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sh-Ambra1 inhibits IRS-1/PI3K/Akt signalling pathway to reduce autophagy in gestational diabetes

Xin Qu[1, 2], Xiao-yan Li[1]*, Yan Feng[3], Xiao-li Wang[1], Lei Li[1], Yu-ping Wang[1], Yong-li Chu[1, 2]*

[1]*Department of Obstetrics and Gynaecology, Yantai Yuhuangding Hospital, Yantai, China*
[2]*Cheeloo College of Medicine, Shandong University, Jinan, China*
[3]*Department of Clinical Nutrition, Yantai Yuhuangding Hospital, Yantai, China*
**These authors contributed equally.*

Abstract

Introduction: Gestational diabetes mellitus (GDM) is the most common metabolic disease in pregnancy. However, studies of activating molecule of Beclin1-regulated autophagy (Ambra1) affecting the insulin substrate receptor 1/phosphatidylinositol 3 kinase/protein kinase B (IRS-1/PI3K/Akt) signalling pathway in GDM have not been reported. The aim of the study was to detect the difference of Ambra1 expression in the placenta of normal pregnant women and GDM patients.

Material and methods: An *in vitro* model of gestational diabetes mellitus was established by inducing HTR8/Svneo cells from human chorionic trophoblast layer with high glucose. The changes of cell morphology were observed by inverted microscope, and the expression levels of Ambra1 gene and protein in model cells were detected. After this, Ambra1 gene was silenced by shRNA transfection, and PI3K inhibitor was added to detect changes in Ambra1, autophagy, and insulin (INS) signalling pathways.

Results: The protein expression levels of Ambra1, Bcl-2 interacting protein (Beclin-1), and microtubule-associated proteins 1A/1B light chain 3B (LC3-II) in the placentas of GDM pregnant women were higher than those of normal pregnant women. High glucose induces morphological changes in HTR8/Svneo cells and increases Ambra1 transcription and translation levels. sh-Ambra1 increased survival of HTR8/SvNEO-HG cells and inhibited Ambra1, Beclin1, and LC3-II transcription and translation levels. Also, sh-Ambra1 increased IRS-1/PI3K/Akt protein phosphorylation levels and inhibited the IRS-1/PI3K/Akt signalling pathway and its resulting autophagy.

Conclusions: sh-Ambra1 increased IRS-1/PI3K/Akt protein phosphorylation levels to reduce autophagy in gestational diabetes.

Key words: *gestational diabetes mellitus; autophagy; Ambra1; IRS-1/PI3K/Akt*

Introduction

Gestational diabetes mellitus (GDM) is diabetes mellitus diagnosed in the middle or late stages of pregnancy, without significant pre-pregnancy diabetes. GDM is common during pregnancy, and it can affect maternal health and foetal development. It is the most common metabolic disease of pregnancy and is defined as "glucose intolerance that occurs in mid- and late pregnancy, resulting in hyperglycaemia of varying severity". The current treatment of GDM focuses on glycaemic control. Although medications can improve blood glucose to some extent, there are still patients with adverse pregnancy outcomes [1]. The growing prevalence of obesity and increasing maternal age have led to a progressive increase in the global prevalence of GDM, which poses a significant economic burden on public health care systems [2]. Pregnancy is associated with insulin resistance (IR) and hyperinsulinaemia, which may predispose some women to develop diabetes [3]. During normal pregnancy, IR begins in mid-pregnancy and develops mainly in late gestation [4]. Among other things, hormones and adipokines secreted by the placenta (including human placental lactogen and human placental growth hormone) may be responsible for the development of IR in women during pregnancy. In addition, the increase in oestrogen, progesterone, and cortisol during pregnancy leads to a disruption of glucose insulin homeostasis [5]. To compensate for peripheral IR during pregnancy, insulin secretion in the female pancreas increases, and GDM occurs when the insulin secreted by the female pancreas is not sufficient to compensate for the metabolic stress of IR.

Insulin substrate receptor 1 (IRS-1) plays an important role in insulin resistance by activating 2 major pathways [phosphatidylinositol 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK)] to deliver insulin signals in cells. Of these, PI3K is primarily involved in metabolic signalling, and it catalyses

Yong-li Chu M.D., Chair and Clinic of Cheeloo College of Medicine, Shandong University; Department of Obstetrics and Gynaecology, Yantai Yuhuangding Hospital; No. 20 East Yuhuangding Road, Yantai 264000, China. phone: 13573590999; e-mail: sq20090412@163.com

the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate, which initiates a kinase cascade reaction that activates protein kinase B (Akt), which in turn induces glucose transporter type 4 (GLUT4) transfer from intracellular vesicles to cellular membrane myosomes, thereby increasing the rate of glucose uptake [6]. Akt is a signalling protein that responds to growth factors or insulin and is thought to promote a variety of cellular functions, including nutrient metabolism, cell growth, and apoptosis [7]. It has been demonstrated that Akt can play a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis, and it may also be a key mediator of insulin signalling [8]. Meanwhile, abnormal Akt signalling was found to be associated with insulin secretion and insulin resistance in type 2 diabetes mellitus (T2DM), obesity, and cardiovascular disease [9]. It was shown that the phosphorylation expression levels of Akt and glycogen synthase kinase-3 beta (GSK-3β) were significantly reduced in skeletal muscle tissue of GDM patients relative to healthy controls [10]. In summary, attention to IRS-1/Akt abnormalities is crucial for glucose metabolism and insulin resistance in GDM patients.

Autophagy is a catabolic process involved in the maintenance of energy homeostasis, and its dysregulation is associated with the development of metabolic diseases, including diabetes [11]. Autophagy plays an important role in embryogenesis, implantation, and maintenance of pregnancy; this role includes supporting the invasion of the extra villous trophoblast (EVT) into the metaplasia (endometrium) up to the anterior third of the myometrium and migration along the lumen of the small spiral arteries during the hypoxic and hypotrophic conditions of early pregnancy [12]. It has been found that autophagy is significantly enhanced in patients with GDM, and high glucose levels enhanced autophagy and apoptosis and reduced cell proliferation and invasion in *in vitro* studies [13]. In *in vivo* studies, women with GDM from older gestational age infants had heavier placentas, reduced levels of Bcl-2 interacting protein (Beclin-1) and damage regulated autophagy modulator (DRAM), reduced cytokeratin 18 neoepitope (M30), reduced poly-ADP-ribose polymerase (PARP) immunoreactivity, and increased proliferating cell nuclear antigen-67 (Ki-67) immunoreactivity, compared to placentas from normal pregnant women, and these changes were associated with increased β cell lymphoma/leukemia × long form (Bcl-xL) and reduced Bcl2 antagonist/killer (Bak) levels [14]. Therefore, autophagy plays an important role in the development and progression of the disease process in GDM patients.

