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SIRT2 plays a novel role on progesterone, estradiol and testosterone synthesis via PPARs/LXRα pathways in bovine ovarian granular cells

Dejun Xu, Huanshan He, Xiaohan Jiang, Rongmao Hua, Huali Chen, Li Yang, Jianyong Cheng, Jiaxin Duan, Qingwang Li*

Northwest A&F University, College of Animal Science and Technology, Yangling, Shaanxi, 712100, China

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ABSTRACT

SIRT2 has been shown to possess NAD^+-dependent deacetylase and desuccinylase enzymatic activities, it also regulates metabolism homeostasis in mammals. Previous data has suggested that resveratrol, a potential activator of Sirtuins, played a stimulation role in steroidogenesis. Unfortunately, to date, the physiological roles of SIRT2 in ovarian granular cells (GCs) are largely unknown. Here, we studied the function and molecular mechanisms of SIRT2 on steroid hormone synthesis in GCs from Qinchuan cattle. Immunohistochemistry and western blotting showed that SIRT2 was expressed not only in GCs and cumulus cells, but also in oocytes and theca cells. We found that the secretion of progesterone was induced, whereas that of estrogen and testosterone secretion was suppressed by treatment with the SIRT2 inhibitor (Thiomyristoyl or SirReal2) or siRNA. Additionally, the PPARs/LXRα signaling pathways were suppressed by SIRT2 siRNA or inhibitors. The mRNA expression of *CYP17*, *aromatase* and *StAR* was suppressed, but the abundance of *CYP11A1* mRNA was induced by SIRT2 inhibition. Furthermore, the PPARα agonist or PPARγ antagonist could mimic the effects of SIRT2 inhibition on hormones levels and gene expression associated with steroid hormone biosynthesis. In turn, those effects were abolished by the LXRα agonist (LXR-623). Together, these data support the hypothesis that SIRT2 regulates steroid hormone synthesis via the PPARs/LXRα pathways in GCs.

1. Introduction

Steroid hormones, including progesterone (P4), estradiol (E2) and testosterone (T), are essential for the maintenance of normal reproductive function and bodily homeostasis. Steroidogenesis is controlled by multiple signaling pathways. Previous studies have demonstrated that cyclic adenosine monophosphate/protein kinase C (cAMP/PKC) [1], mitogen-activated protein kinases/extracellular signal-regulated kinase1/2 (MAPK/ERK1/2) [2] and arachidonic acid (AA-mediated) [3] signaling stimulate steroidogenesis via increasing StAR expression and stabilizing StAR protein activity. However, in females, the levels of steroid hormones and the P4/E2 ratio change along with estrus and gestation. Thus, the mechanism of steroidogenesis is more complicated than previously thought. Thus far, little has been discovered about this topic. Increasing evidence has recently shown that peroxisome proliferator activated receptors/ liver X receptors (PPARs/LXRs) signaling might regulate steroidogenesis. The cholesterol efflux is stimulated by the PPARγ/LXRs signaling pathway [4]. Additionally, steroidogenesis was inhibited by PPARs in rat ovarian granulosa cells (GCs) [5]. However, in a different strain of rats and a different culture model, PPARγ improved estradiol secretion [6]. In addition, LXRs also stimulated ovarian steroidogenesis in vivo [7]. Those studies indicate that PPARs/LXRs affect the steroidogenesis of GCs, but the regulation mechanism of that hormone synthesis remains unclear.

Sirtuins are an evolutionarily conserved family of NAD^+-dependent primary deacetylase proteins that participate in many biological functions, and include, histones [8], transcription factors [9], and metabolic enzymes [10]. Seven members of the Sirtuins family can be found in mammals and are named SIRT1-7. These proteins are expressed in specific tissues and subcellular localizations [11]. SIRT1, 6 and 7 have been observed in the nucleus, and SIRT3, 4, 5 are located in the mitochondria. SIRT2 is the only member of the Sirtuins family that primarily exists in a cytoplasmic isoform and can also be found in the nucleus [11]. Sirtuins were originally reported to display deacetylase activity, but a growing body of evidence suggests that they might perform other modifications or as-yet-unidentified actions to catalyze substrate proteins. A recent study showed that SIRT2 (likely including SIRT1 and SIRT3) possesses efficient demyristoylase activity [12]. In

* Corresponding author.
E-mail addresses: -xudejun2010@163.com (D. Xu), 751268753@qq.com (H. He), 1193926410@qq.com (X. Jiang), 1215842456@qq.com (R. Hua), 714812721@qq.com (H. Chen), 21362323@qq.com (L. Yang), 1540265595@qq.com (J. Cheng), 280041939@qq.com (J. Duan), nwxudejun@sina.com (Q. Li).

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Table 1
Sequences for primers used in quantitative real-time RT-PCR.

Genes	Primer sequences (5'–3')	Accession no.	Fragment size (bp)
GAPDH	Forward:CACCCTCAAGATTGTCAGCA Reverse:GGTCATAAGTCCCTCCACGA	NM_001034034	103
ESR1	Forward:CCAACCAGTGCACGATTGAT Reverse:TTCCGTATTCCGCCTTTCAT	NM_001001443.1	100
ESR2	Forward:ACCTGCTGAATGCTGTGAC Reverse:GTTACTGGCGTGCCTGAC	NM_174051	128
PGR	Forward:TCCCCCCACTGATCAACTTG Reverse:TCCGAAAACCTGGCAGTGA	NM_001205356.1	171
AR	Forward:CCTGGTTTTCAATGAGTACCGCATG Reverse:TTGATTTTTCAGCCCATCCACTGGA	NM_001244127	172
LXRα	Forward:CATCAACCCCATCTTCGAGTT Reverse:CAGGGCCTCCACATATGTGT	NM_001014861.1	163
LXRβ	Forward:TCAGTGCTTGGGACATCAGG Reverse:TCAGTGCTTGGGACATCAGG	NM_001014883	201
StAR	Forward:CCCAGCAGAAGGGTGTCATC Reverse:TGCGAGAGGACCTGGTTGAT	NM_174189.2	157
CYP11A1	Forward:GCTCCAGAGGCAATAAAGAAC Reverse:GACTCAAAGGCAAAGTGAAACA	NM_176644.2	149
CYP17A1	Forward:CCATCAGAGAAGTGCTCCGAAT Reverse:GCCAATGCTGGAGTCAATGA	NM_174304.2	80
Aromatase	Forward:GTGTCCGAAGTTGTGCCTATT Reverse:GGAACCTGCAGTGGGAAATGA	NM_174305.1	148
HSD3β1	Forward:GTTCTACTACATCTCAGACGACACG Reverse:GGCGGTTGAAGCAAGGGTTAT	NM_174343.3	197
HSD17β1	Forward:TGTGGTACTCATTACCGGCTGTT Reverse:CAGCGTGGCATACACTTTGAA	NM_001102365.1	100

