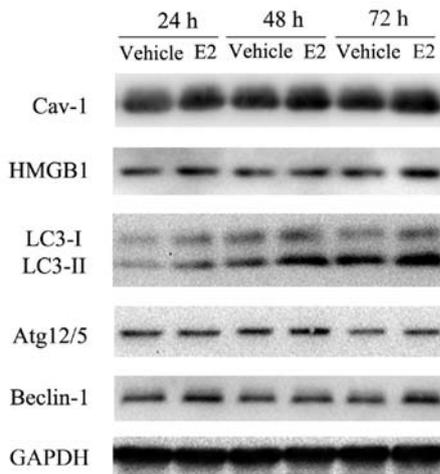


Figure 1. E2 promotes the autophagy of BT474 cells in a time-dependent manner. Western blot analysis was used to determine the effects of E2 on the expression of caveolin-1, HMGB1 and autophagy-related proteins in BT474 cells. Compared to the vehicle-treated group, E2 significantly increased the protein expression of caveolin-1, HMGB1 and autophagy-related proteins (LC3-II, Beclin-1 and Atg12/5) in a time-dependent manner. Experiments were performed in triplicate. E2, 17 β -estradiol; Cav-1, caveolin-1; HMGB1, high mobility group box 1; LC3, light chain 3; Atg, autophagy-related gene.

IL, USA). The Student's t-test was used to determine the differences between the groups. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

E2 increases the expression of caveolin-1, HMGB1 and induces autophagy in BT474 cells. To investigate the involvement of E2 in the autophagy of breast cancer cells, the BT474 (ER⁺) cells were pre-incubated with serum-free RPMI-1640 medium for 24 h to induce autophagy. The cells were then treated with 200 ng/ml E2 or 0.02% ethanol as the vehicle, for various periods of time (24, 48 and 72 h). The expression of caveolin-1, HMGB1 and autophagy-related proteins (Beclin-1, LC3-II and Atg12) was detected by western blot analysis. Our results revealed that compared with the vehicle-treated group, E2 induced a time-dependent increase in the overall expression of caveolin-1, HMGB1 and autophagy-related proteins (Beclin-1, LC3-II and Atg12/5) in the BT474 cells (Fig. 1).

Knockdown of caveolin-1 inhibits E2-induced autophagy in BT474 cells. To further assess the association between caveolin-1 and HMGB1 and autophagy-related proteins, caveolin-1 siRNA was used to knock down caveolin-1 expression. At approximately 48 h following transfection, 200 ng/ml E2 or 0.02% ethanol were added to the medium for a further 24 h. The results revealed that caveolin-1 knockdown inhibited the E2-induced upregulation of HMGB1, LC3-II and Atg12/5, but did not affect the E2-induced upregulation of Beclin-1 (Fig. 2). These results indicate that E2-induced autophagy is regulated by caveolin-1 by affecting the expression of LC3-II and Atg12, but not Beclin-1.

HMGB1 is the link between caveolin-1 and LC3-II expression in BT474 cells. As caveolin-1 siRNA also decreased HMGB1

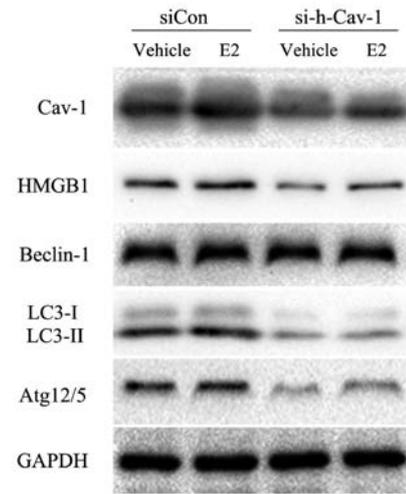


Figure 2. Caveolin-1 knockdown inhibits E2-induced autophagy in BT474 cells. Western blot analysis was performed to determine the effects of caveolin-1 knockdown on the protein expression of HMGB1 and autophagy-related proteins induced by E2. The results revealed that caveolin-1 knockdown markedly attenuated E2-induced LC3-II and Atg12/5 protein expression, and also suppressed HMGB1 protein expression. However, the protein expression of Beclin-1 was not affected. Experiments were performed in triplicate, and results were compared with those of the group transfected with siCon. Cav-1, caveolin-1; si-h-Cav-1, small interfering RNA against human caveolin-1; siCon, control siRNA; E2, 17 β -estradiol; HMGB1, high mobility group box 1; LC3, light chain 3; Atg, autophagy-related gene.

