C28-induced autophagy of female germline stem cells in vitro and its potential mechanisms

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Abstract

Background

There are few studies indicating that small molecular compounds affect the proliferation, differentiation, apoptosis, and autophagy of female germline stem cells (FGSCs). However, the epigenetic regulatory mechanism of small molecular compounds that induce autophagy in FGSCs remains unknown.

Results

In this study, we found that C28 reduced the viability and proliferation of FGSCs, respectively. Additionally, western blotting showed that the expression of autophagy marker light chain 3 beta II (LC3B-II) was significantly increased and expression of sequestosome-1 (SQSTM1) was significantly reduced in C28-treated groups. Immunofluorescence showed that, in C28-treated groups, the number of LC3B-II-positive puncta was increased significantly. These results indicated that C28 induced autophagy of FGSCs in vitro. ChIP-seq data showed that autophagy-related biological processes such as regulation of mitochondrial membrane potential, Golgi vesicle transport, and cellular response to reactive oxygen species were enriched. In addition, RNA-Seq showed that the expression of genes (Trib3, DDIT3, and ATF4) related to endoplasmic reticulum (ER) stress was enhanced by C28.

Conclusion

C28 could induce FGSC autophagy in vitro leading to a decrease in the number of FGSCs. H3K27ac and ER stress might play roles in C28-induced autophagy of FGSCs in vitro.

Background

Female germline stem cells (FGSCs) have been isolated from ovarian tissues of neonatal and adult mice to successfully establish cell lines in vitro[1-5]. FGSCs can restore the fertility ability of infertile female mice[6]. Successively, FGSCs were successfully isolated from rats, pigs, and humans, and cultured in vitro[7-11]. Successful cultivation and establishment of FGSC lines may be promising for clinical therapies of infertility, as well as provide a new strategy to study germ cell development and its mechanism. Some studies have reported on the proliferation, differentiation, apoptosis, and autophagy of FGSCs[12-14]. These studies support the clinical application of FGSCs.
Autophagy is a metabolic process through which macromolecules and organelles are recycled to maintain cell self-stabilization in eukaryotes[15]. Additionally, autophagy participates in many important physiological processes of cells, such as apoptosis, proliferation, and stemness maintenance[16-18]. Extensive autophagy has generally been considered to result in a particular mode of cell death called autophagic cell death or the second type of programmed cell death[19-23]. Many studies have reported that treatment of diseases and conditions can be achieved by regulating autophagy, such as cancer, ageing, and neurodegenerative diseases[24-27]. Autophagy is upregulated in response to unfavorable conditions such as an increase of reactive oxygen species (ROS), decrease of the mitochondrial membrane potential, growth factor deprivation, pathogen infection, and endoplasmic reticulum (ER) stress[28]. Studies concerning the effect of autophagy on FGSC development have been reported[13, 29], but the epigenetic regulatory mechanism of autophagy in FGSCs remains to be explored.

Epigenetic modification is a type of gene regulation, which is very important to maintain normal life activities of mammals. It includes DNA modification, histone variants, and histone modification[30-32]. Biga et al. profiled the distribution of histone 3 lysine 27 acetylation (H3K27me3), H3k9me3, and H3K4me3 along with LC3B, ATG4B, and SQSTM1 loci in starved zebrafish myocytes and linked these modifications with the levels of related transcripts that were used to describe potential epigenetic mechanisms of autophagy regulation in zebrafish[33]. Histone modification upregulates or downregulates gene expression depending on its modification degree. Epigenetic modifications are highly correlated with stem cell differentiation and somatic cell reprogramming. Zhang et al. revealed the unique chromatin status and its biological significance in FGSCs by detection of epigenetic modifications[34].

Small molecular compounds are characterized by high cell permeability, low antigenicity, rapidity, reversibility, an ability to regulate multiple cellular signaling pathways, ease of epigenetic modification, and subtle regulation of their effects by changing their concentrations and combinations. It has been reported that small molecular compounds affect reprogramming, self-renewal, differentiation, apoptosis, and autophagy of stem cells[35-37]. Benzoxaboroles were first
synthesized in 1957[38], which can be used for antimicrobial therapy by inhibiting *Trypanosoma brucei* (Tb) LeuRS[39]. Compound 28 (C28) synthesized from 6-formylbenzoxaborol acts as a Tb LeuRS inhibitor and can be used as a potential novel antitrypanosomal agent. Two benzoxaboroles, C89 and ZCL-082, induce FGSC autophagy in vitro, and the molecular mechanism has been preliminary elucidated[13, 29]. However, the epigenetic mechanism of benzoxaborole-induced autophagy of FGSCs and the effects of C28 on FGSCs are largely unknown.