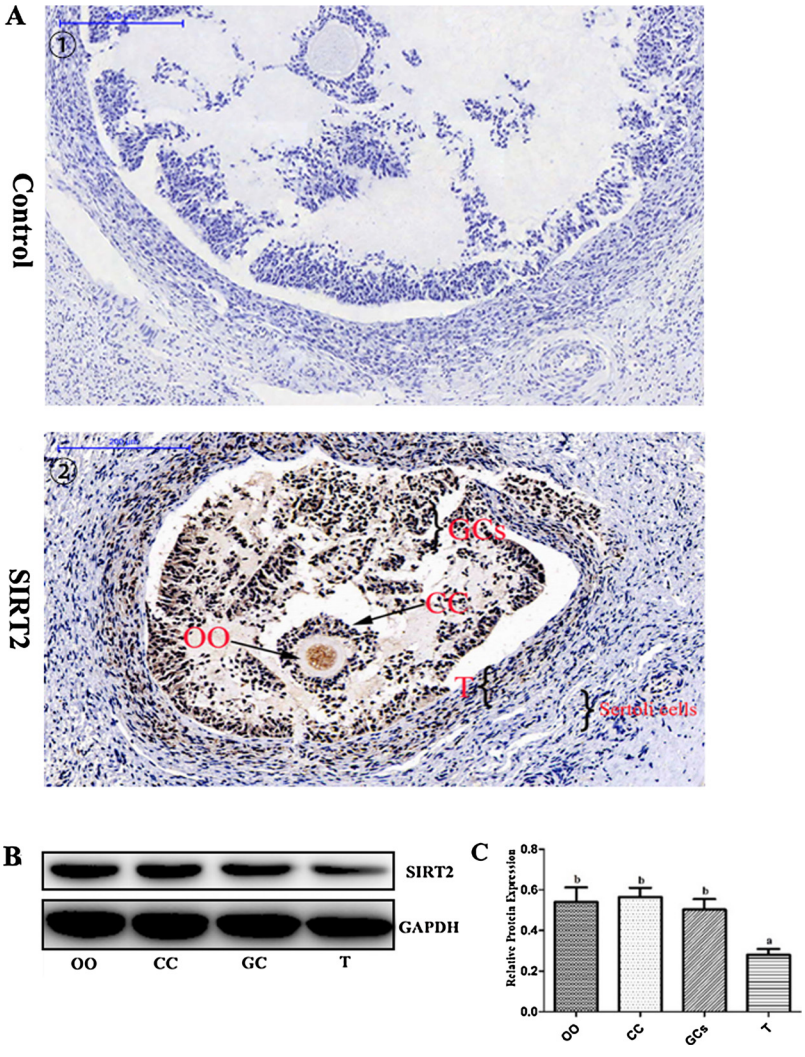

Fig. 1. Expression of SIRT2 in bovine ovaries. (A) SIRT2 localization in a bovine ovarian sample identified via immunochemistry. Bovine ovarian samples were examined using antibodies against SIRT2 (A, 2) or no primary antibodies but rabbit IgG (A, 1). Immuno-specific staining was brown that indicate immunopositive cells. Original magnification × 100. Bar = 200 μm. SIRT2 was detected in granular cells (GCs), oocyte (OO), cumulus cells (CC), theca cell (T) and Sertoli cells. Immunohistochemistry was performed on three different ovarian slides from each of three bovines. (B) SIRT2 protein abundance in OO, CC, GCs and T. The protein expression of SIRT2 was examined by a Western blot analysis. Band intensities normalized to GAPDH are shown. (C) The SIRT2 protein semiquantitative abundance was analyzed by ImageJ software. Data are shown as the means ± SEM of four independent replicates. Bars with different letters (a, b) indicate significant differences, $P < 0.05$.

addition, SIRT4 [13] and SIRT6 [14] were reported to transfer an ADP-ribose group onto the protein targets. SIRT5 was recently shown to primarily possess NAD+-dependent demalonylase and desuccinylase activities [15], although it was initially reported as displaying deacetylase activity. Therefore, Sirtuins possess different biological functions because of the diversity of modifications type and subcellular localizations. To date, the biological functions of SIRT1 are more widespread than those of SIRT2. However, many studies have also demonstrated that SIRT2 is involved in multiple basic processes of cell life activities, including mitosis [16], energy metabolism [17], autophagy [18] and cell cycle progression [19].

The reproductive physiological characteristics of bovine are similar to those of human (e.g., pregnancy cycle, ovarian structure). Thus, bovine is a suitable animal model to study the physiological mechanism of human ovarian. Although SIRT2 is expressed in a wide range of tissues in bovine, including adipose tissue, heart, liver and lung tissue [20], few SIRT2-mediated functions have been reported to date for bovine, as most researches have focused on rat and human models [21]. Unfortunately, the role of SIRT2 and its mechanism in steroidogenesis are unknown. However, there is growing evidence that Sirtuins might be closely related to steroidogenesis. In some studies, Resveratrol was originally found to be a potential activator of Sirtuins, and it suppressed the expression and promoter activity of StAR in mastocytes [22]. In turn, progesterone secretion was induced by Resveratrol via stimulating the expression of StAR and aromatase in rat GCs [23]. Furthermore, we had previously discovered that SIRT2 was largely expressed in bovine ovarian GCs. However, it remained unclear whether steroidogenesis was regulated by SIRT2, a member of the Sirtuins family. Interestingly, the complicated relationships between SIRT2 and PPARs were evaluated in recent studies [24]. Those findings hinted that a redundancy of functions might exist between SIRT2 and PPARs in steroidogenesis. To verify this hypothesis, we investigated the effects of SIRT2 on P4, E2 and T secretion in bovine ovarian GCs. We also studied the cross-talk between SIRT2 and PPARs/LXRs. We further explored the mechanisms underlying SIRT2′s actions on the steroid hormone synthesis pathway and found for the first time that SIRT2 stimulated LXRα to regulate steroid hormone secretion in GCs. This information may be useful for human reproductive health.

2. Methods

2.1. Chemicals

Thiomyristoyl, SirReal2, Palmitoylethanolamide (PAE) and T0070907 were purchased from Selleck chemicals (USA), whereas LXR-623 was purchased from ApexBio (USA). Rabbit polyclonal anti-SIRT2 (OM105774), anti-LXRβ (OM112190), anti-PPARα (OM269304) and anti-PPARγ (OM269319) were purchased from Omnimabs (CA, USA). Rabbit polyclonal anti-LXRα (ab3585), anti-FSH receptor (ab113421), anti-GAPDH (ab190304) and anti-acetyl lysine (ab21623) antibodies were purchased from Abcam (Cambridge, UK). The other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.2. Primary cell cultures and treatment

Bovine ovaries were obtained from a local abattoir (Shanxi, China) and transported immediately to the laboratory within 8 h in phosphate-buffered saline (PBS) containing penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 27 ~ 30 °C. The bovine granulosa cells were isolated from follicles 2 to 8 mm diameter as described previously [25] with some modifications. Viability of the GCs was assessed using the trypan blue dye exclusion method [26]. The granulosa cells were judged to be > 97% based on the expression of FSHR as determined by IF. The cells were seeded into 24-well tissue culture plates (SarstedtInc., Newton, NC) at a density of 10^6 in 1 ml of DMEM/F12 medium supplemented with 5% FBS without antibiotics for plasmid transfection. The cultures were maintained at 37.0℃ in 5% CO2, 95% air for 24 h. After this time, the GCs were washed twice with PBS and cultured for 48 h in serum-free medium (1 ml) containing 0.1% bovine serum albumin (BSA) (w/v), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), transferrin (2.5 mg/ml), epidermal growth factor (EGF) (5 ng/ml), bovine insulin (10 ng/ml), nonessential amino acid mix (1 ×), FSH (0.1 U/ml) penicillin (100 U/ml), and streptomycin (100 mg/ml). Treatments were applied when the 100% serum-free medium was replaced.