Ambra1 (activating molecule of Beclin1-regulated autophagy) is a highly intrinsically dysregulated and vertebrate-conserved macromolecular bridging protein with a WD40 structural domain at its amino terminus, which is involved in the regulation of autophagy as part of the autophagic signalling network, and which plays a key role in embryogenesis [15, 16]. Ambra1 regulates Beclin-1 or Bcl-2, which promotes the formation of autophagic vesicles and can determine the death or survival of the resulting cells by controlling the switch between autophagy and apoptosis [17]. As an important regulator of autophagy, Ambra1 interacts with Beclin1 through the target lipid kinase vacuolar protein sorting 34 (Vps34)/class III phosphatidylinositol 3-kinase (PI3KC3) to assemble class III PI3K complexes and positively regulate the formation of autophagic vesicles [18]. The role of Ambra1 in autophagy and regulation in embryonic stem cells has been reported [15], but its regulatory role in GDM has been rarely reported. It has also been shown that there is an important link between the PI3K/Akt signalling pathway and autophagy [19, 20], which can regulate cellular autophagy by inhibiting the expression of this signalling pathway. However, whether Ambra1 can affect the IRS-1/PI3K/Akt signalling pathway in GDM has not been reported.

Therefore, in this paper, we propose to examine the expression levels of Ambra1 and its downstream autophagy-related proteins in the placental villi and chorionic trophectoderm of GDM patients by examining clinical samples of placentas from normal pregnant women and from GDM patients. We also studied the cell proliferation before and after Ambra1 interference by culturing human chorionic villus trophoblast HTR8/Svneo cells and by constructing a high glucose injury model *in vitro*. We also examined the expression levels of autophagy-related genes and proteins, and the expression of insulin signalling pathway proteins, to clarify the relationship between Ambra1 and IRS-1/PI3K/Akt signalling pathway in GDM.

Material and methods

Patients

We collected placentas from 10 pregnant women with GDM and 10 healthy pregnant women in Yantai Yuhuangding Hospital, Shandong Province. Informed consent was obtained from all pregnant women. The statistical characteristics of the 20 pregnant women are listed in Table 1. The blood was rinsed under sterile conditions within 10 minutes of delivery, an appropriate amount of placenta was cut for homogenization and crushing, the supernatant solution was collected and the Ambra1 protein expression level was detected, and the rest of the placenta was stored at –80°C.

Reagents

RPMI-1640 (Hyclone, SH30809.01B), Opti-MEM (31985-062) and foetal bovine serum (FBS) (10270-106) were obtained from Gibco, cell

Table 1. *The clinicopathological factors of healthy pregnancies and gestational diabetes mellitus (GDM) patients*

Characteristics	Healthy	GDM
Subjects [n]	10	10
Age [years]	32 ± 3.69	31.4 ± 2.65
BMI [kg/m^2]	28.74 ± 2.93	33.46 ± 3.69
Gestation [weeks]	38.59 ± 0.65	38.42 ± 0.74
Foetal birth weight [g]	3195 ± 454.67	4020 ± 614.08
FBG [mmol/L]	4.24 ± 0.45	5.34 ± 1.36
1-h OGTT [mmol/L]	6.73 ± 0.54	13.27 ± 1.77
2-h OGTT [mmol/L]	6.17 ± 0.57	11.09 ± 1.89

Data presented as mean ± standard deviation. All pregnancies are primipara. FBG — fasting blood glucose; OGTT — oral glucose tolerance test

counting kit 8 (CCK8) (CA1210), TOP10 competent cells (C1200), PBS (P1010), and 0.25% trypsin (T1350) were obtained from Solarbio, Lipofectamine 2000 (Invitrogen, 11668-027), AgeI (R3352) and EcoRI (R3101) from NEB, T4 DNA ligase (TaKaRa, 2011A), glutaraldehyde (30092436), alcohol (10009218), and acetone (40064460) were obtained from Sinopharm Chemical Reagent Co., Ltd., osmic acid (GP18456) and lead citrate (GA10701) were from Beijing Zhongjing Science and Technology Co., Ltd., uranyl acetate from Jingzhou Dingtian Chemical Technology Co., Ltd., D-(+)-glucose (G116307) and mannitol (M108831) were from Aladdin (China), LY-294002 (MCE, HY-10108).

Cell model of inducing gestational diabetes mellitus (HTR8/Svneo-HG)

An *in vitro* model of gestational diabetes mellitus was established by inducing HTR8/Svneo cells from human chorionic trophoblast layer with high glucose [21]. Cells in the control group (Con) were added with 5 mmol/L D-(+) -glucose, the high-glucose group (HG) with 30 mmol/L D-(+)-glucose, and the osmotic (OSM) group with 5 mmol/L glucose and 25 mmol/L mannitol. Cells in all groups were cultured normally for 24 h. The purpose of adding mannitol was to exclude the influence of hypertonic factors in a high-glucose environment.

Western blot

The protein levels of Ambra1, LC3-II, Beclin1, p-Akt, p-IRS-1, and p-PI3K were analysed by western blot. Cells were homogenized in radio-immunoprecipitation assay (RIPA) lysate (solarbio, R0010) containing protease inhibitor at 4°C and centrifuged at 12,000 g for 15 min. The concentration of the proteins was measured using BCA Protein Assay Kit (solarbio, PC0020). Proteins (20 μg) were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (IPVH00010, Millipore). The membranes were blocked with 5% skimmed milk for 2 h at room temperature in Tris-buffered saline and incubated with primary antibodies against p-Akt (CST, 4060T); p-IRS-1 (ab109543) and p-PI3K (ab 182651) from Abcam (U.K); IRS-1 (PAB36510), PI3K (PAB30084), Akt (PAB30596), Ambra1 (PAB30582), LC3-II (PAB30656), Beclin1 (PAB35215), β-Actin (PAB36265) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (PAB36269) from Bioswamp (China). overnight at 4°C. β-Actin and GAPDH antibody were selected as an internal reference. All of the primary antibody dilution ratios were 1:1000. The membranes were then washed with Tris-buffered saline and incubated in Goat anti-Rabbit IgG secondary antibody (Bioswamp, SAB43714, 1:10,000) for 2 h at room temperature. Immunoreactivity was visualized by colorimetric reaction using Immobilon Western HRP (Millipore, WBKLS0500). Membranes were scanned with an automatic chemiluminescence analyser (Tanon, Tanon-5200).