In this study, we explored the effects of C28 on FGSC development and the underlying epigenetic regulatory mechanism. We found that C28 induced autophagy of FGSCs in vitro, and that H3K27ac and ER stress might play roles in C28-induced autophagy of FGSCs. These findings may facilitate treatment of female infertility.

**Results**

**C28 reduces the number, viability, and proliferation of FGSCs in vitro**

To confirm the effect of C28 on FGSCs, we cultured FGSCs with various concentrations of C28 for 24 and 48h in vitro. The number of FGSCs was significantly lower in 0.5, 1, 2, and 4μM C28-treated groups at 24h compared with the controls (DMSO treatment) (p < 0.05) (Fig. 1B and C).

Subsequently, cell activity and proliferation were detected by CCK8 and EdU assays. Exposure for 24 and 48h to 0.5, 1, 2, and 4μM C28 significantly decreased cell viability in a concentration-dependent manner according to CCK8 assay results (Fig. 1D and E). EdU assays also revealed a statistically significant decrease in the proliferation of FGSCs after exposure to 0.5, 1, 2, and 4μM C28 for 24 and 48h (Fig. 1F and G). These results showed that C28 reduced the number, viability, and proliferation of FGSCs in vitro. In all subsequent experiments, 0.5μM C28 was used to treat FGSCs.

**C28 has no effect on differentiation or apoptosis of FGSCs in vitro**

To explore the mechanisms of C28 decreasing the number of FGSCs, differentiation-related marker genes of FGSCs, *stra8* and *sycp3*, were detected by RT-PCR. There was no expression of *stra8* or *sycp3* in C28-treated groups (Fig. 2A). Next, we examined the apoptosis rate by flow cytometry and found no significant difference in the percentage of apoptotic cells between C28-treated groups and the controls at 3 and 24h (Fig. 2B–E). These results indicated that C28 had no effect on differentiation.
or apoptosis of FGSCs in vitro.

**C28 induces FGSC autophagy in vitro**

Next, FGSC autophagy was investigated after treatment with C28. Western blot results showed that expression of LC3B-II was significantly increased and SQSTM1 expression was significantly decreased compared with the control at 3h (Fig. 3A-D). Furthermore, immunofluorescence showed that the number of LC3B-II-positive vesicles was significantly increased in FGSCs exposed to C28 for 3h compared with the control (Fig. 3E, F). These results showed that C28 induced FGSC autophagy at 3h. Therefore, 3h was selected to treat FGSCs with C28 for the following experiments. Taken together, the results indicated that C28 induced autophagy of FGSCs in vitro.

**Genome wide profiling of H3K27ac indicates its role in C28-induced autophagy of FGSCs**

To explore the epigenetic mechanism of C28-induced FGSC autophagy, H3K27ac was investigated by ChIP-Seq in C28-treated FGSCs and the control (Fig. 4). Before analyzing the ChIP-seq data, we applied FastQC v0.11.5 software to determine the data quality and analyzed several related variables[42]. The analysis indicated that the sequencing quality was very high (Additional file 2 Fig. S1 and S2). As shown in Fig. 4a, we found that three H3K27ac-marked regions were clustered by analyzing ChIP-Seq data with cluster 2 and 3 marked by H3K27ac less in C28-treated FGSCs. To understand how the different enrichments of enhancer signatures contributed to FGSC phenotypes induced by C28, we performed GO analysis with GREAT. The results showed that enrichment terms in clusters 2 and 3 were related to autophagy, such as regulation of mitochondrial membrane potential, Golgi vesicle transport, and cellular response to reactive oxygen species (Fig. 4b, c). These results suggested that H3K27ac played an important role in C28-induced FGSC autophagy.