To assess the roles and mechanism of SIRT2 on steroid hormone synthesis in GCs, the cells were treated for 24 h with inhibitors or agonists on day 3 in the serum-free medium. The SIRT2 inhibitors were Thiomyristoyl (0.1, 1, 2 μM) and SirReal2 (1, 2, 5 μM), inhibitor of SIRT2, and the PPARγ inhibitor was T0070907 (2 μM). Palmitoylethanolamide (20 μM) was the agonist of PPARα, and LXR-623 (5 μM) was the agonist of LXRα. The inhibitors and agonists were dissolved in dimethyl sulfoxide (DMSO) and added directly to the serum-free medium. After 24 h, the cells were harvested for mRNA and protein extraction, and the media from GCs the cultured was collected for an ELISA assay.

2.3. Measurement of cell viability

The viability of GCs was determined with the Cell Counting Kit-8 (Beyotime, China). The GCs were seeded in 96-well plates at a density of 5 × 103 cells per well with 100 μL of culture medium. Briefly, at different time points (0, 24, 48, 72, 96 and 120 h), the GCs were treated with 10 μL of CCK-8 solutions for 1 h at 37 ℃. Then, the optical density (OD) values at 450 nm were measured by a microplate reader.

2.4. siRNA and plasmid transfection

Four siRNAs were designed from the coding sequence of bovine SIRT2 (NM_001113531.1) according to the siRNA target sites at positions 121, 226, 345 and 609. The sequences of the siRNA targets were: 5′-CTGCGGAATTTCTTCTCCCAGACTCTGGG-3′ for SIRT2 siRNA-1; 5′-TGTCGCAGGGTCATCTGTTTGGTGGGAGC-3′ for SIRT2 siRNA-2; 5′-GGAGGCCATCTTTGAAATCAGCTACTTCA-3′ for SIRT2 siRNA-3; and 5′-GTACTCACTAAGCTGGATGAAAGAGAAGA-3′ for SIRT2 siRNA-4. The control siRNA sequence was 5′-TTCTCCGAACGTGTCACGT-3′. The ds-siRNA sequence was inserted into the BbsI and SwaI sites of the pilenti-siRNA-GFP vector. The plasmids of SIRT2 siRNA were recycled using the Plasmid Maxi kit (OMEGA).

When the GCs were seeded in 24 well plates with 70–80% confluence, the cells were transfected with plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche) (3:2 Ratio). After 24 h, the green fluorescent protein (GFP) of the transfected cells was assessed using a fluorescence microscope. At this time, the transfection medium was changed into serum-free medium. The GCs were harvested for RNA or protein extraction, at 48 h or 72 h. In some experiments the culture medium was collected for the detection of steroid hormones after transfection for 72 h.

2.5. Immunofluorescence

After the cells grew on the glass slide, they were fixed in 4% paraformaldehyde for 30 min and washed three times in PBS. The cells were permeabilized with 0.2% Triton X-100, and then blocked using 10% normal serum in 1% BSA in TBS (10 mM Tris − HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The cells were incubated with anti-FSHR antibodies (diluted 1:200) in 1% BSA in TBS overnight at 4 °C. The slides of the cells were washed twice for 5 min each time, followed by incubation with the AlexaFluor 488-labeled goat anti-rabbit IgG for 60 min. The nuclei were identified by 4,6-diamidino-2-phenylindole (DAPI) staining. Nonimmune rabbit IgG was used as a

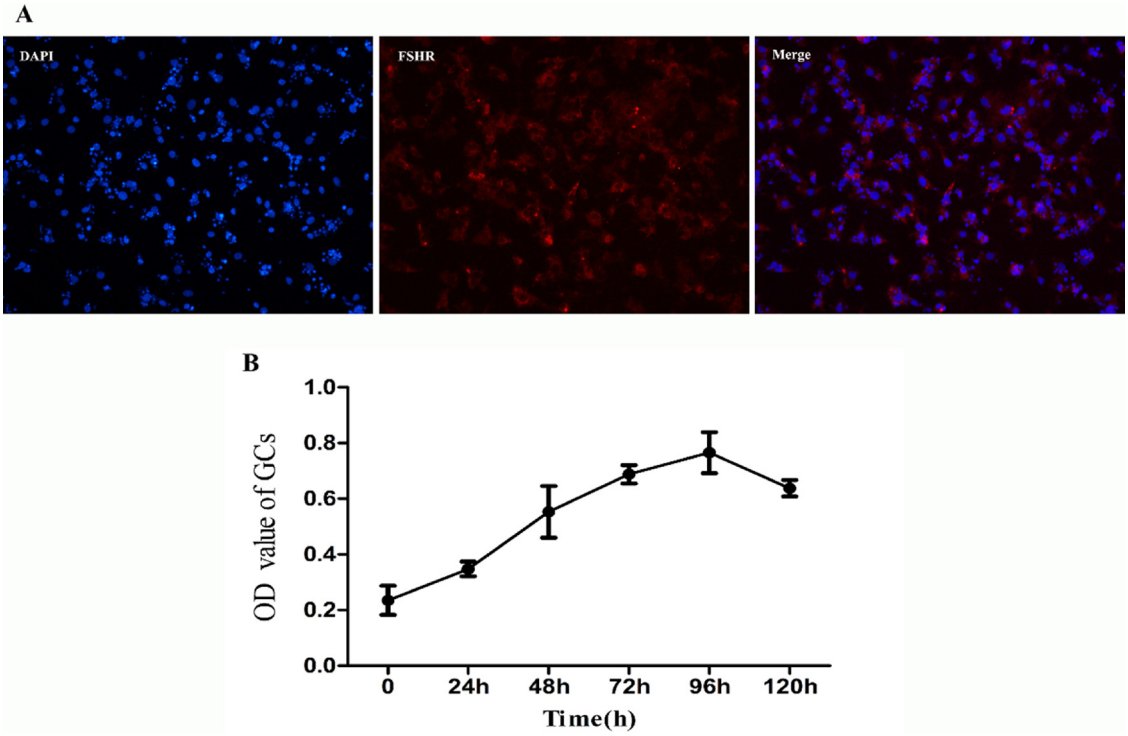

Fig. 2. The identification and viability of bovine ovarian primary granular cells. (A) The identification of granular cells (GCs) was examined using antibodies against FSHR (diluted 1:200). Blue indicates the cell nucleus with DAPI staining. Red indicates FSHR positive cells with immunofluorescence staining. Original magnification × 200. Immunofluorescence was performed on four independent replicates, and cells were counted on 3 independent areas per microscopic field in each replicate immunofluorescence assay. FSHR positive cells VS total cells = 2447/2521, in cell counting. (B) The viability of GCs is shown by CCK-8 assay at different cell culture time points of 0, 24, 48, 72, 96 and 120 h. The experiments were repeated four times (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

negative control. The slides were imaged using a Nikon DS-Ri1 digital camera (Nikon, Tokyo, Japan).

2.6. RT-PCR and quantitative real-time PCR

At 24 or 48 h after the treatments, the GCs were washed in cold PBS and total RNA was extracted using Trizol reagent (Takara, JP) according to the manufacturer's instructions. Total RNA was quantified by NANOROP 2000 (Thermo, USA). The first-strand cDNA was synthesized by subjecting 1 μg of total RNA with a PrimeScript TM RT Reagent Kit with genomic DNA Eraser (Takara, JP) on ice. After reverse transcription of the total RNA, RT-PCR was used to detect *StAR*, *CYP11*, *CYP17*, *Aromatase*, *3βHSD* and *17βHSD* expression in GCs. Template DNA (100 ng), forward primer (200 nM), reverse primer (200 nM), 2 X PCR Taq MasterMix/ with dye (Abm, Canada 25 μL) and nuclease-free H_2O were added to a sterile 0.2 ml RCR tube sitting in a final volume of 50 μL. The samples were incubated at 94 ˚C for 3 min., and then 30 cycles of PCR amplification were performed as follows: denaturing at 94 ˚C for 30 ss, annealing at 60 ˚C for 30 ss, and extending 72 ˚C for 1 min, with a final extension step for 5 min at 72 ˚C. The PCR products were migrated on 2.0% agarose gel stained with HydraGreen™ safe DNA Dye (HydraGene, USA), and exposed to X-ray film. A negative control reaction (omitting template DNA) should always be performed in tandem with the sample PCR to confirm the absence of DNA contamination.

