UFL1 Relieves Cisplatin-Induced Premature Ovarian Failure by Reducing Endoplasmic Reticulum Stress in Granulosa Cells

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Research

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UFL1 Relieves Cisplatin-Induced Premature Ovarian Failure by Reducing Endoplasmic Reticulum Stress in Granulosa Cells

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Abstract

Background: Ubiquitin-like modifier 1 ligating enzyme 1 (UFL1), the ligase of the Ufmylation system, has recently been reported to be involved in apoptosis and endoplasmic reticulum stress (ER stress) in a variety of diseases. Premature ovarian failure (POF) is a gynecological disease that severely reduces the fertility of women, especially in female cancer patients receiving chemotherapy drugs. Whether UFL1 is involved in the protection from chemotherapy-induced POF and its mechanism remains unclear.

Methods: In this study, we examined the function of UFL1 in ovarian dysfunction and granular cells (GCs) apoptosis induced by cisplatin through histological examination and cell viability analysis. We used western blot, quantitative real-time PCR (qPCR) and immunofluorescence (IF) to detect the expression of UFL1 and the level of ER stress specific makers. Enzyme-Linked Immunosorbent Assays were used to detect the level of Follicle-Stimulating Hormone (FSH) and Estrogen (E2) in ovaries and GCs. In addition, we knocked down or overexpressed UFL1 in ovaries or GCs through infected with lentiviral particle suspensions, respectively.

Results: Our data showed that the expression of UFL1 was reduced in POF model ovaries and was accompanied by the occurrence of ER stress. In vitro, cisplatin induced a stressful increase of UFL1 expression in GCs, and enhanced ER stress, which was aggravated by UFL1 knockdown and alleviated by UFL1 overexpression. Furthermore, the data showed that UFL1 knockdown resulted in a decrease of ovarian follicles number, an

increase of atretic follicles, and a decreased expression of AMH and FSHR. Conversely, overexpression of UFL1 reduced the damage of cisplatin to the ovary in vitro.

Conclusions: Our research proved that UFL1 regulates cisplatin-induced ER stress and apoptosis of GCs, and participates in the protection from cisplatin-induced POF, providing a potential therapeutic target for clinical prevention of chemotherapeutic drug-induced POF.

Keywords: UFL1; POF; ER stress; cisplatin; apoptosis; GCs

1. Introduction

Ubiquitin-like modifier 1 ligating enzyme 1 (UFL1), also known as KIAA0776, RCAD, NLBP and Maxer, which molecular weight is approximately 90kDa composed of 794 amino acids, is the only identified E3 ligase in Ufmylation modification system(1-3). In addition to playing a crucial role in the ubiquitin-like system, UFL1 is also involved in various processes such as endoplasmic reticulum stress (ER stress), apoptosis, autophagy, inflammation, and oxidative stress in some organs such as hematopoiesis, heart, breast, and small intestine(4). Li et al. found that the loss of UFL1 weakened the protein kinase-like endoplasmic reticulum kinase (PERK) signal in unfolded protein response (UPR) and aggravated the occurrence of ER stress, while the up-regulation of UFL1 in cardiomyocytes could maintain ER homeostasis and prevent heart exhaustion(5). Zhang et al. discovered that UFL1 exhaustion would lead to abnormal activation of transformation related protein 53 (P53) and autophagy degradation which increased cell death, finally leading to embryo damage and hematopoietic defects(6). Cai et al. revealed that the deletion of UFL1 contributed a large loss of intestinal Paneth cells and goblet cells, which changed the intestinal tract bacteria and caused susceptible to enteritis(7). Therefore, previous researches proved that UFL1 can maintain the ER homeostasis of cells and play an important role in embryonic development and some disease progression. Interestingly, granular cells (GCs) proliferation or differentiation is closely related to ER stress(8), however, whether UFL1 can influence follicular development and protect ovarian function via relieving ER stress is still unclear.

The ER is the main organelle responsible for the biosynthesis of lipids or sterols, maintaining calcium homeostasis, and managing protein synthesis, folding and secretion into other organelles(9). However, various pathological conditions, such as hypoxia, starvation, and calcium depletion, hinder the properly fold and modification of protein and finally trigger ER stress(9-11). The specific markers of ER stress are Glucose Regulated Protein 78 (GRP78), the spliceosome of X-box Binding Protein 1 (XBP1s), C/EBP Homologous

Transcription Factor (CHOP), which level upregulation indicate the aggravation of ER stress(12). The proliferation and differentiation of GCs in the follicle regulates the maturation of oocytes and impact the female reproductive function so that the occurrence of severe ER stress in GCs may lead to ovarian dysfunction and infertility(11, 12).

Premature ovarian failure (POF) is an ovarian functional defect before the age of 40 years, which characterized by amenorrhea, hypergonadotropicemia and estrogen deficiency(13-15). There are several factors to cause POF, chemotherapeutics is one of the important reasons but the molecular mechanism is unknown(16, 17). The maturation of oocytes needs GCs provide nutrients and growth factors(18), so that the apoptosis or damage of GCs may be the main reason of follicular atresia and POF. Studies verified the excessive ER stress can initiate apoptotic cell death via the up-regulation of the UPR transcription factor CHOP(19). Here, we hypothesis that chemotherapy drugs causing GCs apoptosis may be through activating severe ER stress pathway. Previous studies have shown that UFL1 can regulate ER stress in cardiomyocytes and bone marrow cells(5, 6), however, whether UFL1 can alleviate ER stress in GCs to rescue POF remains to be explored.

In this study, we explored the function of UFL1 to protect follicle and GCs via constructed a POF model and primary culture GCs treated with cisplatin. Our research demonstrated that UFL1 expression increased obviously all types stage of follicles and rescued ovarian function. The results showed that UFL1 survives cisplatin-induced ovarian GCs apoptosis by relieving the ER stress, and alleviates POF in some extent. Our study suggests that UFL1 may be a molecular target to relieve ovarian injury induced by the chemotherapy drug and provide a new clinical treatment strategy in future.

2. Materials and Methods

2.1. Animals and Treatment

The 6~8 weeks old Kunming mouse used in the experiment were purchased from the Department of Animal Science of Nanchang University Jiangxi Medical College. All mouse were provided with plenty of food and tap water and were intraperitoneally injected with cisplatin (2.5mg/kg and 5.0mg/kg, sigma, USA) for 10days to construct the POF model(20, 21). The study was approved by the Animal Care Committee of Nanchang University Jiangxi Medical College (Animal protocol: NCDXSYDWFL-2015097).

2.2. Primary Ovarian Granulosa Cells Isolation and Culture

The ovaries were collected and GCs were isolated mechanically under aseptic conditions after 48h of 20U Pregnant Mare Serum Gonadotropin (PMSG) injection. The GCs were put into a culture plate containing 10%(v/v) FBS (Gibco, Staley Rd Grand Island, NY, USA), FSH 100U and 1% antibiotic in DMEM/F12 1X (1:1) (Gibco, USA),

and were incubated at 37℃ with 5% CO2 for 48 hours(22). After incubation, the appropriate number of GCs were planted in culture plates after count using a hemocytometer according to each experimental requirement.