Plasmid construction

Ambra1 Gene synthesis (Gene ID: 55626, GI No.1905520465), polymerase chain reaction (PCR) amplification, AgeI and EcoRI enzyme-cut, T4 DNA ligase connect gene and vector, the DNA fragments were added into TOP10 competent cells, heat shock transformation was performed at 42°C, antibiotic plate screening was performed, and bacterial fluid was collected for sequencing.

Cell culture, transfection

HTR-8/Svneo (Procell Life Science & Technology Co., Ltd., CL-0599, China) cells were cultured in 10% FBSin RPMI-1640 (Dulbecco's Modified Eagle Medium) at 37°C, 5% carbon dioxide (CO_2) in an incubator.

shRNA transfection was utilized to silence Ambra1 (si-Ambra1) in HTR8/Svneo cells. 5×10^5 cells were inoculated in 6-well plates 24 h before transfection. 100 pmol miRNA was diluted in 250 μL OPTI-MEM, and gently soiled 5 times. 5 μL Lipofectamine® RNAiMAX was diluted in 250 μL OPti-MEM, then gently smoothed 5 times, and left to stand for 5 min at room temperature. The 2 solutions were gently homogenized 5 times and incubated at room temperature for 20 min to obtain the complex. 500 μL compound was added to the cells and the plates were shaken. After 4 h culture, the new medium was replaced and cultured for 24 h to detect the expression of transferred genes in real time quantitative polymerase chain reaction (RT-qPCR), U6 as internal gene.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol (Ambion, 15596026), and DNA was eliminated using DNase Free Water (solarbio, R1600). The obtained RNA was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (TAKARA, RR036Q) and amplified using the SYBR Green PCR kit (KAPA Biosystems, KM4101). Reaction conditions were as follows: initial denaturation at 95°C for 3 min; 39 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s, and extension at 72°C for 25 s; and final extension at 65°C for 5 s and 95°C for 50 s. The primers were synthesized by Wuhan Tianyi Huayu Gene Technology Co., LTD. The sequences were as follows: Ambra1 forward: 5′-AACCAGTGCCTCTTCGG-3′, reverse: 5′-GAGCCATTCCCTCCATCT-3′; Beclin1 forward: 5′-CCGTGGAATGGAATGAGA-3′, reverse: 5′-CGTAAGGAACAAGTCGGTATC-3′; LC3-II forward: 5′-GAGAGCAGCATCCAACCA-3′, reverse: 5′-CTGTGTCCGTTCACCAACA-3′; β-actin forward: 5′-ACACTGTGCCCATCTACG-3′, reverse: 5′-TGTCACGCACGATTTCC-3′; β-actin served as endogenous controls of Ambra1, respectively. The data were analysed using the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit 8 (CCK8)

A total of 3×10^3 cells /100 μL were added to each well of the 96-well plates, and the cells were cultured overnight to allow the cells to adhere to the wall. The cells were treated according to different groups and cultured for 24 h. 10 μL CCK8 solution was added to each well and cultured for 4 h. The absorbance value of each well was measured at 450 nm, and the cell survival rate was calculated.

Flow cytometry (FCM)

In total, 1×10^6 cells were resuspended in medium and centrifuged at 400 g, 4°C, for 5 min. The cells were then resuspended in 200 μL of PBS and stained with 10 μL of Annexin V-fluorescein isothiocyanate (BD) and 10 μL of propidium iodide (BD) in the dark for 30 min. After adding 300 μL of PBS, flow cytometry was performed using a NovoCyte apparatus (ACEA Biosciences).

Transmission electron microscope

A total of 1 × 10^7 cells were fixed with glutaraldehyde followed by osmic acid. After being washed with PBS, the cells were dehydrated in alcohol series, embedded in acetone and epoxy resin, sectioned at a thickness of 60 nm, and stained with uranyl acetate and lead citrate. The resultant sections were subjected to examination for ultrastructure using a transmission electron microscope (H-7700, Hitachi).

Statistical analyses

Statistical analyses were performed using SPSS23.0 software. All data were presented as mean ± standard deviation (SD) in Prism 8 software. One-way ANOVA was used to compare differences among 3 or more groups, and post-hoc Fisher's least significant difference (LSD) test was used for the individual group comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Differences in Ambra1 in pregnant women and GDM patients

In the placentas of 10 normal pregnant women (PW) and 10 gestational diabetes mellitus (GDM) patients, the protein expression of Ambra1, Beclin1, and LC3-II differed; the protein expression levels of Ambra1, Beclin1 and LC3-II in the placentas of GDM pregnant women were higher than those of normal pregnant women (Fig. 1). The clinicopathological factors of healthy pregnancies and GDM patients are shown in Table 1.