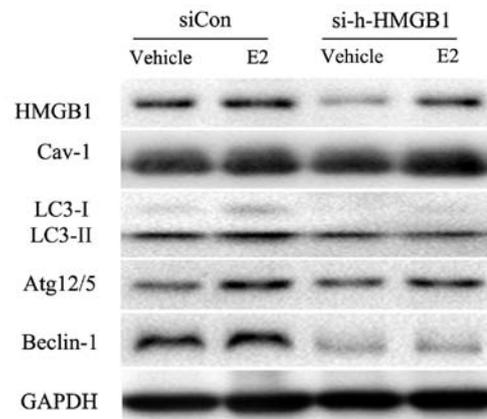


Figure 3. HMGB1 is the link between caveolin-1 with LC3-II expression and autophagosome formation in BT474 cells. Western blot analysis was used to assess the effects of HMGB1 knockdown in the expression of caveolin-1 and autophagy-related proteins induced by E2. The results demonstrated that HMGB1 knockdown decreased the expression of LC3-II and Beclin-1 induced by E2, but did not affect the expression of caveolin-1 and Atg12/5. Experiments were performed in triplicate and the results were compared with those of the group transfected with siCon. HMGB1, high mobility group box 1; si-h-HMGB1, small interfering RNA against human HMGB1; siCon, control siRNA; Cav-1, caveolin-1; E2, 17 β -estradiol; LC3, light chain 3; Atg, autophagy-related gene.

expression, which is deeply related to autophagy, we then determined whether caveolin-1 regulates autophagy by mediating HMGB1 expression. HMGB1 siRNA was constructed and used to knock down the expression of HMGB1. We found that the knockdown of HMGB1 resulted in the inhibition of LC3-II and Beclin-1 expression (Fig. 3). However, caveolin-1 expression was not impaired by HMGB1 knockdown (Fig. 3), suggesting that HMGB1 may be involved in the E2/caveolin-1-induced LC3-related autophagy.