**ER stress is involved in C28-induced FGSC autophagy**

The quality of RNA-seq reads was examined using FastQC. High quality clean reads were obtained from raw reads by removing the adaptor sequences, reads with >5% ambiguous bases, and low quality reads. The clean reads were then aligned to the mouse genome (version: mm10_GRCm38) using the Hisat2 program. DEG analysis was performed using DESeq2. Significance and false discovery rate (FDR) analyses were conducted according to the following criteria: i) fold change >1.5,
<1.5 or \( P < 0.05 \) and ii) FDR < 0.05 (Additional file 2 Fig. S3A). Moreover, the correlation coefficient of each replicated sample showed high consistency (Additional file 2 Fig. S3B). Transcriptome sequencing was performed in C28-treated groups and the control. DESeq2 was used to analyze differentially expressed genes (DEGs). The DEGs between the C28-treated group and control were directly reflected by hierarchical clustering (Fig. 5a) and a volcano plot (Fig. 5B). Principal component analysis (PCA) indicated that similar within-sample and different between-sample expression patterns (Fig. 5C). Functional annotation analysis revealed upregulation of some ER stress- and autophagy-related genes (\textit{Trib3}, \textit{DDIT3}, and \textit{ATF4}) (Fig. 5D). qPCR results confirmed that the expression of these was genes significantly increased in the C28-treated group compared with the control and were consistent with RNA-Seq data (Fig. 5E). These results revealed that ER stress was involved in the process of C28-induced autophagy of FGSCs.

**Discussion**

Presently, infertility is a severe problem worldwide. Successfully isolating FGSCs from postnatal mammalian ovaries and culturing them in vitro may facilitate treatment of female infertility[43, 44]. Many studies have provided convincing evidence that small molecular compounds affect the physiological functions of mammalian cells, such as proliferation, differentiation, autophagy, and apoptosis[13,14, 29,45]. In this study, we found that C28 induced significant autophagy, and H3K27ac and ER stress might play roles in C28-induced autophagy of FGSCs in vitro.

Autophagy is a self-digestion process in cells, which plays an essential role in cell development and the cellular response to stress[46]. However, excessive autophagy triggers apoptosis-mediated cell death (also a known type of programmed cell death)[47]. Our results showed that the expression of autophagic marker LC3B-II was significantly increased and that of SQSTM1 was significantly reduced in FGSCs after treatment with C28 for 3h, which indicated that C28 induced FGSC autophagy.

To explore the underlying mechanism of C28-induced autophagy in FGSCs, we performed genome wide profiling of H3K27ac and RNA-Seq analysis in the C28-treated group. Epigenetics mediate the occurrence of a single event such as autophagy, proliferation, apoptosis, and differentiation[48]. It has been reported that epigenetic modifications and autophagy are closely related. Deoxynivalenol
induces autophagy/apoptosis and affects epigenetic modifications in porcine oocytes[34]. a-Solanine affects histone methylation, increases oocyte death, and induces autophagy and apoptosis[49]. Histone acetylation is one of the key histone modifications of chromatin and widely regulates gene expression and silencing. Spermidine affects deacetylation of H3 by suppression of histone acetyl transferases to induce autophagy in yeast cells. In addition, epigenetic activation of autophagy genes is a conserved process in the oxidative stress response of flies, worms, and humans[50, 51]. Our ChIP-Seq results showed that C28-treated FGSCs had lower levels of H3K27ac at enriched genes related to regulation of the mitochondrial membrane potential, Golgi vesicle transport, and cellular response to reactive oxygen species. A decrease of the mitochondrial membrane potential and opening of mitochondrial permeability transition pores induce mitochondrial autophagy[52]. In our study, the autophagy induced by C28 in FGSCs might be mitochondrial autophagy. Autophagy is an important evolutionary conserved process in eukaryotes, which degrades intracellular materials. In this process, lysosomes are vesicles produced by Golgi. In the present study, the low levels of H3K27ac at genes enriched for Golgi vesicle transport in C28-treated FGSCs might lead to cell death. ROS play an important role in cellular metabolism, such as apoptosis[53, 54] and autophagy[55]. Autophagy reduces the cytotoxic damage of ROS to protect cells by clearing organelles and proteins[55]. Our ChIP-Seq results showed that exposure of FGSCs to C28 may induce the cellular response to ROS, leading to autophagy.