2.3. Cell Treatment

GCs were treated with different concentrations of cisplatin (0, 5, 10, 20 and 40μM) to screen for appropriate dosing concentrations. Then GCs were treated with different times of 40μM cisplatin (0h, 3h, 6h, 12h, to 24h) to screen the appropriate time of drug action. The changes of ER stress markers were detected after treatment with 20uM cisplatin for 12h, and apoptosis proteins and cell viability were detected after treatment with 20uM cisplatin for 24h.

2.4. Cell Transfection

We collected lentivirus particle suspension by co-transfecting pSPAX2, pVSVG and target plasmid into 293T cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect cells or ovaries according to the instructions. The plasmid was extracted with glycerol broth (GenePharma, Shanghai, China) according to the instructions. The amplified product was purified and the UFL1 gene was cloned into the pEX-3 vector, and it was then transferred into competent cells. The GCs were seeded into six-well plates and infected lentivirus at a density of 60%–70% for 48h. We used two pairs of UFL1 shRNA. One of the UFL1 shRNA sequence is 5′-GAAACACTTCTGTGTCAGAAA-3′, and the antisense sequence is 3′-GCTCTGGAACATGGGTTGATA-5′. The sequence of another one is 5′-GCAGCAGAAGCTTGTGATATT-3′, and the antisense sequence is 5′-TATCACAAGCTTCTGCTGCTT-3′.

2.5. Ovary Extraction and Culture

The ovaries were dissociated and placed in pre-cooling PBS solution and as much surrounding tissue was removed as possible. Ovaries were placed in the Transwell chamber (Millicell, Darmstadt, Germany) in Waymouth medium (Sigma, USA) containing 10% (v/v) FBS (Gibco, Staley Rd Grand Island, NY, USA), 0.23 mM sodium pyruvate, and penicillin and streptomycin (P/S) (Solarbio, Beijing, China)(23). For in vitro ovarian experiments, ovaries were co-cultured with shRNA or OE-UFL1 lentivirus particles suspension for 7 days which would be replaced every 24h.

2.6. HE Staining and Follicle Counting

Ovaries were immediately fixed overnight with 4% paraformaldehyde at room temperature and then embedded in paraffin. According to the manufacturer's instructions, the paraffin sections of the ovarian tissue were

stained with hematoxylin and eosin solution (G1001 and G1004, Servicebio, Wuhan, China) to observe the pathological structure of the ovary and to calculate the number of follicles at all levels. Follicular counts were performed according to the method proposed in previous studies.

2.7. Cell Proliferation Assay and Colony Formation Assay

GCs were spread on a 96-well plate with 2000 cells per well and treated with cisplatin for different concentration (0, 5, 10, 20 and 40μM) or different time (0h, 3h, 6h, 12h, to 24h). A Cell Counting Kit (CCK8, Transgen BioTECH, Beijing, China) was used to detect cell proliferation activity. In addition, 1000 GCs were seeded in 6-well plates and cultured for 7 days under shRNA lentivirus treatment. Crystal violet was used to stain and count the number of cellular colonies.

2.8. Immunoblotting, Immunohistochemistry, and Immunofluorescence

Immunoblotting (IB), immunohistochemistry (IHC), and immunofluorescence (IF) were performed as described previously(24). Images were acquired using a NIKON Eclipse 80i microscope. The primary antibodies used in this study include beta-Tubulin (10094-1-AP, Proteintech, Wuhan, China), UFL1 (ab226216, Abcam, Cambridge, UK), XBP1 (ab37152, Abcam, Cambridge, UK), GRP78 (66574-1-lg, Proteintech, Wuhan, China), CHOP (15204-1-AP, Proteintech, Wuhan, China), BCL-2 (26593-1-AP, Proteintech, Wuhan, China), BAX (WL01637, Wanleibio, Shenyang, China), Caspase3/cleaved-Caspase3 (WL02117, Wanleibio, Shenyang, China), Caspase-3 (WL04004, Wanleibio, Shenyang, China), AMH (HA500137, HUABIO, Hangzhou, China), FSHR (22665-1-AP, Proteintech, Wuhan, China). All HRP- and fluorophore-conjugated secondary antibodies were obtained from Elabscience company. The EdU kit was purchased from Keygen BioTECH (KGA337-1000).

2.9. Quantitative Real-Time PCR

Total mRNA was extracted from tissue or cell samples using Trizol reagent. The cDNA was obtained by reverse transcription of mRNA according to the instructions of the PrimeScriptRT kit for subsequent testing. TB Green Mix was used for real-time quantitative PCR. The reference gene is β-actin, the PCR primer sequences are shown in Table 1, and are used only for PCR amplification of specific segments of the gene of interest.

Table 1. Sequences used for quantitative real-time PCR.

Gene Name	**Primer Sequence：5’-3’**	**Gene ID**
UFL1	Forward: TGGCTATCTAGAATTTGACGCT Reverse: CATAGCACATCTTCAACTGAC	NM_001355512.1

GRP78	Forward: ATGATGAAGTTCATGTGGTGG	NM_001163434.1
	Reverse: CTGATCGTTGGCTATGATCTCC	
ATF4	Forward: AGTTTAGAGCTAGGCATGAAG	NM_001287180.1
	Reverse: CATACAGATGCCATGTCATTG	
CHOP	Forward: CTCGCTCTCCAGATTCCAGTC	NM_001290183.1
	Reverse: CTTCATGCGTTGCTTCCCA	
ACTB	Forward: CTACCTCATGAGATCCTGACC	NM_007393.5
	Reverse: CACAGCTTCTCTTTGATGTCAC	

2.10.Mitochondrial Membrane Potential Measurement

The JC-1 apoptosis detection kit (KeyGen BioTECH, China) was used to detect mitochondrial membrane potential. GCs were washed three times in sterile PBS, and JC-1 working solution was added to each well and incubated for 30 min under 5% CO2 and 37 °C conditions. Hoechst 33342 (Solarbio B8040) was used to stain the nuclei. After incubation, the FGSCs were washed with PBS three times and observed under a NIKON Eclipse 80i fluorescence microscope. The green fluorescent channel image (FL1) and the red fluorescent channel image (FL2) were analyzed by ImageJ software, and the ratio of FL2 to FL1 was calculated to reflect the mitochondrial membrane potential.