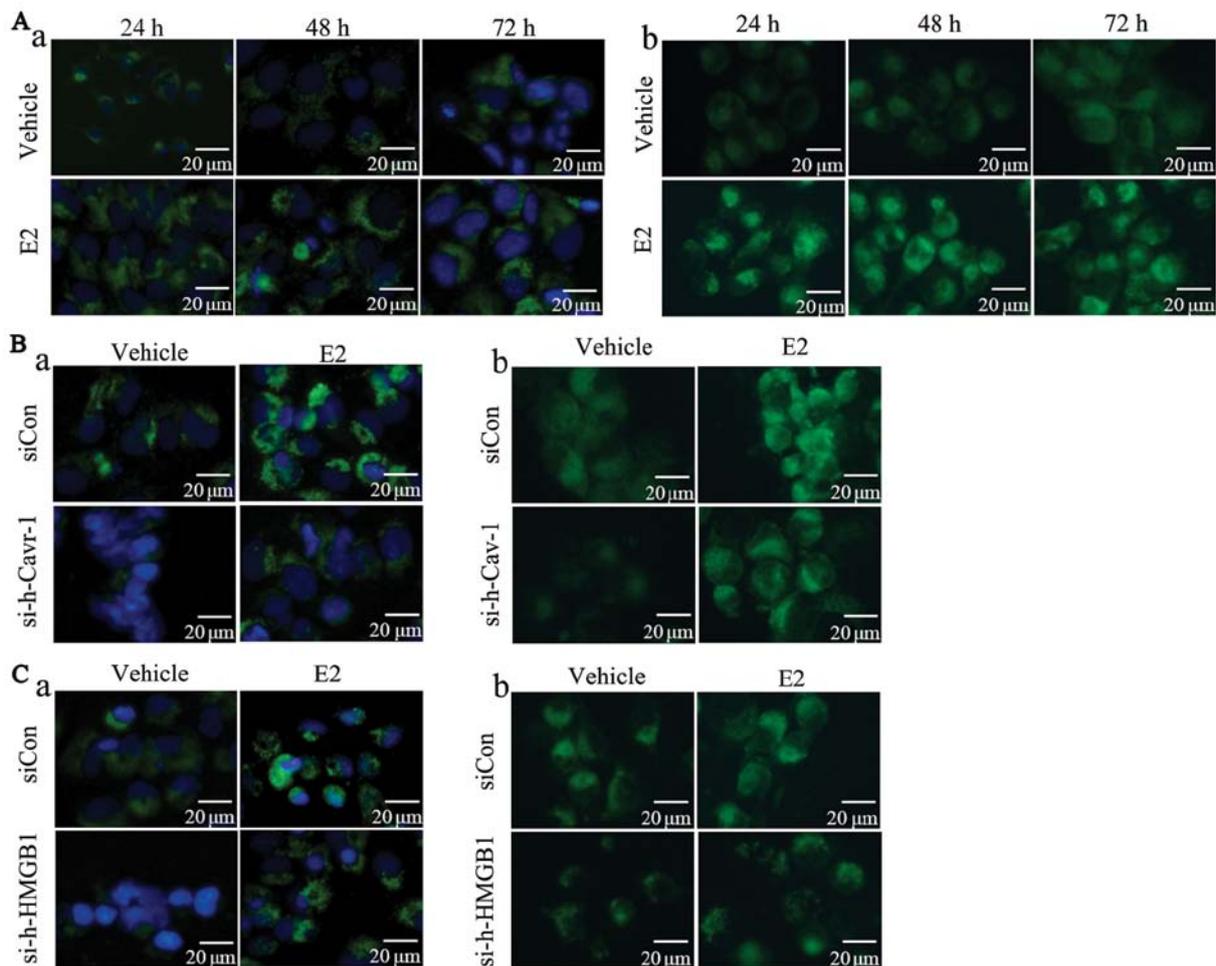


Figure 4. Caveolin-1 or HMGB1 blockade attenuates the E2-induced formation of LC3 punctates and autophagosomes. Immunofluorescence staining for LC3 was used to detect the LC3 punctates and MDC staining was performed to evaluate the autophagosome formation in BT474 cells. (A-a) Compared with the vehicle-treated group, LC3 punctates were induced by E2 in a time-dependent manner. (A-b) Compared with the vehicle-treated group, E2 promoted autophagosome formation. (B-a) The results revealed that caveolin-1 knockdown inhibited E2-induced LC3 punctate formation compared with the siCon group. (B-b) Caveolin-1 knockdown suppressed autophagosome formation compared with siCon group. (C-a) Compared with the siCon group, HMGB1 knockdown decreased E2-induced LC3 punctate formation. (C-b) Compared with the siCon group, HMGB1 knockdown attenuated E2-induced autophagosome formation. Experiments were performed in triplicate. E2, 17 β -estradiol; Cav-1, caveolin-1; si-h-Cav-1, small interfering RNA against human caveolin-1; siCon, control siRNA; HMGB1, high mobility group box 1; si-h-HMGB1, small interfering RNA against human HMGB1; LC3, light chain 3; MDC, monodansylcadaverine.

The blockade of caveolin-1 or HMGB1 attenuates E2-induced autophagosome formation. To evaluate autophagy, the LC3 punctate was directly detected by immunofluorescence staining for LC3, and MDC staining was further performed to detect autophagosome formation. Our results revealed that compared with the vehicle-treated group, E2 promoted the formation of both LC3 punctates (Fig. 4A-a) and autophagosomes (Fig. 4A-b). Compared with the group transfected with negative control siRNA (siCon), caveolin-1 or HMGB1 knockdown markedly decreased the E2-induced formation of LC3 punctates (Fig. 4B-a and C-a) and autophagosomes (Fig. 4B-b and C-b).

Caveolin-1 or HMGB1 knockdown promotes apoptosis in BT474 cells. We then explored the regulation of caveolin-1 and HMGB1 knockdown in the apoptosis and E2-induced cell growth of BT474 cells. Flow cytometric analysis and immunofluorescence staining for cleaved caspase-3 were used to assess the apoptosis of BT474 cells, and CCK-8 assay was performed in order to evaluate the viability of the BT474 cells. The results

demonstrated that compared with the vehicle-treated group, E2 inhibited apoptosis and the formation of cleaved caspase-3 punctates (Fig. 5A and B-a). In addition, compared with the siCon group, caveolin-1 or HMGB1 knockdown markedly promoted apoptosis which had been inhibited by E2 (Fig. 5A and B-b). The results from the evaluation of cell viability by CCK-8 assay revealed that caveolin-1 or HMGB1 knockdown markedly suppressed the E2-induced cell growth (Fig. 5C).