The targeted cDNA was quantified by CFX96TM Real-Time PCR (Bio-Rad, USA) with the SYBR Premix Ex TaqII (2×) Reagent Kit (Takara, JP). The bovine-specific primers for target genes for real-time PCR are listed in Table 1. The thermal cycling parameters were as follows: 30 s at 95 ℃; 40 cycles of 5 s at 95 ℃ and 30 s at 60 ℃; this protocol was used to amplify each targeted cDNA. After the real-time PCR reactions melting curve analyses were performed to verify the PCR product purity. The expression of each gene was calculated according to the threshold cycle (CT) value. Each sample was expressed relative to GAPDH as the housekeeping gene. Then, the relative normalized expression was estimated using the △△Ct method.

2.7. Western blotting

After treatments, the cells were washed with cold PBS, and then lysed in 100 μl/well cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and protease-inhibitor cocktail) containing 1 mM PMSF (Beyotime, Hangzhou, China) for 30 min on ice. The supernatants of the homogenate were recovered by centrifugation at 12,000 × g, for 10 min at 4 ℃. The total protein concentrations were measured using a BCA protein assay kit (Beyotime, China), and mixed with SDS-PAGE sample loading buffer and boiled for 5 min. Twenty micrograms of total protein was submitted to gel electrophoresis, and then separated by 10% SDS-PAGE. The protein was electrophoretically transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA) with transfer buffer (39 mM glycine, 48 mM Tris-base, 1% SDS, 20% methanol, pH 8.3). The membranes were then blocked in TBST containing 5% non-fat dry milk with slight shaking for 60 min at room temperature. After being washed by TBST the membranes were incubated overnight at 4 ℃ with primary antibodies (anti-SIRT2, 1:800; anti-LXRα, 1:1000; anti-LXRβ, 1:500; anti-PPARα, 1:500; anti-PPARγ, 1:1000 and anti-GAPDH, 1:10,000) diluted in 5% non-fat dry milk in TBST. The membranes were washed three times with TBST for 5 min each time, followed by a 1 h incubation in goat anti-rabbit IgG(H + L)-HRP (Sungene Biotech, China 1:5000) with slight shaking for 60 min at room temperature. After being washed four times for 5 min, the protein bands were exposed to X-ray film for visualization using ECL (Millipore, USA). The band intensities were measured using ImageJ software (NIH).

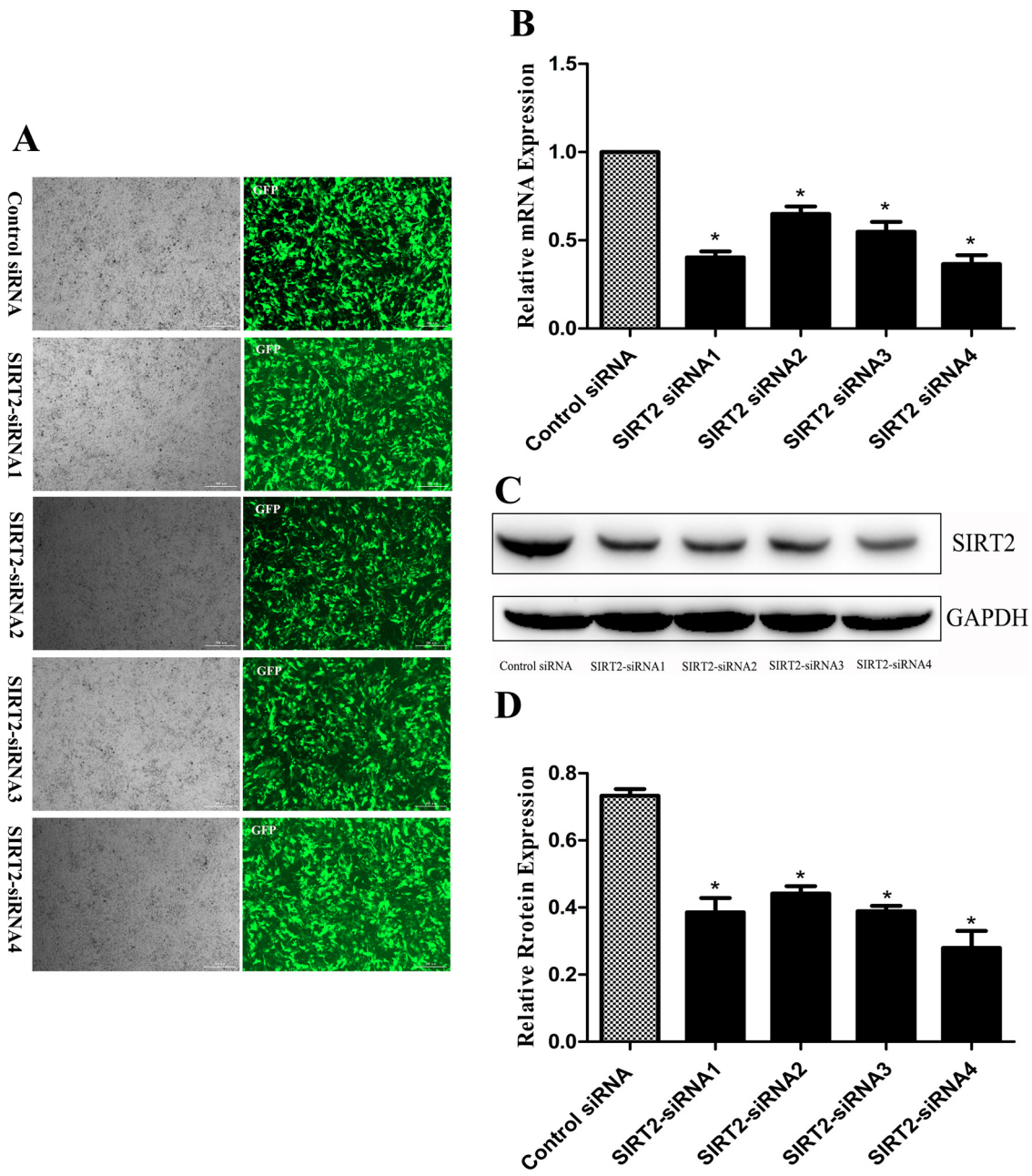

Fig. 3. The transfection and knockdown efficiency of SIRT2-siRNA plasmids. (A) Five piLenti-siRNA-GFP plasmids, named control siRNA, SIRT2-siRNA 1, SIRT2-siRNA 2, SIRT2-siRNA 3 and SIRT2-siRNA 4, were transfected in primary bovine GCs, respectively. Twenty-four hours after transfection, the expression of GFP in the piLenti-siRNA-GFP plasmids is shown. Original magnification × 100. Bar = 300 μm. (B) The mRNA level of the SIRT2 gene was detected after transfection with the piLenti-siRNA-GFP plasmids for 48 h. (C) Western blot for SIRT2 is shown after transfection with the piLenti-siRNA-GFP plasmids for 48 h. (D) The relative protein expression of SIRT2 was semi quantitatively analyzed using ImageJ software. The data are shown as the means ± SEM of four independent replicates. "*" = $P < 0.05$ on each column identifies statistically significant differences.