The results of RNA-Seq showed that ER stress-related genes (Trib3, DDIT3, and ATF4) were highly expressed in C28-treated FGSCs and qPCR validated this result. According to previous reports, DDIT3 is an ER stress-related gene that induces ER stress to inhibit the mTOR pathway by regulating TRIB3 and ATG5[56-58]. It has been revealed that the ATF4-DDIT3/CHOP-TRIB3-AKT1-mTOR pathway induces autophagy[57, 59]. Additionally, the ATG12-ATG5-ATG16 complex mediates covalent binding of LC3B-I and phosphatidy lethanolamine to form lipid-soluble LC3B-II, thereby participating in extension of the autophagic bilayer membrane and the formation of autophagosomes[60, 61]. Our study showed that C28 upregulated the expression of ER stress-related genes Trib3, DDIT3, and ATF4 in FGSCs, and the protein expression of LC3B-II was also upregulated in FGSCs treated with C28.
These results suggested that C28 activates ER stress by upregulating ER stress-related genes in FGSCs, inhibiting the mTOR pathway to induce autophagy and decreasing of the number of FGSCs.

Conclusions
We found that C28 might induce autophagy by changing epigenetics and upregulating ER stress in FGSCs (Fig. 6), leading to a decrease in the number of FGSCs. This study provides evidence to support the novel finding that C28-induced autophagy is related to epigenetic regulatory mechanisms and ER stress. Our study also provides the framework for clinical application of C28.

Materials And Methods

Animals
Five-day-old C57BL/6 mice were purchased from SLAC Laboratory Animal Co. (Shanghai, China). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Chemical compound
C28 was synthesized by Professor Huchen Zhou from the Schools of Pharmacy and Medicine, Shanghai Jiao Tong University. The chemical structure of C28 is shown in Figure 1A.

FGSC culture in vitro
The mouse FGSC line used in this study was characterized and established previously [40]. FGSCs were cultured on mitotically inactivated SIM mouse embryo-derived, thioguanine and ouabain-resistant (STO) cells. The culture medium for FGSCs was alpha-minimum essential medium (Invitrogen Life Sciences) supplemented with 10% fetal bovine serum (Life Technologies), 30 mg/ml pyruvate (Amresco), 2mM L-glutamine (Amresco), 0.1mM β-mercaptoethanol (Biotech), 1mM non-essential amino acids (Invitrogen Life Sciences), 20ng/ml mouse epidermal growth factor (PeproTech), 10ng/ml human basic fibroblast growth factor (PeproTech), 10ng/ml mouse glial cell line-derived neurotrophic factor (PeproTech), 10ng/ml mouse leukemia inhibitory factor (Santa Cruz Biotechnology), and 15mg/ml penicillin (Amresco). The medium was changed every 2–3 days. Cells were passaged at ratios of 1:2 or 1:3 by enzymatic digestion every 4–5 days. The cells cultured at 37°C with 5% CO₂.
**CCK8 assay**

Cell viability was assessed by cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). FGSCs were seeded at approximately 2000 cells/well in 96-well plates and grown at 37°C with 5% CO₂ for 24h. The cells were treated with 0, 0.25, 0.5, 1, 2 and 4µM C28 for 24 and 48h, and then reacted with 10µL CCK8 solution for 1h. Then, the absorbance was measured at 450nm using a microplate reader (Bio-Tek Instruments, Thermo Fisher Scientific, Winooski, VT, USA). All assays were conducted in triplicate.

**EdU assay**

A Cell-Light EdU Apollo® 567 in vitro imaging kit (RiboBio, Guangzhou, China) was used to assay cell proliferation, according to the manufacturer’s protocol. The cells were incubated with 50µM EdU for 2h at 37°C after C28 treatment for 24h. Then, the cells were fixed in 4% paraformaldehyde for 20min at room temperature and washed with 2mg/ml glycine for 5min on a shaker. Permeabilization was conducted by incubation in 0.5% Triton X-100 for 1h. Then, 1×Apollo was added, followed by incubation for 30min on a shaker. Subsequently, cells were washed three times with PBS. Then, 1×Hoechst 33342 was used to stain nuclei. Images were obtained under a DM2500 microscope (Leica, Germany) and analyzed by Image J.