2.11. Hormone Measurement with Enzyme-Linked Immunosorbent Assays

For serum sample collection, at the end of the experiment, a blood sample was collected from the eyeball vein and centrifuged at 3000rpm for 15 minutes. For in vitro cultured ovarian tissue samples and granulosa cells, ovarian tissue homogenate and GCs suspension homogenate were collected after the required experimental treatment. FSH (E-EL-M0511c, Elabscience, Wuhan, China) or AMH (E-EL-M3015, Elabscience, Wuhan, China) levels in the samples were measured using ELISA kits according to the manufacturer's instructions.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software, and one-way analysis of variance was used to detect statistical differences between multiple sets of data. P values <0.05 was considered statistically significant. All data are expressed as the mean ± standard error of at least three independent experiments

3. Results

3.1. The Expression of UFL1 Decreases in POF Model Mouse Ovaries

Firstly, we identified the expression of UFL1 in ovaries at different stages of development. During mouse development from 1D to 10M, the expression of UFL1 increased from 1D to 2M and tended to decrease at 10M in protein and mRNA level (Figure 1A and Figure S1A), indicating that the abundance of UFL1 is associated with ovarian aging. We constructed mouse models of POF by intraperitoneal injection of cisplatin (Figure S1B-F). The expression of UFL1 protein was significantly decreased in the ovary of POF model mice (Figure 1B), and IHC analysis showed that the decrease of UFL1 protein mainly occurred in ovarian GCs (Figure 1C). Therefore, we isolated primary ovarian GCs (Figure S1G, H) and determined the expression and localization of UFL1 by IF in GCs (Figure 1D). Next, we treated GCs with a gradient concentration of cisplatin, the results discovered that the protein expression of UFL1 increased within a certain concentration range (<15μM), whereas showed a decrease at 20μM (Figure 1E). Meanwhile, the level of UFL1 protein was elevated at 12h and reduced at 24h when treated with 20μM cisplatin (Figure 1F, G). In short, the expression of UFL1 was weaken in POF ovaries, and instantaneously upregulated in stress and finally descended in GCs under cisplatin treatment.

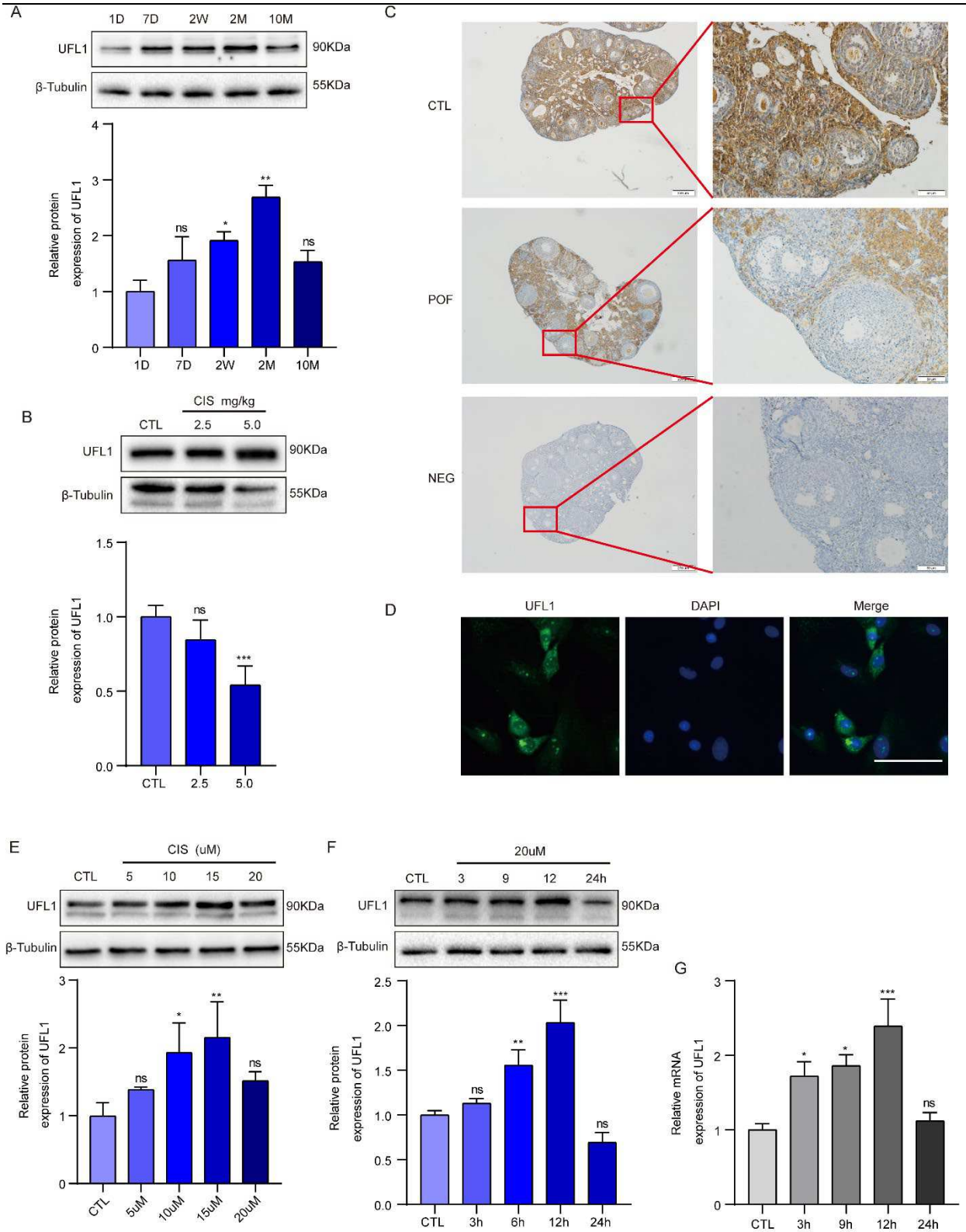

Figure 1. The expression of UFL1 decreased in POF model mouse ovaries and GCs. (A) The protein level of UFL1 in mouse ovaries at 1D, 7D, 2W, 2M, and 10M. (B) The UFL1 protein level in POF ovaries. (C) IHC detected the UFL1 in POF ovaries. Bar, 50μm. (D) IF detected the UFL1 in primary GCs. Bar, 20μm. (E) The level of UFL1 in GCs treated with a gradient concentration of cisplatin. (F, G) The protein and mRNA level of UFL1 in GCs treated with 20μM cisplatin at different time points. * p <0.05; ** p <0.01; *** p <0.001.

3.2. Cisplatin Treatment Triggers ER Stress in GCs and Ovaries

To observe the damage effect of GCs caused by cisplatin, CCK8 analysis was used to detect the activity of GCs. The results showed that the cell proliferation was inhibited by cisplatin with time and gradient dependence (Figure 2A, B). We next determined the occurrence of ER stress in POF GCs and ovaries, respectively. When treated with a gradient concentration of cisplatin, the protein expression of ER stress specific markers GRP78 and XBP1s in GCs increased within 15μM and decreased at 20μM while CHOP was persistently increased (Figure 2C). Then, we chose 20μM cisplatin to analyze the changes of ER stress markers in GCs with different time points, and the data discovered that the level of GRP78 and XBP1s increased within 12h and weakened after 24h while CHOP raised slightly within 12h and upregulated significantly after 24h (Figure 2D, E). As shown in Figure 2F, a similar trend of ER stress markers was observed in different dosage cisplatin treated ovaries. Furtherly, we tried to explore whether alleviating ER stress could reduce the damage of cisplatin to GCs. The results of IF showed that the fluorescence intensity of GRP78 protein increased after cisplatin treatment and was inhibited by 4-phenylbutyric acid (4-PBA, an ER stress inhibitor) (Figure 2G). Meanwhile, the level of Estrogen (E2) secreted by GC in the 4-PBA-treated group was higher than that of the cisplatin-treated group (Figure 2H), suggesting that inhibiting ER stress may be helpful to alleviate the damage of GCs. In a word, cisplatin induced time - and concentration-dependent ER stress in ovary and GCs, and the functional damage of GCs can be improved by inhibiting ER stress.