2.8. Co-immunoprecipitation

The freshly obtained samples were lysed with cold RIPA buffer. The 5% lysates were analyzed by western blotting with anti-Ac-Lys (1:1000), anti-PPARα (1:500), anti-PPARγ (1:1000), respectively. No-specific background was eliminated with protein A agarose and normal rabbit IgG (Beyotime, China). The supernatants were recovered by centrifugation at 12,000 × g for immunoprecipitation. The protein concentration was quantified using a BCA protein assay kit. The rabbit polyclonal anti-Ac-Lys (1 μg) antibodies were added to the lysate sample (500 μl). Then the antibody/lysate samples were incubated together overnight at 4 °C. The immune complexes were captured by protein A agarose with gently shaking for 3 h at 4°C. After washing 5 times with cold lysis buffer, the immune complexes were collected and mixed with SDS-PAGE sample loading buffer and boiled for 5 min A western blot analysis was performed using the final indicated antibodies.

2.9. ELISA for measurements of steroid hormones

The GCs' culture medium was collected to measure the concentrations of estradiol, progesterone and testosterone with a competitive ELISA Kit (Cayman, USA). The estradiol ELISA kit had a range from 6.6 to 4,000 pg/ml and a sensitivity of approximately 15 pg/ml. The

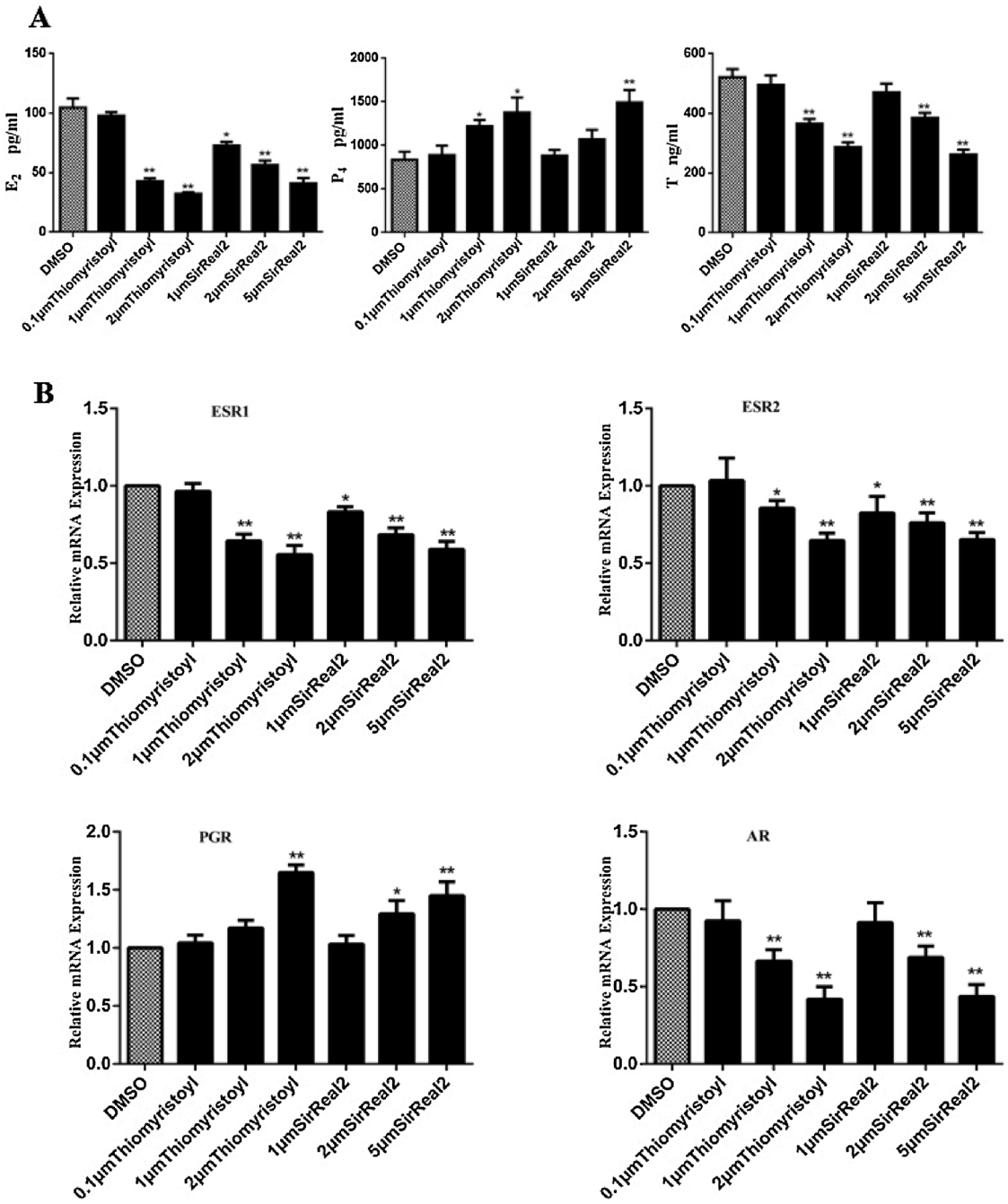

Fig. 4. SIRT2 inhibition blocked the secretion of estradiol (E2) and testosterone (T), whereas induced the secretion of progesterone (P4). (A) SIRT2 inhibition regulated the secretion of steroid hormone. The concentrations of P4, E2 and T in GCs serum-free culture medium were measured using ELISA assays after treatment with Thiomyristoyl (0.1, 1, 2 μM) or SirReal2 (1, 2, 5 μM) for 24 h. The data are shown as the concentration (pg/ml) of steroids secreted. (B) SIRT2 inhibition regulated the expression of hormone receptor. The mRNA levels of *PGR*, *ESR1*, *ESR2* were investigated with a RT-qPCR assay after treatment with Thiomyristoyl (0.1, 1, 2 μM) or SirReal2 (1, 2, 5 μM) for 24 h in GCs. The mRNA level of the control groups was arbitrarily set at 1.0, and that of the treatment groups was estimated relative to the control value. Data are shown as the means ± SEM of four independent replicates. "*" = $P < 0.05$, "**" = $P < 0.01$ on each column identifies statistically significant differences.

progesterone ELISA kit had a range from 7.8 to 1,000 pg/ml and a sensitivity of approximately 10 pg/ml. The testosterone ELISA kit had a range from 3.9 to 500 pg/ml and a sensitivity of approximately 6 pg/ml. The ELISA procedure was performed according to Cayman's ELISA kit instructions.

2.10. Statistical analysis

The statistical data were analyzed with Graphpad Prism version 5 and SPSS version 20 software. A one-way ANOVA test and Duncan's tests were used to analyze the main effects of treatments. Unless otherwise specified, the data were represented as mean ± standard error of the mean (SEM), and $P < 0.05$ was used to determine significant differences between treatments.