**Apoptosis analysis**

Apoptotic cells were quantified using an annexin V-FITC/PI detection kit (Invitrogen, Carlsbad, CA) and flow cytometry. Briefly, FGSCs were treated with 0.5µM C28 for 3 and 24h, washed with PBS, collected, and resuspended in 1×Binding Buffer. The cells were then stained for 15min at room temperature with annexin V and propidium iodide, and analyzed by flow cytometry (BD FACSCalibur, BD Biosciences) to quantify apoptosis.

**Reverse transcription-polymerase chain reaction and quantitative real-time polymerase chain reaction**

FGSCs were cultured in vitro and treated with 0.5µM C28 for 24 and 48h. Total RNA was extracted from ovarian tissues of neonatal mice and FGSCs treated with C28 using TRIzol reagent (Life
Technologies, CA), according to the manufacturer’s instructions. RNA quantity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). HiScript II Q RT SuperMix for qPCR kit (Vazyme Biotech) was used to obtain cDNA. Approximately 1µg RNA was used to synthesize cDNA in a 20µl reaction volume. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with Taq DNA polymerase. The RT-PCR products were separated on 1% agarose gels and imaged with a bioimaging system (Alpha Innotech). RT-PCR products were confirmed by sequencing.

Quantitative real-time polymerase chain reaction (qPCR) was conducted with SYBR Premix Ex Taq (Takara, Shanghai, China) and an ABI 7500 Real-Time PCR System (Applied Biosystems) to measure expression levels of Trib3, DDIT3, and ATF4. The qPCR reaction volume of 20µl included 10µl SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 1µl cDNA template, 2µl primer mixture, and 7µl water. qPCR conditions were as follows: 95°C for 30s 40 cycles of 95°C for 15s and 60°C for 60s, and then then 95°C for 15s, 60°C for 60s, and 95°C for 15s. All assays were repeated three times. Fold changes in expression were calculated using the \(2^{-\Delta \Delta Ct}\) method. \(\Delta \Delta Ct = \Delta Ct\) experimental group - \(\Delta Ct\) control group, and \(\Delta Ct = Ct\) target gene – Ct Gapdh. Primers are listed in Additional file 1: Table S1.

**Western blotting**

FGSCs were cultured in vitro and treated with 0.5µM C28 for 3 and 6h. Cells were lysed with RIPA buffer (Shanghai Yeasen Biotechnology Co., Ltd) containing a protease inhibitor cocktail. Protein concentrations were measured using the bicinchoninic acid (BCA) assay. The western blotting procedure was as follows. A total of 20µg protein was separated by 15% w/v sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Then, the membranes were blocked with 5% dry nonfat milk in Tris-buffered saline with Tween 20 (TBST) at a room temperature for 1h, followed by overnight incubation with anti-LC3 (1:1000; Abcam), anti-SQSTM1 (1:1000; Abcam), and anti-β-tubulin (1:6000; Abcam) primary antibodies in 5% nonfat milk in TBST for 1h. Membranes were washed three times with TBST for 10min each wash. Subsequently, the membranes were incubated with the secondary antibody (1:2000; Proteintech) in 5% dry nonfat milk
in TBST at room temperature for 2h, and then washed three times with TBST for 10min each wash. Finally, the protein bands were scanned with a chemiluminescence imaging system (ProteinSimple, Santa Clara, CA, USA). Densitometry of the bands was performed by Quantity One software.

**Immunofluorescence**

FGSCs were treated with 0.5µM C28 for 3h, washed with 1× phosphate-buffered saline (PBS), fixed in 4% formaldehyde for 30min at room temperature, and then washed three times with PBS. Subsequently, the cells were blocked in goat serum at 37°C for 10min, incubated overnight with the primary rabbit anti-LC3B antibody (1:150; Abcam) at 4°C, and then washed three times with PBS. Then, the cells were incubated with the tetramethylrhodamine isothiocyanate-conjugated secondary antibody (1:150, goat anti-rabbit IgG; ProteinTech). Finally, the cells were washed with PBS three times and stained with 4′, 6-diamidino-2-phenylindole at room temperature for 5min. Images were acquired using a Leica digital camera under a fluorescence microscope (DMI3000B; Leica). Various fields of view (>three regions) were analyzed for each labeling condition, and representative results are shown.