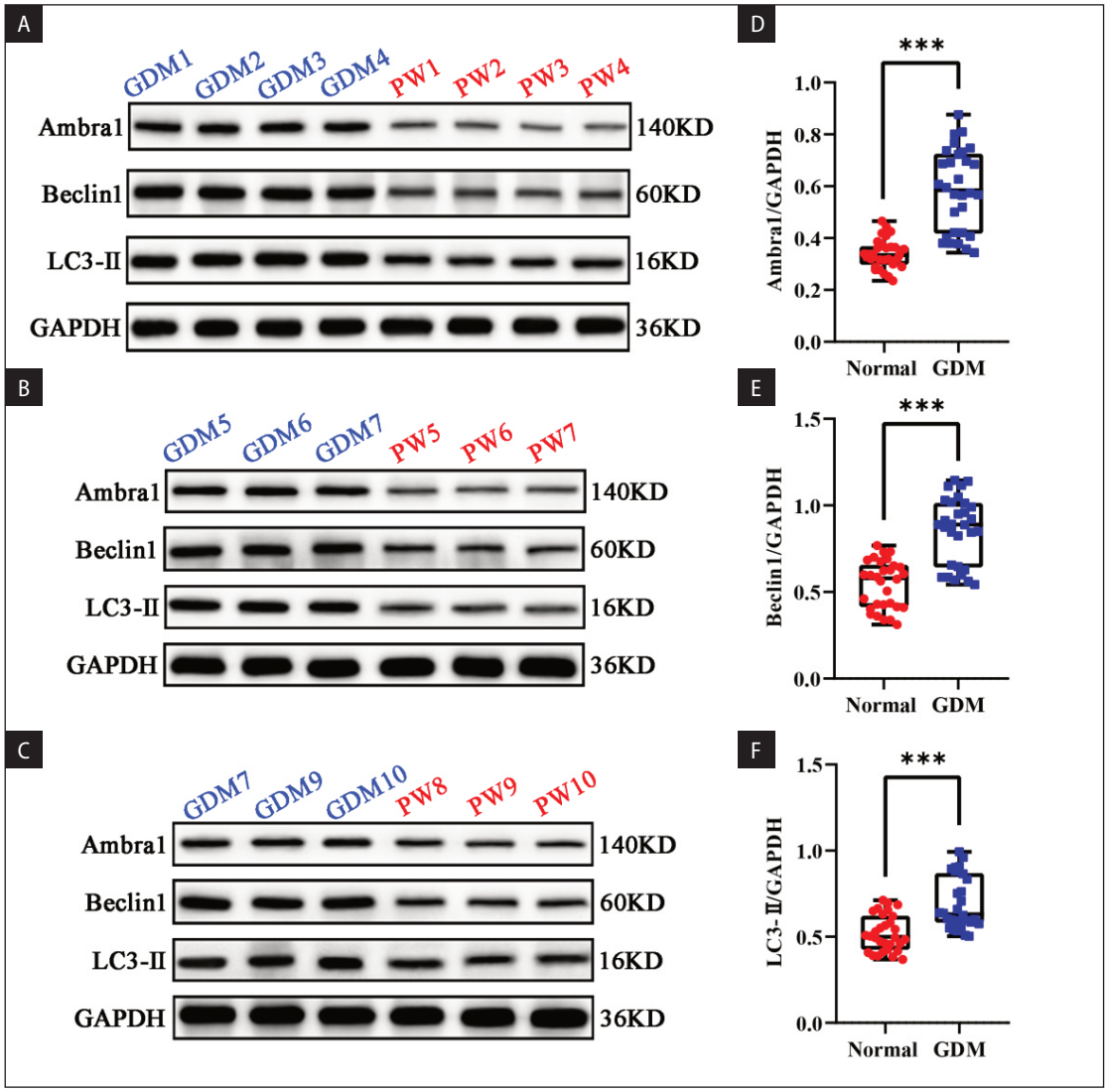

Figure 1. *The difference of activating molecule of Beclin1-regulated autophagy (Ambra1), Bcl-2 interacting protein (Beclin1), and microtubule-associated proteins 1A/1B light chain 3B (LC3-II) expression level in normal (red) pregnant women (PW) and gestational diabetes mellitus (GDM, blue).* **A.** *The difference of Ambra1, Beclin1, and LC3-II protein expression level in normal PW 1–4 and GDM pregnant women 1–4;* **B.** *The difference of Ambra1, Beclin1, and LC3-II protein expression level in normal PW 5–7 and GDM pregnant women 5–7;* **C.** *The difference of Ambra1, Beclin1, and LC3-II protein expression level in normal PW 8-10 and GDM pregnant women 7, 9, 10;* **DF.** *The difference of Ambra1, Beclin1, and LC3-II protein expression level in normal PW 1-10 and GDM pregnant women 1–10. n = 10; GAPDH — glyceraldehyde 3-phosphate dehydrogenase. ***p < 0.001*