3. Results

3.1. SIRT2 expression in bovine ovaries

SIRT2 was expressed in granular cells (GC), oocytes (OO), cumulus

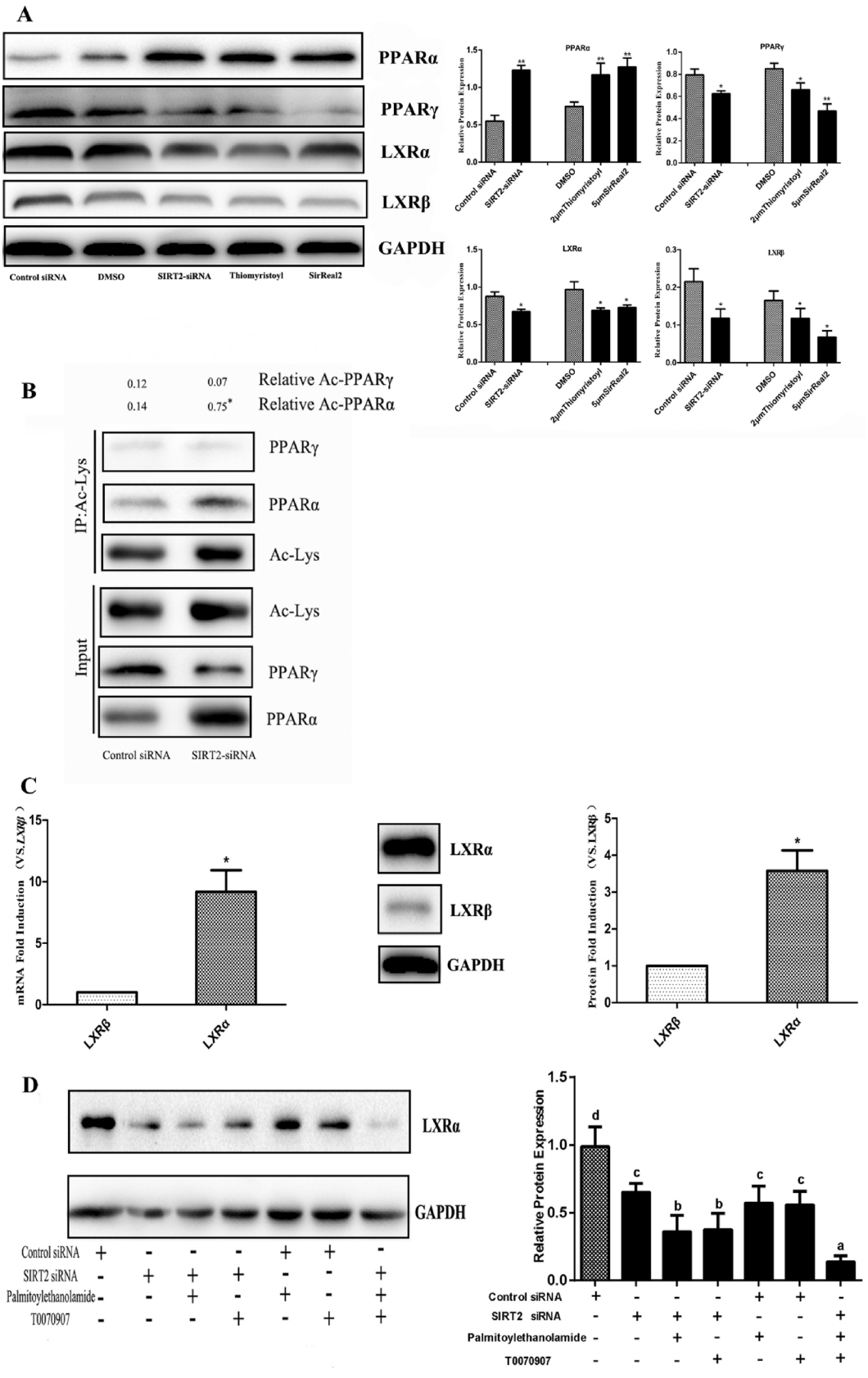

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Fig. 5. SIRT2 inhibition mediated PPARs/LXRs expression. (A) SIRT2 knockdown blocked PPARγ and LXRα, promoted PPARα. The GCs were transfected with SIRT2-siRNA 4 plasmids for 48 h and treated with the SIRT2 inhibitors Thiomyristoyl (2 μM) and SirReal2 (5 μM) for 24 h. Western blotting for PPARα, PPARγ, and LXRα are shown. According to the western blot results, the protein ratios were analyzed in treatment groups relative to the GAPDH protein abundance. (B) SIRT2 knockdown increased Ac-PPARα. Coimmunoprecipitation (coIP) for Ac-PPARα and Ac-PPARγ are shown by using Ac-Lys antibodies. The immunoprecipitates were analyzed by western blotting with the indicated antibodies. IP, immunoprecipitation. (C) LXRα predominantly expressed in GCs, but not LXRβ. The mRNA and protein abundance of LXRα and LXRβ were respectively detected by RT-qPCR and western blot in primary GCs. The expression level of LXRβ was arbitrarily set at 1.0, and the mRNA and protein expression ratios of LXRα/ LXRβ are shown. (D) SIRT2 knockdown blocked the expression of LXRα via PPARs. The primary GCs were pretreated with SIRT2-siRNA or control siRNA for 48 h, and then treated with or without the PPARα agonist Palmitoylethanolamide (20 μM) or the PPARγ antagonist T0070907 (2 μM) for 24 h. The LXRα protein abundance was measured by western blotting. The protein ratios were analyzed in treatment groups relative to the GAPDH protein abundance. Data are shown as the means ± SEM of four independent replicates. "*" = $P < 0.05$, "**" = $P < 0.01$ and bars with different letters (a, b, c) indicate significant differences, $P < 0.05$.

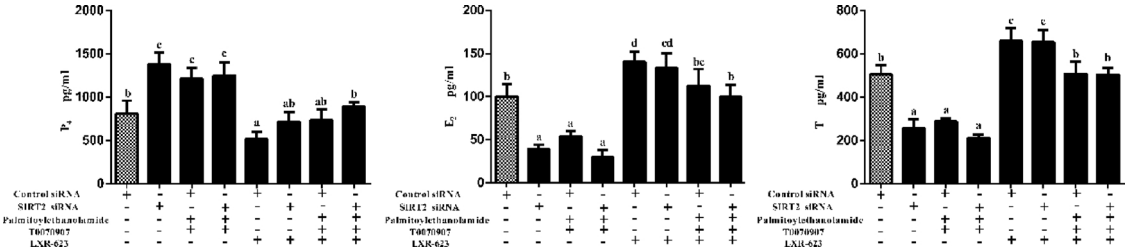

Fig. 6. SIRT2 regulated P4, E2, and T secretion via the PPARs/LXRα pathways. The primary GCs were treated with the PPARα agonist Palmitoylethanolamide (20 μM), the PPARγ antagonist T0070907 (2 μM) and the LXRα agonist LXR-623 (5 μM) for 24 h with or without pretreatment with SIRT2-siRNA. The concentrations of P4, E2 and T in GCs in serum-free culture medium were detected by ELISA assays. Data are shown as the means ± SEM of four independent replicates. Bars with different letters (a, b, c, d) indicate significant differences, $P < 0.05$.

cells (CC) and theca cells (T) by immunohistochemistry with bovine ovarian sections (Fig. 1A). However, SIRT2 was rarely found in Sertoli cells (Fig. 1A). There was an abundance of the SIRT2 protein in GC, OO and CC; however, there was a reduced expression in T as detected by a western blotting assay ($P < 0.05$; Fig. 1B, C). The results indicate that SIRT2 might play an important role in the development and maturation of bovine ovarian follicles.