Discussion

Autophagy and apoptosis are two key regulatory mechanisms for cell survival or death. The balance between autophagy and apoptosis determines the cell status. Previous studies have revealed that E2 and estradiol analogues affect cell death and metastasis through the regulation of autophagic and apoptotic pathways under various conditions, such as starvation, ischemia and hypoxia (21-23). In those studies, it was demonstrated that E2 promoted cell survival by increasing autophagy and inhibiting apoptosis. However, the exact molecular and func-

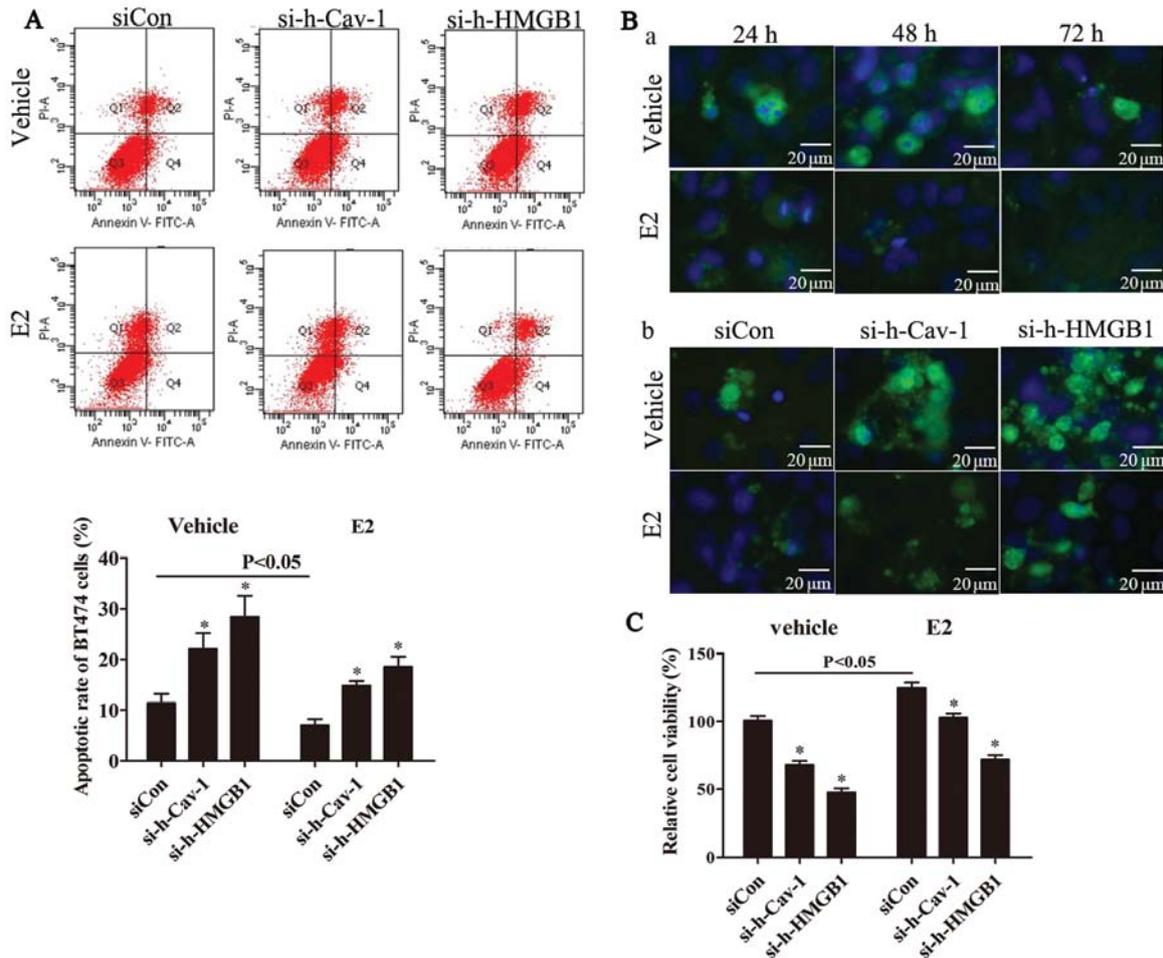


Figure 5. Caveolin-1 or HMGB1 knockdown promotes apoptosis. (A) Flow cytometric assay was used to detect the apoptotic rate in BT474 cells. The results revealed that compared to the vehicle-treated group, E2 inhibited cell apoptosis. Compared to the siCon group, caveolin-1 knockdown or HMGB1 knockdown markedly augmented apoptosis, suppressing the E2-induced inhibition of apoptosis. (B) Immunofluorescence staining for cleaved caspase-3 was performed to detect the formation of cleaved caspase-3 punctates in BT474 cells. (a) The results revealed that compared to the vehicle-treated group, E2 decreased cleaved caspase-3 punctate formation. (b) Caveolin-1 knockdown or HMGB1 knockdown increased the formation of cleaved caspase-3 punctate compared with the siCon group. (C) Viability of BT474 cells was analyzed by CCK-8 assay. The results revealed that E2 promoted cell growth and caveolin-1 or HMGB1 knockdown suppressed cell growth. Error bars represent the means \pm standard deviation. Experiments were performed in triplicate. * $P < 0.05$. E2, 17 β -estradiol; si-h-Cav-1, small interfering RNA against human caveolin-1; siCon, control siRNA; HMGB1, high mobility group box 1; si-h-HMGB1, small interfering RNA against human HMGB1.

tional mechanisms of E2-mediated autophagy and apoptosis have not yet been elucidated.

LC3, Atg12 and Beclin-1 have been identified as autophagy markers. LC3 is an autophagy-related protein and LC3-I is changed to LC3-II in the process of autophagy. LC3-II aggregates on the autophagosome membranes and the ratio of LC3-II/LC3-I represents a marker for autophagy (24). Autophagosome formation demands a ubiquitin-like conjugation system. In this system, Atg12 is bound to Atg5 to target the autophagosome vesicles (25). Beclin-1 is the mammalian ortholog of yeast Apg6/Vps30 and plays a crucial role in the formation of autophagosomes (26).