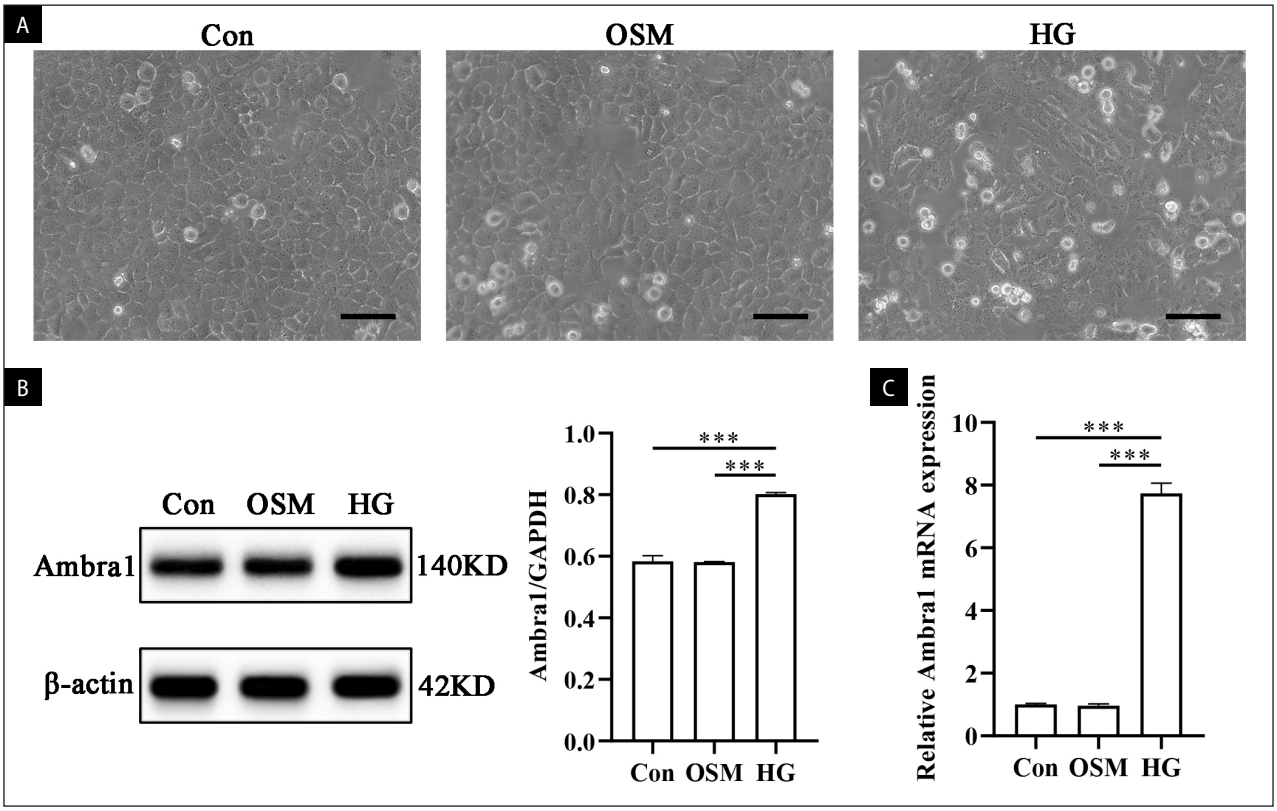

Figure 2. *High glucose induced changes in an HTR8/Svneo cell model.* **A.** *Microscopic observation of HTR8/Svneo cell model morphology. Scale bar, 1000 μm;* **B.** *Western blot detection of activating molecule of Beclin1-regulated autophagy (Ambra1) protein expression level.* **C.** *Real-time quantitative polymerase chain reaction (RT-qPCR) detection of Ambra1 mRNA expression level. Con — control group; HG — the high-glucose group; OSM — the osmotic group; GAPDH — glyceraldehyde 3-phosphate dehydrogenase. ***p < 0.001*

High glucose induced HTR8/Svneo cell model (HTR8/Svneo-HG) and Ambra1 change

After 30 mM D-(+)-glucose induced HTR8/Svneo cells for 24 h, the morphology of HTR8/Svneo cells changed; the number of cells decreased, the number of cells floating after death increased, and the cell membrane ruptured. However, at the same osmotic pressure of 5 mM D-(+)-glucose versus 25 mM mannitol treated HTR8/Svneo cells for 24 h, normal cell morphology, the cell membrane was intact, and the number of floating cells was normal. Therefore, the high glucose induced lesions in HTR8/Svneo cells, which was used as an *in vitro* model for pregnant diabetic patients (Fig. 2A). In the *in vitro* model of gestational diabetes, the transcriptional and translational levels of Ambra1 were increased (Fig. 2BC) — a result consistent with the high expression of Ambra1 protein in the placenta of pregnant women with GDM.

Effects of sh-Ambra1 on proliferation of HTR8/ SvNEO-HG cells

Both Ambra1 mRNA and protein expression levels were inhibited after interference with 3 different targets on the Ambra1 gene (Fig. 3AB). Ambra1 mRNA and protein expression levels were significantly reduced in the sh-RNA1 group compared with the control and sh-RNA NC groups, so sh-RNA1 was selected for subsequent experiments. In addition, the survival rate of HTR8/SvNEO-HG cells in the sh-Ambra1 group were significantly higher than that in the model group and sh-NC group (Fig. 3C).

Effects of sh-Ambra1 on autophagy and INS pathways in HTR8/ SvNEO-HG cells

After silencing the Ambra1 gene, the mRNA expression levels and protein expression levels of Ambra1, Beclin1, and LC3-II in the sh-Ambra1 group were lower than those in the model and sh-NC groups. At this time, in HTR8/ SvNEO-HG cells, the mRNA expression levels and protein expression levels of Ambra1, Beclin1, and LC3-II were significantly increased compared with HTR8/SvNEO cells (Fig. 4ABC), which were the same as those detected in clinical samples, so we speculated that gestational diabetes can lead to autophagy activation. Ambra1 is involved in regulating autophagy while also playing a role in regulating autophagy activation. In addition, the p-IRS-1/p-PI3K/p-Akt signalling pathway was inhibited in HTR8/SvNEO-HG cells, and p-IRS-1, p-PI3K, and p-Akt protein expression levels were sig-

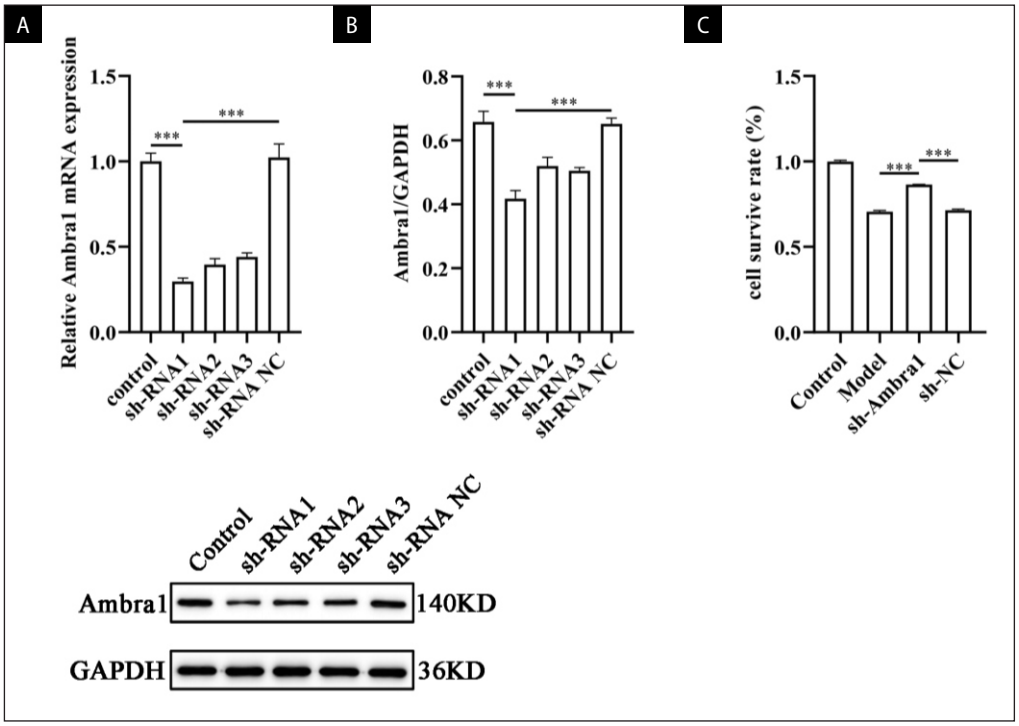

Figure 3. *Effects of sh-Ambra1 on proliferation of HTR8/SvNEO-HG cells.* **A.** *Transfection efficiency identification by real-time quantitative polymerase chain reaction (RT-qPCR);* **B.** *Western blot detection of Ambra1 protein expression level.* **C.** *HTR8/SvNEO-HG cells survive rate detected by cell counting kit 8 (CCK8). ***p < 0.001*

nificantly increased in the sh-Ambra1 group compared with the mod and sh-NC groups (Fig. 4DE).