3.2. The identification and viability of bovine ovarian primary granular cells

The bovine GCs were stained to detect the FSHR protein with immunofluorescence staining. The data indicated that more than 97% of cells were ovarian primary granular cells from bovine follicles (Fig. 2A). We further evaluated the viability of GCs and found that the GCs were in the adaptation period before 24 h of culture. The GCs then grew exponentially at exponential stage until 96 h (Fig. 2B).

3.3. SIRT2 was efficiently knocked down by the SIRT2-siRNA

The bovine GCs were transfected with control siRNA plasmids or four piLenti- SIRT2-siRNA-GFP plasmids (the four recombinant plasmids were designated as: SIRT2-siRNA 1, SIRT2-siRNA 2, SIRT2-siRNA 3, and SIRT2-siRNA 4), respectively. After transfection for 24 h, the transfection efficiency of the siRNA plasmids was analyzed by GFP expression in GCs. In this study, the transfection efficiency almost was nearly 80% (Fig. 3A). At 48 h after transfection, SIRT2 expression was significantly reduced by the four siRNA plasmids at the transcriptional and translational levels ($P < 0.05$, Fig. 3B, C, D). The results showed that the expression of SIRT2 was knocked down by the four plasmids with SIRT2-siRNA 4 having the greatest efficiency (61.82 ± 2.54%) in bovine GCs (Fig. 3C, D).

3.4. SIRT2 regulated P4, E2 and T secretion

To examine the role of SIRT2 on steroid hormone synthesis in primary granulosa cells, the concentrations of P4, E2 and T in serum-free culture medium were measured by ELISA assay after treatment with SIRT2 antagonist (Thiomyristoyl or SirReal2) for 24 h. Additionally, the mRNA expression of hormone receptors was measured with a RT-qPCR assay. We first found that the release of P4, E2, T and expression of hormone receptors were regulated by SIRT2. P4 secretion and progesterone receptor (PGR) expression were significantly induced by treatment with the SIRT2 antagonist ($P < 0.05$, Fig. 4A, B). By contrast, the secretion of E2 or T and the mRNA abundance of estrogen receptor (ER) or androgen receptor (AR) were evidently decreased by treatment with the SIRT2 antagonist ($P < 0.05$, Fig. 4A, B).

3.5. SIRT2 inhibition mediated PPARs/LXRs pathways

We determined whether there was a mediating role of SIRT2 on PPARs/ LXRs, which are well known regulators of sterol biosynthesis in GCs. The protein expression of PPARs/ LXRs was analyzed after treatment with the SIRT2 antagonist or siRNA. The results showed that the expression of PPARγ, LXRα and LXRβ was suppressed by SIRT2 knockdown or treatment with SIRT2 inhibitors (2 μM Thiomyristoyl or 5 μM SirReal2). However, a significant increase in the protein levels of PPARα was observed with SIRT2 inhibition ($P < 0.05$, Fig. 5A). The data suggest that SIRT2 plays different mediating roles toward PPARs. Moreover, we found that SIRT2 knockdown increased Ac-PPARα, but had no effect on Ac-PPARγ (Fig. 5B).

In addition, we found that LXRα was expressed at a high level, but LXRβ was weakly expressed in bovine GCs ($P < 0.05$, Fig. 5C). The results implied that the follicular biology function might be predominantly regulated by LXRα, but not by LXRβ. To determine the possible SIRT2 mediated pathway of LXRα, the GCs were challenged by the PPARα agonist Palmitoylethanolamide (20 μM) or the PPARγ antagonist T0070907 (2 μM) for 24 h with or without pretreatment with SIRT2-siRNA. The data showed that the protein expression of LXRα was significantly suppressed after treatment with Palmitoylethanolamide or T0070907 ($P < 0.05$, Fig. 5D). Interestingly, SIRT2 knockdown, Palmitoylethanolamide or T0070907 had a co-suppression effect on the expression of LXRα ($P < 0.05$, Fig. 5D). The results showed that SIRT2 or PPARγ inhibition possessed a down-regulation effect on LXRα expression. Conversely, the down-regulation effect of LXRα was induced by PPARα activation. These data demonstrate that SIRT2 plays a novel stimulation role in the PPARs/ LXRs signaling pathways Fig. 5.

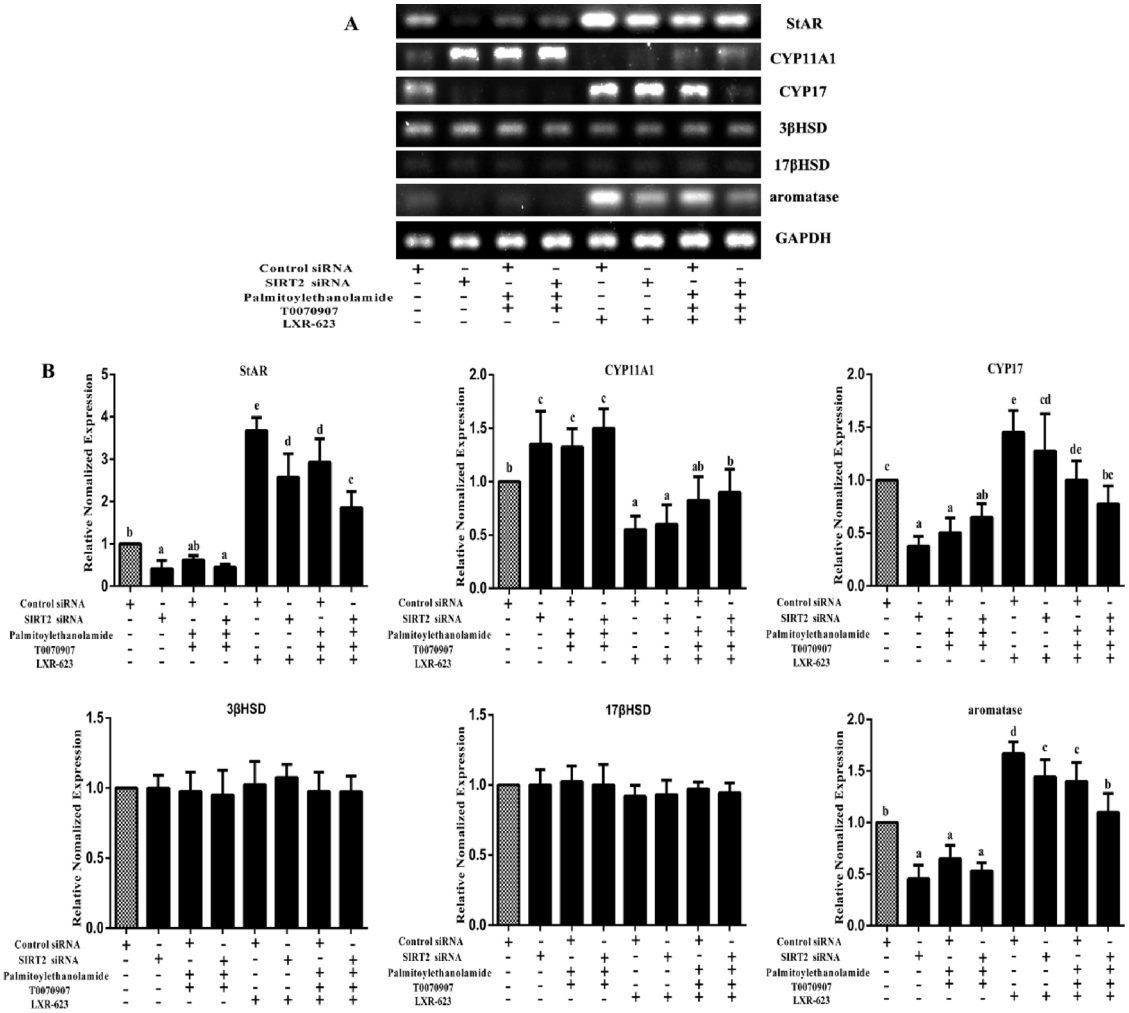

Fig. 7. SIRT2 depended on LXRα to regulate the steroid hormone synthesis pathway. (A) The primary GCs were treated with the PPARα agonist Palmitoylethanolamide (20 μM), the antagonist T0070907 (2 μM) and the LXRα agonist LXR-623 (5 μM) for 24 h with or without pretreatment with SIRT2-siRNA. The mRNA expression of StAR, *CYP11 A1*, *CYP17*, *3βHSD, 17βHSD* and *aromatase* was detected by an RT-PCR assay. (B) The RT-qPCR assay for the mRNA abundance of genes as described above is shown. Data are shown as the means ± SEM of four independent replicates. Bars with different letters (a, b, c, d) indicate significant differences, $P < 0.05$.