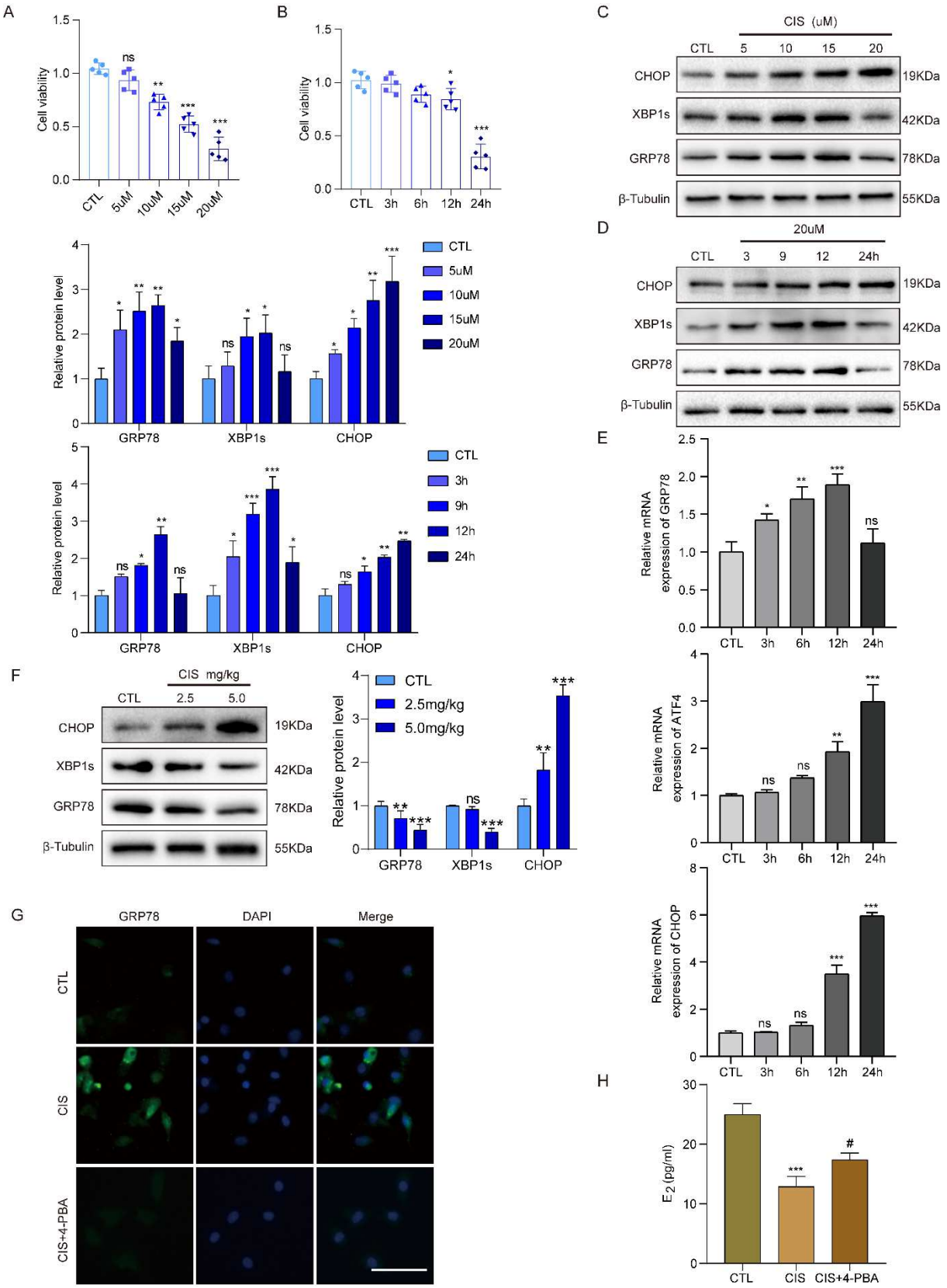

Figure 2. Cisplatin treatment induced an increase of ER stress both in ovaries and GCs. (A) The activity of GCs treated with different concentrations of cisplatin. (B) CCK-8 analysis detected the activity of GCs treated with 20μM cisplatin at different time. (C) The protein levels of UFL1, GRP78, XBP1s and CHOP in GCs treated with different concentrations of cisplatin. (D) The protein levels of

UFL1, GRP78, XBP1s and CHOP in GCs at different time points under 20μM cisplatin treatment. (E) The mRNA levels of UFL1, GRP78, ATF4 and CHOP in GCs at different time points under 20μM cisplatin treatment. (F) The protein levels of GRP78, XBP1s and CHOP in POF ovaries. (G) IF detected the expression of GRP78 in GCs in the control group, cisplatin treatment group, and 4-PBA co-treatment group. Bar, 50μm. (H) ELISA detect E2 level of GCs in the control group, cisplatin treatment group, and 4-PBA co-treatment group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with the control group. # $p < 0.05$, compared with the cisplatin treatment group.

3.3. UFL1 Deficiency Aggravates Cisplatin-Induced ER Stress and Apoptosis in GCs

To confirm whether UFL1 is involved in the process of ER stress and apoptosis under cisplatin treatment, we knocked down the expression of UFL1 with shRNA in GCs (Figure 3A). Compared with the control group, GCs proliferation was weaker in UFL1 knockdown groups (Figure 3B, C), and the expression of the ER stress proteins GRP78, XBP1s and CHOP were enhanced at the same time (Figure 3D, E), which indicating that UFL1 depletion were more vulnerable to ER stress and apoptosis. As shown in Figure 3F and 3G, the level of GRP78, XBP1s and CHOP were significantly increased and a similar trend was observed both in the ratio of BAX/BCL-2 and Cleaved-Caspase 3/Caspase 3 in the UFL1 knockdown + cisplatin group. The IF images showed weaker red JC-1 signal in the UFL1 knockdown +cisplatin groups than in the cisplatin treatment group (Figure 3H), which suggesting that UFL1 deficiency furtherly strengthened mitochondrial membrane potential（MMP）after cisplatin treated. In addition, the alteration of E2 concentration also verified the above results (Figure 3I). Together, our experiments proved that UFL1 expression certainly correlates with ER stress and apoptosis induced by cisplatin.