Effects of IRS-1/PI3K/Akt pathways on autophagy in HTR8/ SvNEO-HG cells

In our previous work, we showed that autophagy-related gene and protein expression levels were significantly increased in both GDM patients and high-glucose-induced GMD cell models, and the IRS-1/PI3K/Akt signalling pathway was inhibited in HTR8/ SvNEO-HG cells, so we speculated whether cellular autophagy levels in GMD are regulated by the IRS-1/PI3K/Akt signalling pathway. By detecting the expression levels of Ambra1, Beclin1, and LC3-II in sh-Ambra1, LY-294002, and sh-Ambra1+LY-294002 (Comb), on HTR8/SvNEO-HG cells, we found that the expression levels of Ambra1, Beclin1, and LC3-II protein were higher in the LY-294002 group than in the sh-Ambra1 group and the model group, but Ambra1, Beclin1, and LC3-II protein expression levels were significantly lower in the Comb group than in the LY-294002 group (Fig. 5A). It can be found that the expression levels of Ambra1, Beclin1, and LC3-II proteins in HTR8/ SvNEO-HG cells were higher than those in the model group after inhibition of IRS-1/PI3K/Akt signalling pathway, and the expression levels of p-IRS-1, p-PI3K, and p-Akt proteins were lower than those in the model group; however, after silencing Ambra1, the inhibitory effect of LY- 294002 was attenuated (Fig. 5B). As shown in Figure 6, under transmission electron microscopy (TEM), mitochondrial structures were observed in the control group; autophagosome vesicle formation and autophagic lysosomes were observed in the model group; compared with the LY-294002 group, autophagosome and autophagic lysosomes were decreased in the sh-Ambra1 group and the Comb group (Fig. 6).

Discussion

In autophagy, cells or organelles undergo lysosomal degradation. As pregnancy progresses placental apoptosis increases, and autophagy plays a role in trophoblast differentiation and invasion. In pregnancy disorders like preeclampsia and intrauterine growth restriction (IUGR), caspase 3, caspase 8, and BAX are high, while Bcl-2 is low [22]. In our study, the survival rate of HTR8/SvNEO-HG cells in the sh-Ambra1 group was found to be higher than that in the model group and the Ambra1-unperturbed group by CCK8. It has been found that DRAM-3 is a regulator of macroautophagy, which has a cytoprotective effect under starvation conditions, and that DRAM-3 inhibits cell death and promotes the survival of cells grown in the absence of glucose, an effect that is not dependent on macro-

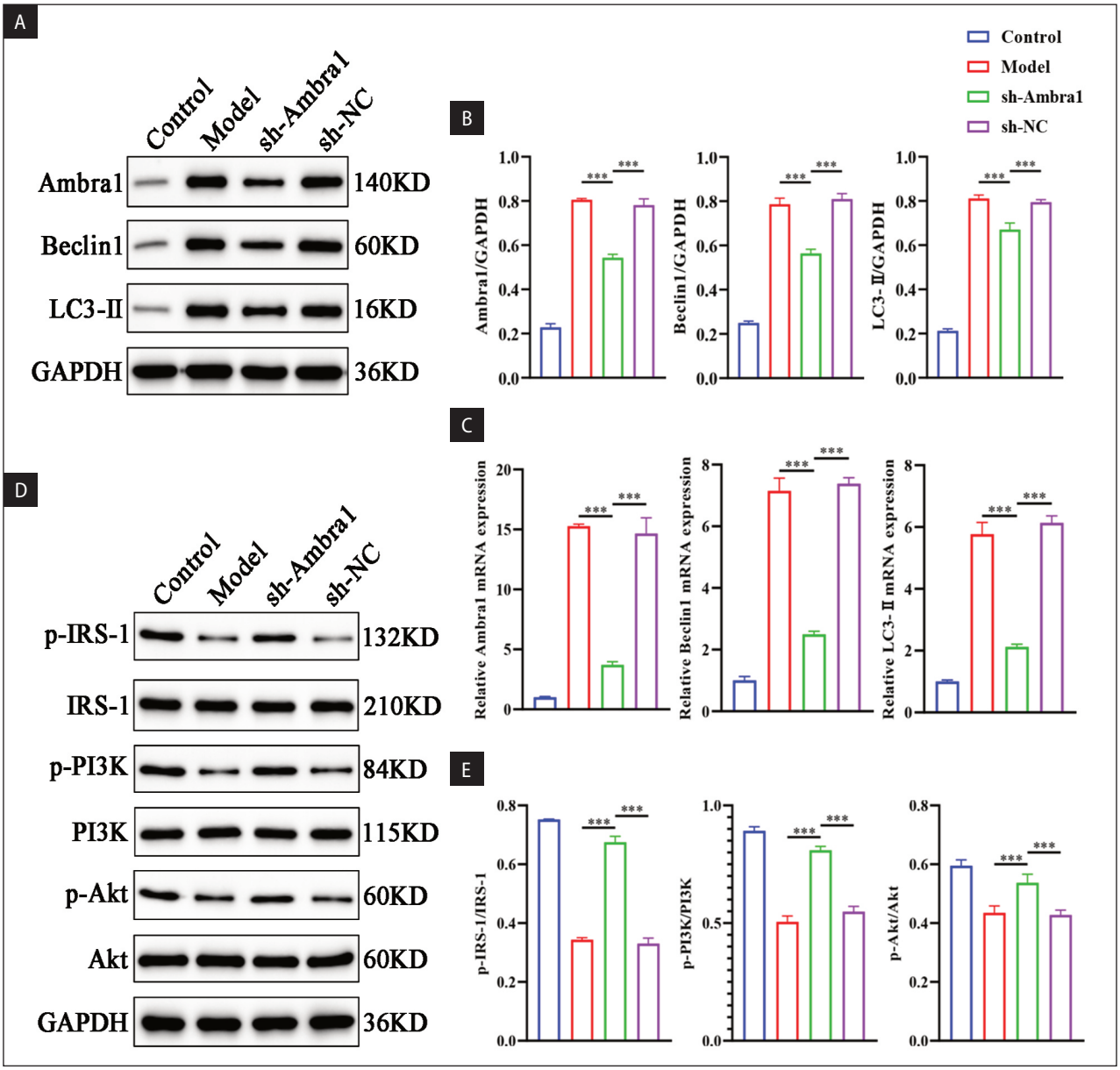

Figure 4. *Effects of sh-Ambra1 on autophagy and insulin (INS) pathways in HTR8/SvNEO-HG cells.* **AB.** *Western blot detection of activating molecule of Beclin1-regulated autophagy (Ambra1), Bcl-2 interacting protein (Beclin1), and microtubule-associated proteins 1A/1B light chain 3B (LC3-II) expression levels.* **C.** *Real-time quantitative polymerase chain reaction (RT-qPCR) detection of Ambra1, Beclin1, and LC3-II mRNA expression levels.* **DE.** *Western blot detection of p-IRS-1/p-PI3K/p-Akt signaling pathway. GAPDH — glyceraldehyde 3-phosphate dehydrogenase. ***p < 0.001*