The role for caveolin-1 in autophagy and apoptosis has been supported by several studies. Recent research has demonstrated that caveolin-1 binds LC3B through its scaffolding domain (27). It has been reported that caveolin-1 depletion increases the expression of autophagy markers (LC3-II, Atg5/12 and p-ULK) (28). In addition, the expression of caveolin-1 abrogates apoptosis in hepatocytes, small cell lung cancer cells and

thymocytes (29-31). However, certain studies have reported that caveolin-1 sensitizes cells to drug-induced apoptosis (32,33). These data suggest that the effects of caveolin-1 on autophagy and apoptosis are dependent on specific cell types and stimuli.

HMGB1 protein, a chromatin-binding factor, presents as a highly conserved nuclear protein and bends DNA. HMGB1 is also involved in various cellular processes, such as cell differentiation, wound healing, cell survival and death, as well as cell apoptosis and autophagy (34-36). Although a number of studies have revealed that HMGB1 is closely related to autophagy and apoptosis, the role of HMGB1 in E2-mediated autophagy remains unclear.

In the present study, we provide a possible explanation for E2-mediated autophagy and apoptotic processes in breast cancer cells. We demonstrate that E2 promotes cell growth by mediating autophagy and apoptosis in BT474 breast cancer cells, which are regulated by the caveolin-1/HMGB1 pathway. We found that E2 promoted the expression of both caveolin-1 and HMGB1, as well as that of autophagy-related proteins

(LC3-II, Beclin-1 and Atg12/5). E2 also inhibited apoptosis and the formation of cleaved-caspase-3 punctates. Caveolin-1 knockdown downregulated HMGB1 and autophagy-related proteins (LC3-II and Atg12/5) and suppressed autophagosome formation, and promoted apoptosis. Furthermore, the blockade of HMGB1 inhibited the expression of autophagy-related proteins (LC3-II and Beclin-1) and significantly promoted apoptosis in the BT474 cells. Based on this evidence, it can be concluded that the caveolin-1/HMGB1 pathway contributes to the E2-mediated pro-autophagy and anti-apoptotic processes.

Taken together, the data from the present study provide further insight into the relationship and interaction between E2 and autophagy and apoptosis. However, the significance of E2 and caveolin-1 in mediating autophagy and the apoptosis of breast cancer cells requires further investigation.

Acknowledgements

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