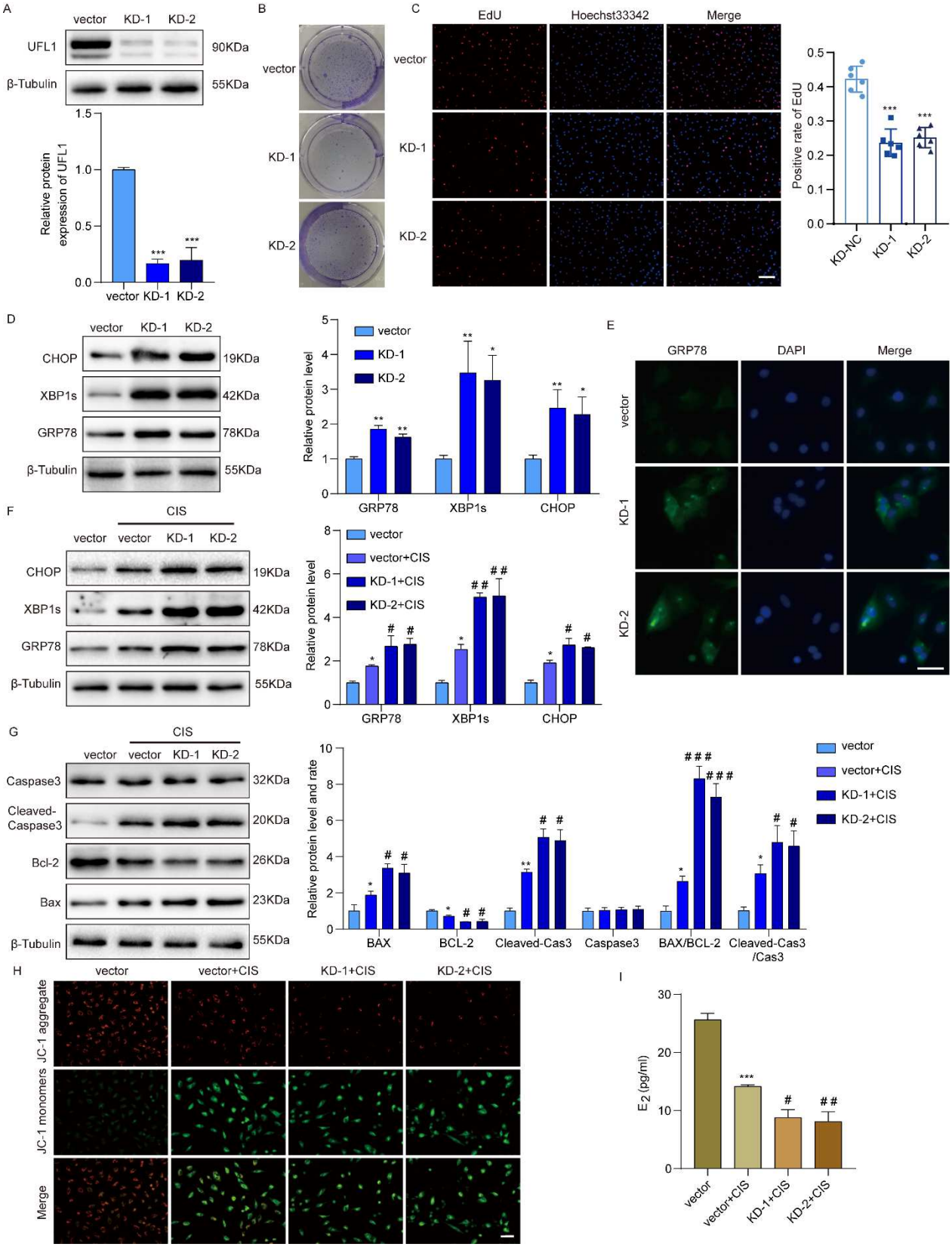

Figure 3. UFL1 depletion aggravates cisplatin-induced ER stress and apoptosis in GCs. (A) The knock down efficiency of UFL1 shRNA. (B) UFL1 knockdown attenuated GCs colony formation. (C) The proliferative activity of GCs was evaluated by EdU staining positive cell rate. Bar, 100μm. (D) Changes in protein expression of GRP78, XBP1s and CHOP with UFL1 shRNA. (E) IF was used

to detect the expression of GRP78 between the control and UFL1 knock down GCs. Bar, 50μm. (F) The protein level of GRP78, XBP1s and CHOP with cisplatin treatment in UFL1 knockdown GCs. (G) The changes of protein BAX, BCL-2, Cleaved Caspase3 and Caspase3 with cisplatin treatment in UFL1 knockdown GCs. (H) JC-1-based immunofluorescence assay of UFL1 knockdown GCs with cisplatin treatment. Red represents JC-1 aggregate signal, and green represents JC-1 monomer signal. Bar, 50μm. (I) E2 secretion from GCs was detected by ELISA kit. ** $p < 0.01$; *** $p < 0.001$, compared with the control group. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared with the cisplatin treatment group.

3.4. Overexpression of UFL1 Resists Cisplatin Induced ER Stress and Apoptosis.

Further, we attempted to investigate the protective effect of UFL1 against cisplatin treatment via overexpressed UFL1 (OE-UFL1) in GCs. As shown in Figure 4A, the level of UFL1 in the OE-UFL1 group was more than two times compared with the control group. The results of CCK-8 analysis and EdU staining showed that cellular viability and proliferation were increased after infected with UFL1 lentivirus particles (Figure 4B, C), and E2 level was increased simultaneously (Figure 4D). Western blot results showed that overexpression of UFL1 decreased the ratios of BAX/BCL-2 and Cleaved-Caspase 3/Caspase 3, and downregulated the expression of Grp78, XBP1s and CHOP in the meantime (Figure 4E-G). In addition, JC-1 staining showed that the level of MMP was lower in the OE-UFL1 group than the cisplatin treatment group (Figure 4H). All in all, the overexpression of UFL1 can protect GCs from cisplatin induced ER stress and apoptosis.