autophagy [23]. Therefore, we speculate that a similar phenomenon occurs in HTR8/ SvNEO-HG cells.

Research of placental antioxidant defences and autophagy-related genes in maternal obesity and gestational diabetes mellitus indicated that, compare with the obese with no comorbidities [OB GDM (–)], PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1) expression increased in OB GDM (+) [24]. PHLPP1 involved in regulation of protein kinase B (Akt) and protein kinase C (PKC) signaling mediates dephosphorylation in the C-terminal domain hydrophobic motif of members of the AGC Ser/Thr protein kinase family. In our study, phosphorylation of IRS-1/PI3K/Akt protein was inhibited by sh-Ambra1, demonstrating that IRS-1/PI3K/Akt also plays a role in regulating cellular autophagy in HTR8/ SvNEO-HG cells.

Li et al. showed that the key autophagy-related genes, autophagy-related 7 (ATG7) and microtubule-associated protein 1A/1B-light chain 3 (LC3), were increased in GDM compared with normal pregnant women [21]. In our study, we also found higher levels of Ambra1, Beclin1, and LC3-II protein expression in

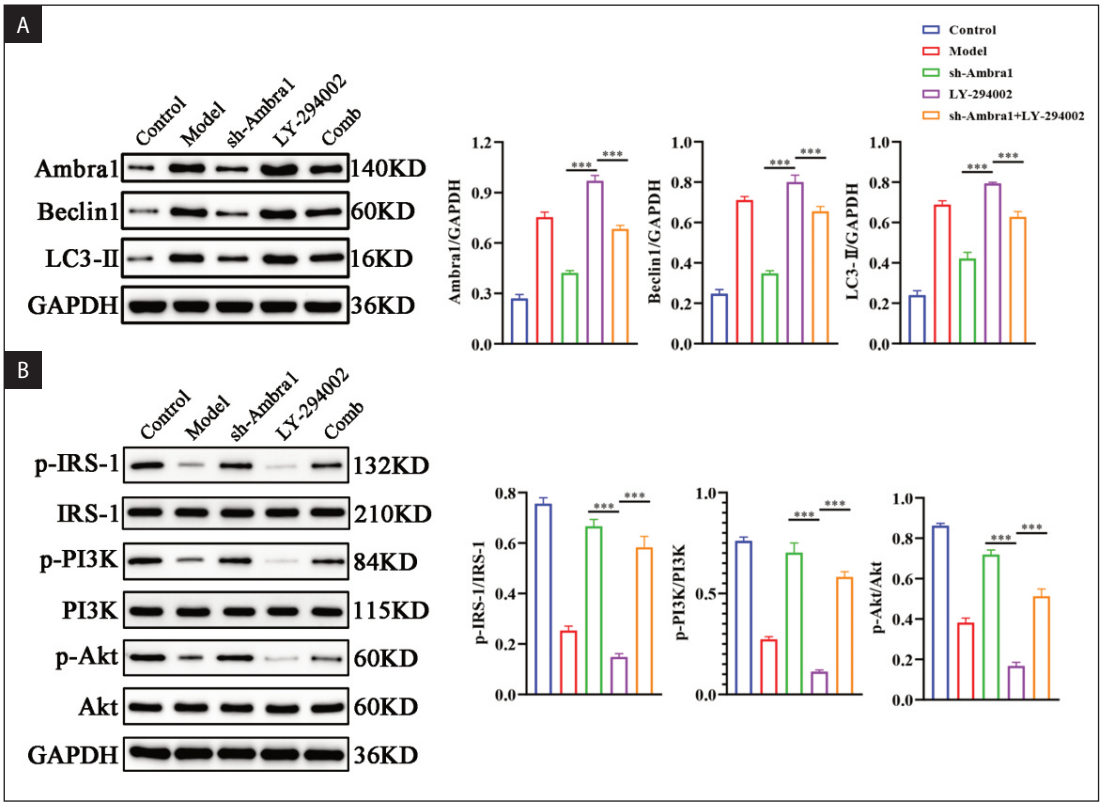

Figure 5. *Effects of the insulin substrate receptor 1/phosphatidylinositol 3 kinase/protein kinase B (IRS-1/PI3K/Akt) pathways on autophagy in HTR8/SvNEO-HG cells.* **A.** *Western blot detection of activating molecule of Beclin1-regulated autophagy (Ambra1), Bcl-2 interacting protein (Beclin1), and microtubule-associated proteins 1A/1B light chain 3B (LC3-II) expression levels.* **B.** *Western blot detection of p-IRS-1/p-PI3K/p-Akt signaling pathway. GAPDH — glyceraldehyde 3-phosphate dehydrogenase. ***$p < 0.001$*

the placenta of pregnant women with GDM than in normal pregnant women.

Luo et al. established a GDM rat model by injecting 1% streptozotocin combined with a high-fat diet and found that hepatocyte apoptosis and hepatic autophagosomes were significantly increased in GDM rats compared with normal rats [25]. In our study, increased autophagosomes in HTR8/ SvNEO-HG cells were found by TEM.

Huang et al. indicated that miR-200b inhibits autophagy and triggers apoptosis by directly targeting the autophagy-related gene Ambra1 (autophagy/Beclin1 regulatory factor 1) [26]. The phosphatidylinositol 3-kinase complex I (PI3K complex I) is a crucial regulator of autophagy, which contains Beclin 1, a long ATG14 isoform of autophagy-related gene 14 (ATG14L), vacuolar protein sorting 34 (VPS34), and Ambra1, and controls autophagosome formation [27].