**RNA sequencing**

Total RNA was extracted as described above. RNA integrity was assessed using the Agilent Bioanalyzer 2100. RNA quantity and concentration were assessed using the NanoDrop 2000 spectrophotometer. The total RNA was treated with DNase to remove genomic DNA contamination. Isolation of mRNA was performed using the Oligotex mRNA Mini Kit (Qiagen GmbH, Hilden, Germany). The mRNA was used for RNA sequencing (RNA-Seq) library preparation with the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). The library was then subjected to Illumina sequencing with paired-end 2 × 150 as the sequencing mode. Raw sequence data were submitted to the NCBI Gene Expression Omnibus under accession number GSE136386.

**Chromatin immunoprecipitation sequencing**

The preparation of chromatin immunoprecipitation (ChIP) and input DNA libraries were performed as described previously. First, cells were crosslinked with 1% formaldehyde for 10min at room
temperature and quenched with 125mM glycine for 5min. Then, the cells were resuspended in cold SDS lysis buffer (140mM NaCl, 1mM EDTA, pH 8.0, 1% TritonX-100, and 0.1% SDS protease inhibitors) and sheared with a Bioruptor water bath sonicator (Diagenode). The chromatin fragments were precleared and then immunoprecipitated with Protein A+G Magnetic beads coupled with anti-H3K27ac (ab4729, Abcam). After reverse crosslinking, immunoprecipitated DNA and input DNA were end-repaired and ligated with adapters using the NEBNext Ultra End-Repair/dA-Tailing Module (E7442, NEB) and NEBNext Ultra Ligation Module (E7445, NEB). High throughput sequencing of the ChIP fragments was performed using Illumina NextSeq 500, following the manufacturer’s protocols. We used bowtie2 to align the reads to the mm9 reference genome. PCR duplicates were removed using Samtools (version 2.0.1). Normalized genome coverage tracks were generated from uniquely mapped reads using deepTools2 (version 3.1).

**Gene Ontology analysis**

Gene Ontology (GO) enrichment was performed using GREAT analysis (version 1.8)[41]. After clustering the active enhancer, the biological processes were determined using the whole genome as the background and positives as the nearest gene within 10kb for Figure 4 and a false discovery rate (FDR) (significant by both) cutoff of 0.05.

**Statistical analyses**

Each experiment was performed three times. Data are expressed as the mean ± SEM. Statistical analysis was performed by the Student’s t-test using SPSS statistics (version 17.0; IBM Corp., Armonk, NY, USA). $P< 0.05$ was considered as statistically significant.

**Abbreviations**

FGSCs: Female germline stem cells; C28: Compound 28; Tb LeuRS: *Trypanosoma brucei* LeuRS; LC3B-II: Light chain 3 beta II; SQSTM1: Sequestosome-1; ER stress: Endoplasmic reticulum stress; GO: Gene ontology; FDR: False discovery rate; ROS: Reactive oxygen species.

**Declarations**

**Ethics approval and consent to participate**

All procedures involving animals were approved by the Institutional Animal Care and Use Committee
of Shanghai and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

JW conceived and designed the experiments; PC, XYZ and GGT performed most of the experiments; XYZ and GGT collected and analyzed the data; PC and XYL investigation; ZZL, XLY, XYP and YRW resourced PC, XYZ and XYY wrote the draft manuscript; XYZ, XYY and JW revised the manuscript; RH, HCZ and JW funding acquisition.