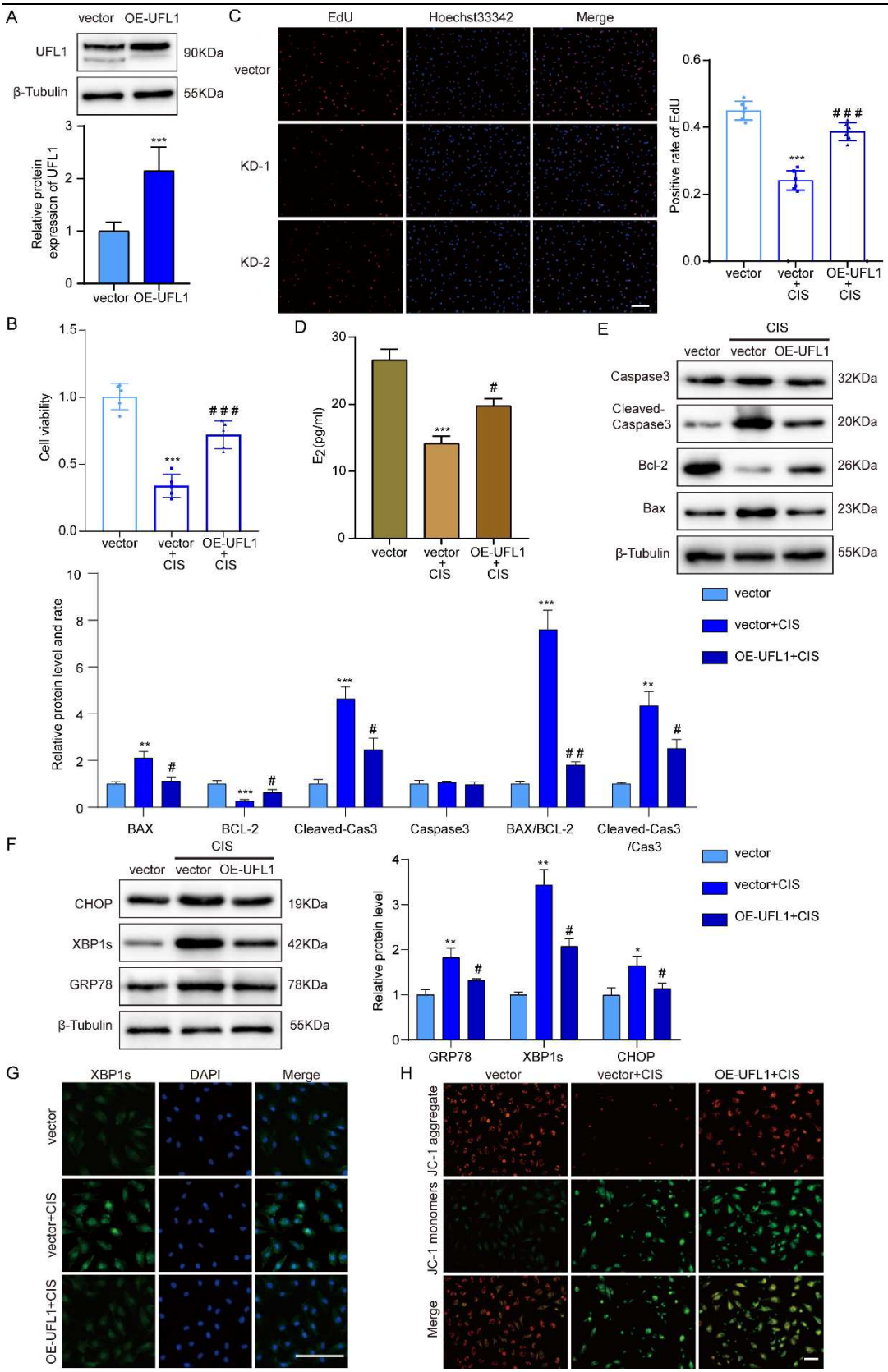

Figure 4. Overexpression of UFL1 alleviated the apoptosis and ER stress induced by cisplatin. (A) Western blot was used to detect UFL1 overexpression efficiency. (B) The cell viability of UFL1 overexpression group was detected by CCK-8. (C) The proliferative activity of GCs was evaluated by EdU staining positive cell rate. Bar, 100μm. (D) E2 levels in the supernatant of granulocyte cell

culture. (E) The changes of protein BAX, BCL-2, Cleaved Caspase3 and Caspase3 with cisplatin treatment in UFL1 overexpression GCs. (F) The protein level of GRP78, XBP1s and CHOP with cisplatin treatment in UFL1 overexpression GCs. (G) The expression of XBP1s in UFL1 overexpression GCs treated with cisplatin was detected by immunofluorescence. Bar, 100μm. (H) JC-1-based immunofluorescence assay of UFL1 overexpression GCs with cisplatin treatment. Red represents JC-1 aggregate signal, and green represents JC-1 monomer signal. Bar, 50μm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with the control group. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared with the cisplatin treatment group.

3.5. The Loss of UFL1 Causes Ovarian Follicular Atresia

To evaluate the role of UFL1 in maintaining ovarian function, we co-cultured ovaries with UFL1 shRNA lentivirus particles in vitro. As shown in Figure 5A, we successfully knocked down the expression of UFL1 in ovaries. The FSHR and AMH represent ovarian reserve function, our results showed that their level were reduced significantly after UFL1 knockdown (Figure 5A), besides the concentration of E2 was also decreased in certain extent (Figure 5B). Compared with the control group, atretic follicles were added while primitive follicles were decreased in the UFL1 knockdown group (Figure 5C-E). Same as the trend in UFL1 knockdown GCs, ER stress markers also showed an obviously increasing in the ovarian UFL1 knockdown group (Figure 5F). In addition, we added UFL1 shRNA in cisplatin group. As a result, the expression ratios of apoptotic protein BAX/BCL-2 and Cleaved-Caspase 3/Caspase 3 were obviously increased compared with only cisplatin group (Fig.5 G), and the FSHR and AMH showed a uniform trend (Figure 5H). Above all, these data indicate that the knockdown of UFL1 in ovaries induced follicular dysfunction, atresia and decline in number, suggesting that UFL1 might play a crucial role in maintaining ovarian function.