In this study, we investigated the changes of autophagy in HTR8/SvNEO-HG cells by inhibiting PI3K/Akt pathways, and found that the expression levels of Ambra1, Beclin1, and LC3-II proteins increased significantly after inhibiting PI3K/Akt pathways, the expression of phosphorylated IRS-1/PI3K/Akt protein was significantly decreased, and autophagosomes and autophagolysosomes increased; however, when Ambra1 was silenced, the effect of inhibitor LY-294002 was reduced. Therefore, it indicates that Ambra1 is not only highly expressed in GDM, but also its ability to reduce the phosphorylation of IRS-1/PI3K/Akt proteins. Combined with Figure 4, it can be found that sh-Ambra1 can activate the phosphorylation of IRS-1/PI3K/Akt proteins, while IRS-1/PI3K/Akt signalling pathway regulates autophagy in HTR8/SvNEO-HG cells. The diagnostic criteria for GDM were adopted from the 2010 International Association of Diabetes and Pregnancy Study Groups (IADPSG), and the tests included fasting glucose and oral glucose tolerance test [28–30]. IRS-1/PI3K/Akt is a part of the INS and therefore failed to detect insulin receptors, and glycaemic differences in GDM patients are a shortcoming of this study.

Conclusion

sh-Ambra1 activates IRS-1/PI3K/Akt protein phosphorylation in GDM, decreases the transcription

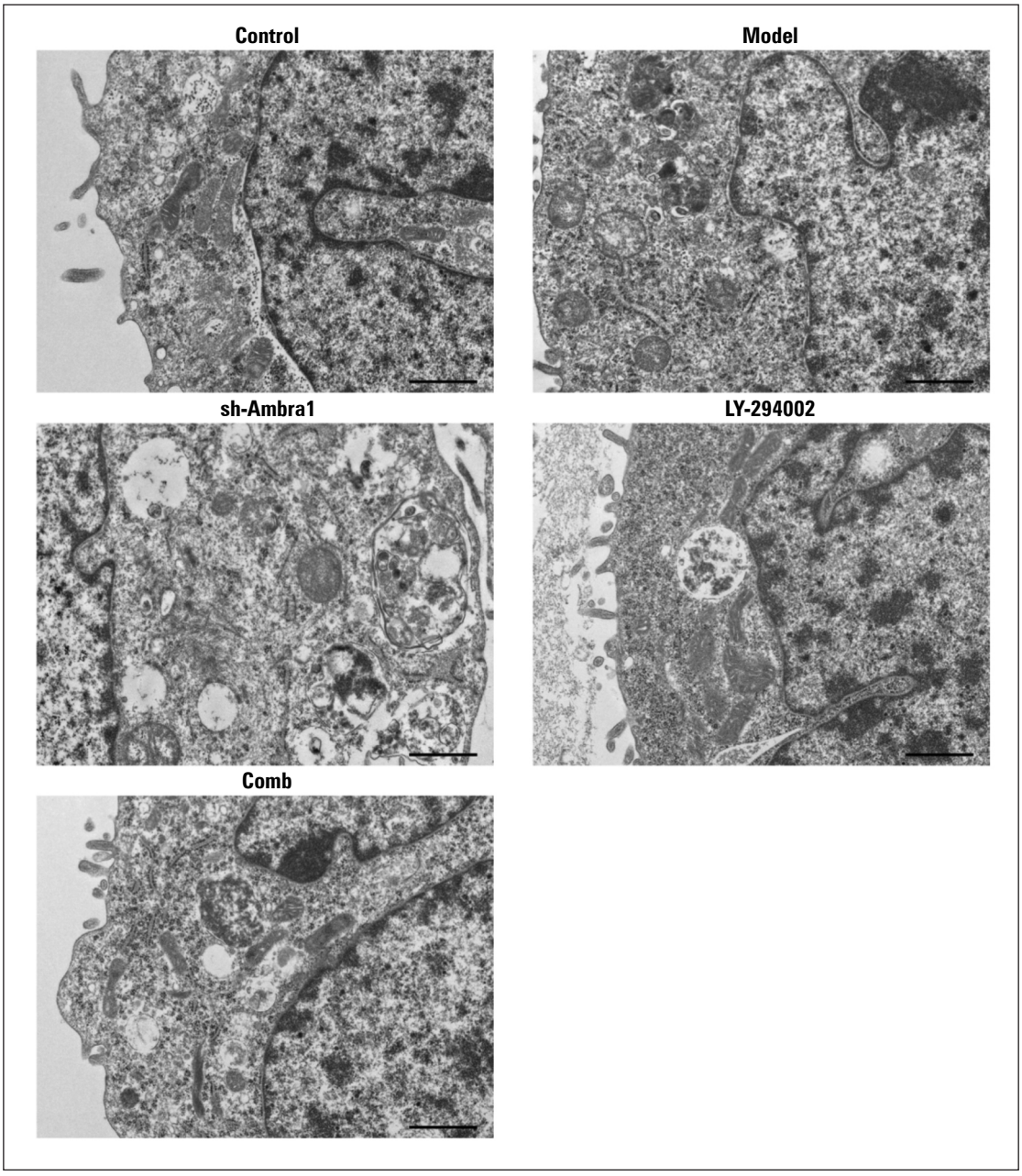

Figure 6. *Transmission electron microscopy (TEM): autophagy in HTR8/SvNEO-HG cells. Scale bar, 1 μm*

and translation levels of autophagy genes Ambra1, Beclin1, and LC3-II, and reduces autophagy in HTR8/SvNEO-HG cells via the IRS-1/PI3K/Akt signalling pathway.

Ethical approval
Approved by the Ethics Committee of the Yantai Yuhuangding Hospital. Approval No. 2021-012. Detailed information and consent to participate in Supplemental Material.

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Conflicts of interest
There are no conflicts of interest.

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Data availability statement
The data used to support the findings of this study are available from the corresponding author upon request.

Ethics statement
Approved by the Ethics committee of the Yantai Yuhuangding Hospital. Approval No. 2021-012. Detailed information and consent to participate in Supplemental Material.

Author contributions
X.Q. and X.Y.L. were responsible for the overall conception, experiment, and paper writing. Y.L.C. was faculty adviser. Y.F.,

X.W., L.L. and Y.W. were responsible for part of the experiment and reviewing paper.

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