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Figures
Figure 1

C28 reduces the number, viability, and proliferation of FGSCs in vitro. A: Chemical structure of compound 28. B: Representative images of FGSCs treated with various concentrations of C28. Scale bar, 50 μm. (i: Control; ii: 0.25 μM; iii: 0.5 μM; iv: 1 μM; v: 2 μM; vi: 4 μM). Control: DMSO. C: Graphic representation of the number of FGSCs treated with various concentrations of C28 per well in 48-well plates. Values are presented as the mean ± SEM of
three biological replicates. **P < 0.01. D: Graphic representation of results from CCK-8 assays to determine cell viability of FGSCs treated with various concentrations of C28 for 24h. Data points are the percentage (% = OD450 treated/OD450 untreated) relative to untreated cells at that time point. E: Graphic representation of results from CCK-8 assays to determine cell viability of FGSCs treated with various concentrations of C28 for 48h. Data points are the percentage (% = OD450 treated/OD450 untreated) relative to untreated cells at that time point. F: Fluorescence images of EdU incorporation in FGSCs treated with various concentrations of C28 for 24h. Cells were stained with Apollo 567 (red) to detect EdU and Hoechst (blue) to visualize nuclei. Both images were merged. Scale bar, 50 μm. (i: Control; ii: 0.25 μM; iii: 0.5 μM; iv: 1 μM; v: 2 μM; vi: 4 μM). Control: DMSO. G: Graphic representation of the number and EdU content of FGSCs treated with various concentrations of C28 for 24h. The percentage of EdU+ cells (EdU+/Hoechst+ x 100%) was determined in four random fields per sample. All data are expressed as the mean ± SEM of values from experiments performed in triplicate. *P < 0.05, **P < 0.01.
Effect of C28 on FGSC differentiation and apoptosis A: RT-PCR analysis of the expression of Syct2, Stra8, and Gapdh in ovaries, FGSCs treated with DMSO for 24 and 48 h (control), and FGSCs treated with C28 for 24 and 48 h (C28). Gapdh was used as a sample loading control. M, 250bp DNA ladder. B: Flow cytometric analysis of FGSCs treated with C28 or DMSO for 3h and then stained with an Annexin V-FITC apoptosis detection kit. C: Quantification of the
apoptosis rate of FGSCs treated with C28 or DMSO for 3h. Data are shown as the mean ±SEM of three biological replicates. P>0.05. D: Flow cytometric analysis of FGSCs treated with C28 or DMSO for 24h and the stained with an Annexin V-FITC apoptosis detection kit. E: Quantification of the apoptosis rate of FGSCs treated with C28 or DMSO for 24h. Data are shown as mean ±SEM of three biological replicates. P>0.05.

Figure 3
C28 induces autophagy in FGSCs A: Western blot analysis to detect protein levels of LC3B and β-tubulin (loading control) in FGSCs treated with 0.5 μM C28 or DMSO (Control) for 3 and 6h. B: Graphic representation of the relative protein expression of (LC3B-II/β-tubulin×100%). Control: DMSO. Data are shown as the mean ± SEM of three biological replicates. **P < 0.01. C: Western blot analysis to detect protein levels of SQSTM1 and GAPDH (loading control) in FGSCs treated with 0.5 μM C28 or DMSO (Control) for 3 and 6h. D: Graphic representation of the protein expression of SQSTM1. Control: DMSO. Data are shown as the mean ± SEM of three biological replicates. **P < 0.01. E: Fluorescence images of LC3B-II puncta in FGSCs treated with 0.5 μM C28 or DMSO (Control) for 3h. F: Graphic representation of the quantification of LC3B-II puncta. Control: DMSO. Data are shown as the mean ± SEM of three biological replicates. **P < 0.01.
Figure 4

Chip-seq analysis of C28-treated FGSCs A: K-means clustering of H3k27ac ChIP-Seq signals in control and C28-treated FGSCs. A window of 10kb (-5 to +5 kb) around the peak center is shown. B: Top10 enrichment scores of GO terms under the downregulated biological process category in cluster 2. C: Top10 enrichment scores of GO terms under the downregulated biological process category in cluster 3.
Transcriptomic analysis of C28-treated FGSCs

A: Hierarchical clustering revealed the DEG expression patterns between the C28-treated group and control. B: DEGs were screened out by volcano plot analysis. C: Principal component analysis (PCA) of all samples. D: Differentially expressed transcription factors related to ER stress and autophagy. E: qPCR validation of dysregulation of three genes identified by RNA-Seq analysis. The fold expression change relative to the mean expression level of C28-treated FGSC and control is shown. Control: DMSO. Error bars represent SEM (*P≤0.05, **P ≤ 0.002, ***P ≤ 0.001)
Proposed model of the regulatory mechanism of C28-induced autophagy C28 induces autophagy in FGSCs by regulating H3k27ac [45-47], and enhances the expression of genes related to ER stress[49-51].

Supplementary Files
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