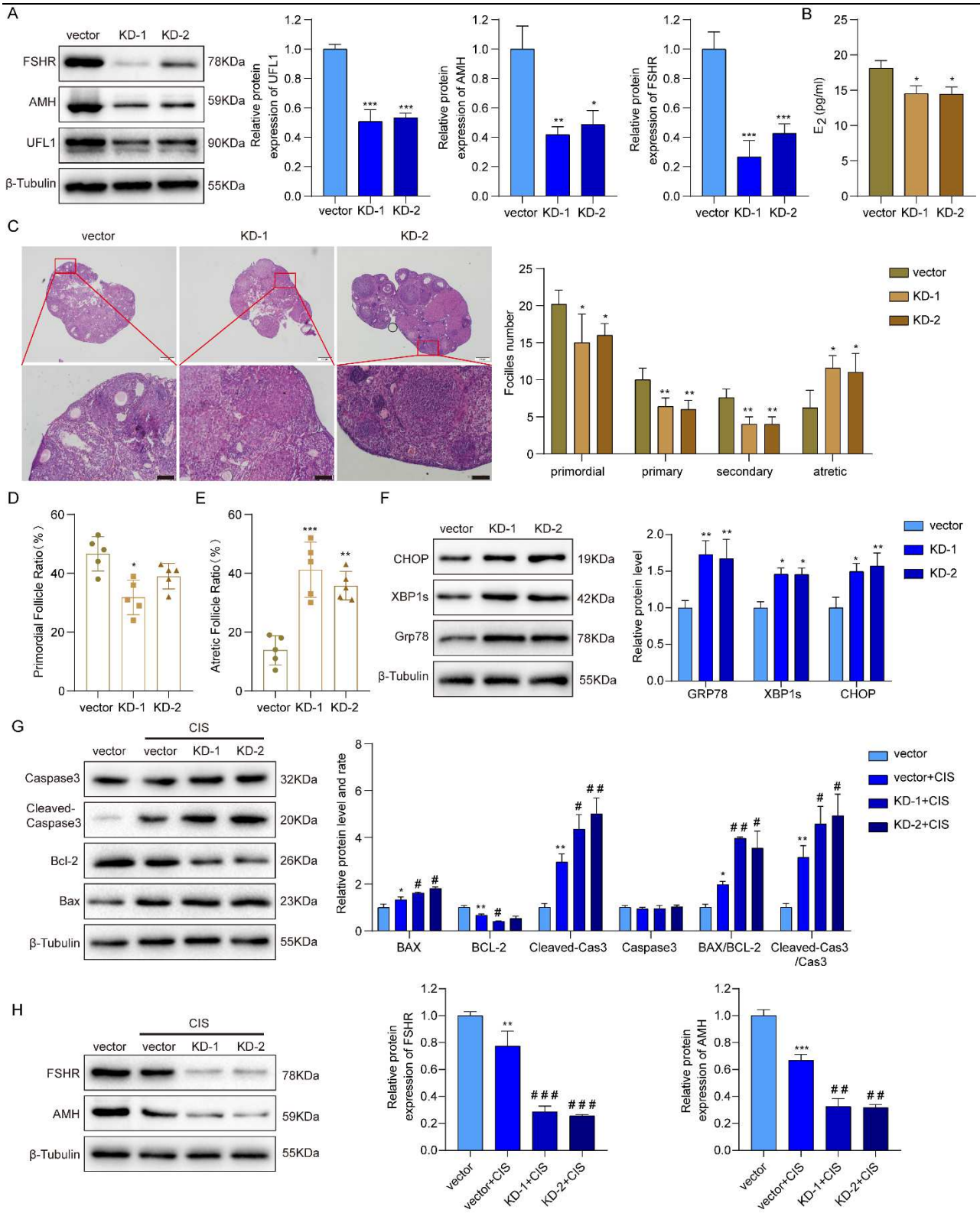

Figure 5. Loss of UFL1 leaded to activation of ovarian ER stress, increased atretic follicles, and decreased ovarian function. (A) The changes of the protein level of UFL1, AMH and FSHR in UFL1 knockdown ovaries. (B) E2 level of ovarian abrasive fluid after knockdown UFL1. (C) The follicles were observed after HE staining and all levels of follicle number were counted by tissue slice. Bar, 200μm and 50μm. (D, E) The rate of follicle atresia and the primordial follicular rate vs. the total number of follicles in each group. (F)

The changes of the ER stress markers GRP78, XBP1s and CHOP in UFL1 knockdown ovaries. (G) The changes of protein BAX, BCL-2, Cleaved Caspase3 and Caspase3 with cisplatin treatment in UFL1 knockdown ovaries. (H) The changes of protein FSHR and AMH with cisplatin treatment in UFL1 knockdown ovaries. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with the control group. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared with the cisplatin treatment group.

3.6.UFL1 Alleviates POF Induced by Cisplatin in Vitro

Next, we co-cultured ovaries with OE-UFL1 lentivirus particles in cisplatin group, the efficiency of OE-UFL1 was shown in Figure 6A. After 7 days co-culture, the number of atretic follicles decreased and primordial follicles increased compared with only cisplatin group (Figure 6B-D), there was also an increased protein expression of AMH and FSHR simultaneously (Figure 6E). Furthermore, the protein level of BAX and Cleaved-Caspase3 were decreased and BCL-2 was increased in the OE-UFL1 group (Figure 6F), and that ELISA results showed an increase of E2 concentration (Figure 6G). Taken together, our data suggests that the overexpression of UFL1 can mitigate POF induced by cisplatin and augment follicle number to some extent.

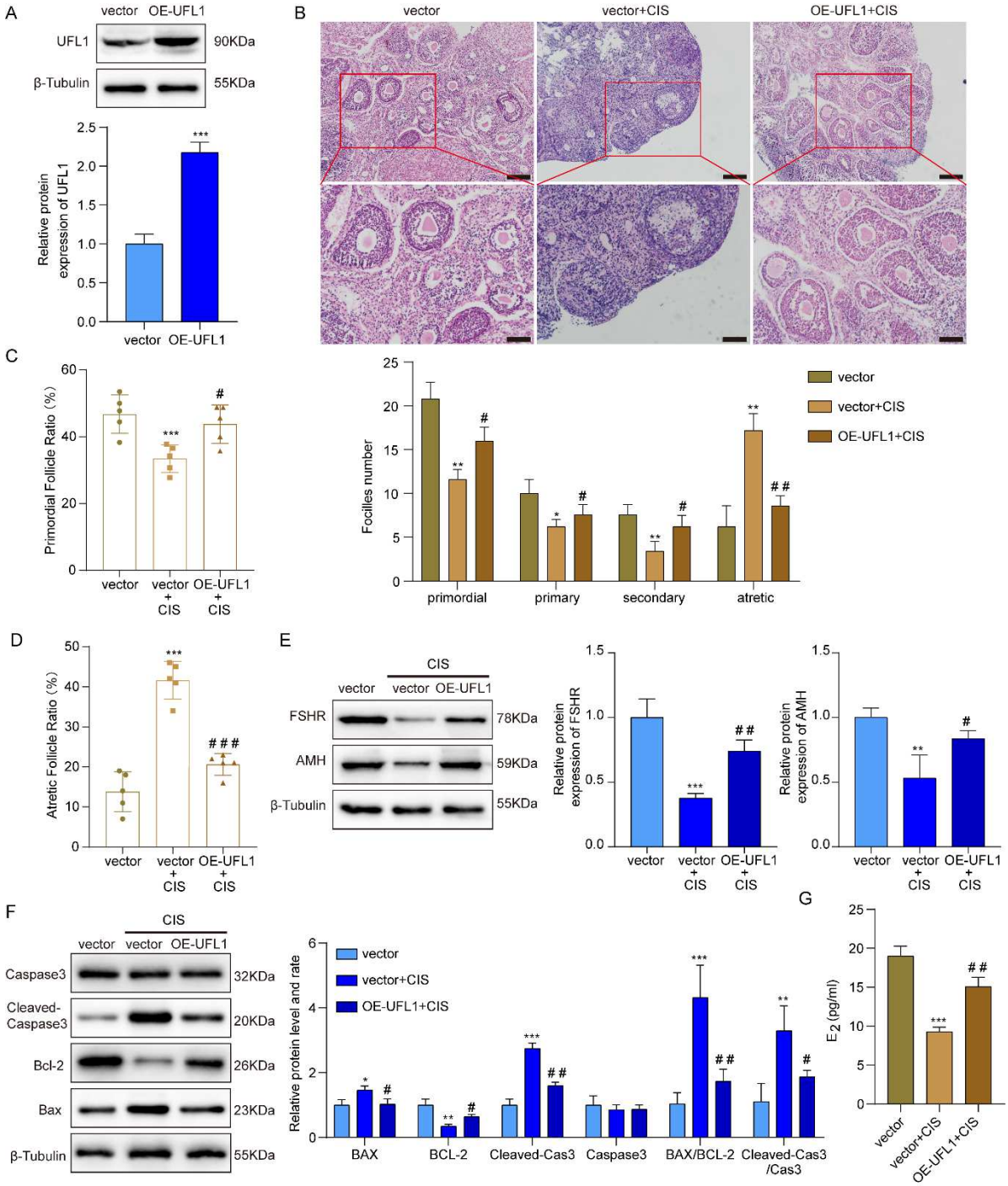

Figure 6. Overexpression of UFL1 alleviated the damage of cisplatin in ovaries in vitro. (A) The efficiency of UFL1 overexpression was detected by western blot. (B) The follicles were observed after HE staining and all levels of follicle number were counted by tissue slice. Bar, 100μm and 50μm. (C, D) The rate of follicle atresia and the primordial follicular rate vs. the total number of follicles in each group. (E) The changes of protein FSHR and AMH with cisplatin treatment in UFL1 overexpression ovaries. (F) The changes of protein BAX, BCL-2, Cleaved Caspase3 and Caspase3 with cisplatin treatment in UFL1 overexpression ovaries. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with the control group. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared with the cisplatin treatment

group.

4. Discussion

Recently researches have confirmed that UFL1 plays a crucial role in some biological process such as cell proliferation, differentiation and embryonic development(25-28), but the function of UFL1 in POF has not been explored. In this study, we firstly revealed that UFL1 is expressed in GCs, oocytes and stromal cells in ovarian tissues. Comparing the expression of UFL1 in the ovaries at different developmental stages, it was found that the level of UFL1 increased significantly along with follicular development, whereas decreased in aging ovaries. Follicular development is accompanied with proliferation and differentiation of GCs, which implies plentiful protein synthesis and posttranslational modification (PTM)(29, 30). Therefore, we speculated that the increased expression of UFL1 contributes to the maintenance of ER homeostasis in GCs, and provides a stable internal environment for follicular development. We detected the UFL1 protein level via constructing a POF model, the results showed that UFL1 was decreased in POF mouse which supports our speculation. Next, we tested the ER stress specifical molecules in POF model, and found that the level of GRP78 and XBP1s were decreased. Interestingly, the expression of CHOP which can activate apoptotic pathway was increased obviously. Previous studies have reported that ER stress can induce the expression of GRP78, XBP1s and other ER molecular chaperones to produce protective effects as well as trigger endogenous cell apoptosis, and ultimately affect the outcome such as adaptation, injury or apoptosis for stressed cells(31, 32). Therefore, we guessed that the mechanism of POF caused by cisplatin is through severe ER stress, which eventually led to apoptosis of GCs, follicular atresia and ovarian dysfunction.

ER stress is a transient and dynamic process, which molecular markers are highly susceptible to other factors(33, 34). In order to avoid stimulation in ER homeostasis during the primary isolation and culture of GCs, we treated normal GCs with cisplatin in vitro to replace primary POF GCs. The data showed that the level of UFL1 increased within a certain treatment time (<12h) in GCs with 20μM cisplatin. The interesting phenomenon was that the changes of GRP78 and XBP1s showed a same trend as UFL1, while CHOP increased with the prolonged treatment time which is consistent with alteration in POF ovaries. Cells occur the accumulation of a large number of misfolded proteins after subjected cisplatin, and finally resulting in the occurrence of ER stress. Researches proved that mild and transient ER stress can be alleviated by activating the UPR pathway while severe and

continuous ER stress can induce the apoptotic pathway(35). In this study, our data also confirms that cisplatin causes apoptosis in GCs via triggering severe ER stress. To verify whether UFL1 plays a protective role in ER stress, we knocked down and overexpressed UFL1 in GCs and ovaries respectively. The results showed that OE-UFL1 can alleviate cisplatin-induced ER stress and apoptosis, reduce atretic follicles and improve ovarian function. Inversely, knockdown of UFL1 aggravate the damage of cisplatin. In summary, our results indicate that UFL1 plays a protective effect on GCs survive, follicular number and against follicle atresia via alleviating ER stress and apoptosis.

In recent years, researches show that UFL1 can protect cells, such as bovine mammary epithelial cells, goat endometrial epithelial cells and human osteoarthritis chondrocytes, from LPS stimulation(7, 25, 36). In this study, we demonstrated that UFL1 protects GCs from cisplatin damage by relieving ER stress, while more research is needed to explore how UFL1 regulates ER stress. Walczak et al. discovered that ribosomes RPL26 is the new substrate of UFL1 conjugation, and the Ufmylation of RPL26 is linked with ER homeostasis(37, 38). Studies showed that UFL1 firstly forms a receptor complex with C53 and DDRGK1, then DDRGK1 recruits UFL1 to the ER surface for Ufmylation-dependent ER autophagy(39), and their binding is necessary for DDRGK1 Ufmylation process(40, 41). And the C53 protein was an ER autophagy receptor, UFL1 and DDRGK1 are co-delivered to vacuoles with C53 and are essential for C53-mediated autophagy(41). Therefore, the above researches suggest that UFL1 changes the ER homeostasis by ER autophagy pathway. Beyond that, UFL1 may induce ER stress by activating ferroptosis. As an important molecule in Ferroptosis, P53 can be covalently modified by UFL1 and depletion of UFL1 can decrease P53 stability(42). Related study has shown that the activator of Ferroptosis induces an increase in ER stress(43). Thus, UFL1 knockout may activate Ferroptosis and induce ER stress by regulating P53 activity. In addition, the latest research has reported that the protein stability of SLC7A11, which is a crucial ferroptosis regulator, can be reduced by inhibiting its Ufmyltion process(44). Thus, we speculate that UFL1 deletion may can reduce the Ufmylation of SLC7A11 to activate ferroptosis related ER stress.

In this study, we focused on whether UFL1 can relieve the POF induced by cisplatin. The results indicated that UFL1 alleviates ovarian dysfunction and GCs apoptosis by reducing ER stress in GCs, but we still need more experiments in vivo to evaluate the potential function of UFL1 as a target to alleviate ovarian aging and prevent POF caused by chemotherapy drugs. In conclusion, our study proved that UFL1 alleviated cisplatin-induced GCs apoptosis and ER stress, and provide a new strategy and perspective to prevent the damage of chemotherapy drugs in ovaries.

5. Conclusions

In conclusion, our study proved that UFL1 alleviated cisplatin-induced GCs apoptosis and ER stress, and provide a new strategy and perspective to prevent the damage of chemotherapy drugs in ovaries.

Abbreviations

UFL1: Ubiquitin-like modifier 1 ligating enzyme 1; ER stress: Endoplasmic Reticulum stress; POF: Premature Ovarian Failure; GCs: Granular Cells; FSH: Follicle-Stimulating Hormone; E2: Estrogen; PERK: Protein kinase-like Endoplasmic Reticulum Kinase; UPR: the Unfolded Protein Response; P53: Transformation Related Protein 53; GRP78: Glucose Regulated Protein 78; XBP1s: the spliceosome of X-box Binding Protein 1; CHOP: C/EBP Homologous Transcription Factor; ATF4: Activating Transcription Factor 4; BCL-2: B-cell lymphoma-2; BAX: BCL2-Associated X; Cleaved Cas3: Cleaved Caspase-3; Cas3: Caspase3; AMH: anti- Mullerian hormone; CIS: Cisplatin; FSHR: Follicle-Stimulating Hormone Receptor; DDRGK1: DDRGK domain containing 1; C53: CDK5 regulatory subunit associated protein 3; SLC7A11: solute carrier family 7 (cationic amino acid transporter, y+ system), member 11.

Supplementary Materials:

Declarations

Ethics approval and consent to participate： The study was approved by the Animal Care Committee of Nanchang University Jiangxi Medical College (Animal protocol: NCDXSYDWFL-2015097).

Availability of data and materials： All data generated through this study are included in this article.

Consent for publication： Not applied.

Competing interests: The authors declare no conflict of interest.

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H.D. and Z.F. accomplished all experiments; J.L., Q.Y. and T.Y. analyzed all data. All authors read and approved the final manuscript.

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