3.6. SIRT2 regulated P4, E2, and T secretion via the PPARs/LXRα pathways

We further studied the concentrations of P4, E2 and T as measured by ELISA assays. As expected, similar to the SIRT2 inhibitor treatment, P4 secretion was increased, but E2 and T secretion were suppressed by SIRT2 knockdown in bovine GCs ($P < 0.05$, Fig. 6). Furthermore, this regulated action of SIRT2 knockdown could be mimicked by treatment with the PPARα agonist or the PPARγ antagonist ($P < 0.05$, Fig. 6). In turn, P4 secretion was inhibited, but E2 and T secretion were stimulated by treatment with the LXRα agonist LXR-623 (5 μM) ($P < 0.05$, Fig. 6). Importantly, the regulation effects of SIRT2 knockdown, PPARα activation and PPARγ inhibition on P4, E2, and T secretion were abolished by the LXRα agonist ($P < 0.05$, Fig. 6). These data strongly support the perspective that SIRT2 regulates steroid hormone synthesis by affecting the activity of the PPARs/LXRα signaling pathways.

3.7. SIRT2 depended on LXRα to regulate the steroid hormone synthesis pathway

We then determined the mRNA levels of steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes. Interestingly, the data showed that the mRNA levels of *17βHSD* and *aromatase* were lower than those of *CYP11 A1* in a vitro culture model of GCs Fig. 7A). This result demonstrated that the concentration of P4 was much higher than that of E2 in our previous studies. In addition, SIRT2 knockdown significantly stimulated the mRNA expression of *CYP11 A1* ($P < 0.05$, Fig. 7A, B), which was important for progesterone synthesis as a rate-limiting enzyme. Conversely, SIRT2 knockdown suppressed the mRNA expression of *aromatase* (Fig. 7A, B), which was vital for estrogen synthesis as a rate-limiting enzyme. Similar down-regulation effects were obtained on *StAR* and *CYP17* ($P < 0.05$, Fig. 7A, B). Moreover, the regulation effects of SIRT2 knockdown on steroid hormone synthesis pathway were mimicked by addition of the PPARα agonist Palmitoylethanolamide (20 μM) or the PPARγ antagonist T0070907 (2 μM), but they were abolished by treatment with LXRα agonist LXR-623 (5 μM) ($P < 0.05$, Fig. 7B). However, the SIRT2-PPARs-LXRα signal axis did not affect the basal mRNA levels of *3βHSD* or *17βHSD* (Fig. 7A, B). Collectively, the results demonstrated that SIRT2 promoted the molecular pathway of E2 and T biosynthesis and suppressed the expression of *CYP11 A1* via LXRα.

4. Discussion

Recently, the possible roles of SIRT2 in the regulation of various metabolic processes have emerged, including adipocyte differentiation

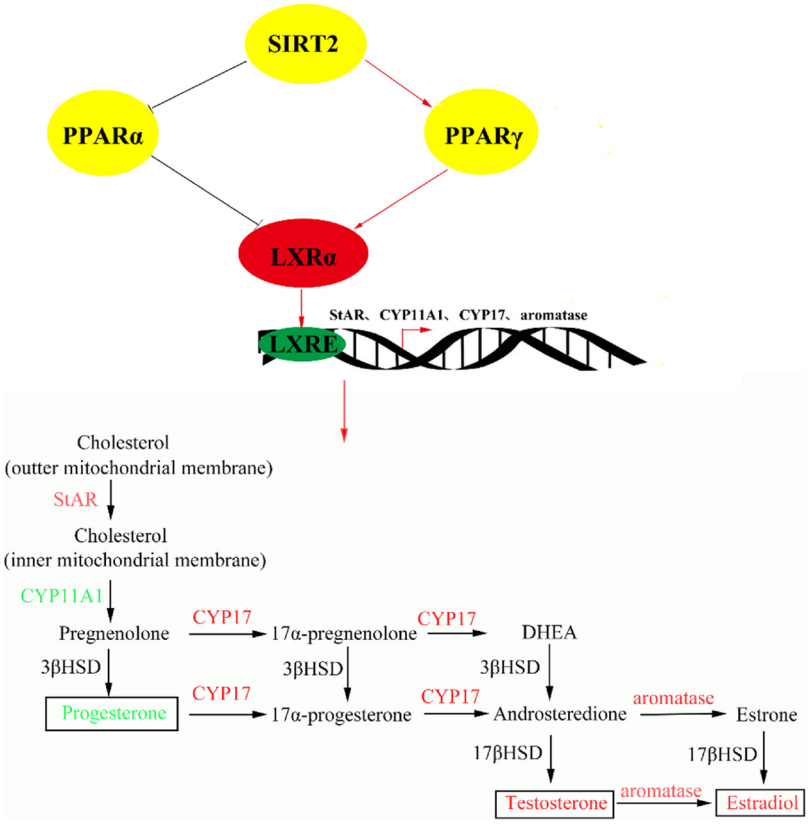

Fig. 8. The molecular mechanism of SIRT2 on steroid hormone synthesis in granular cells. StAR, steroidogenic acute regulatory protein; CYP11 A1, P450 side chain cleavage; CYP17, 17ahydroxylase; 3βHSD, 3β-hydroxysteroid dehydrogenase; 17βHSD, 17β-hydroxysteroid dehydrogenase.

[27], gluconeogenesis [28], and insulin sensitivity [29]. Nevertheless, the biological function of SIRT2 was not still fully understood in ovarian cells, particularly in follicle cells. Previous studies had shown that SIRT2 had been detected in metabolically relevant tissues in particular [30]. We had also shown the abundant presence of SIRT2 in bovine ovarian granular cells. The results indicated that SIRT2 might play an important role in the steroid hormone secretion of bovine GCs. Recent studies have shown that the level of sterol was reduced by the SIRT2 inhibitors AGK2, AK-1 or AK-2 via hindering the nuclear transfer of SREBP-2 and down-regulating the expression of associated genes in nerve cells [31,32]. However, it remained unclear whether SIRT2 regulated P4, E2 and T secretion in bovine GCs. Here, we first surveyed the effects of SIRT2 on steroid hormone biosynthesis in GCs. Interestingly, the results from our works showed that SIRT2 inhibition was not a simple up-regulation or down-regulation of steroid hormone secretion. The secretion of E2 and T was down-regulated by treatment with the SIRT2 inhibitors Thiomyristoyl or SirReal2, but P4 was stimulated by that treatment. Similar results were obtained with SIRT2 knockdown. This evidence suggests that the relationship between SIRT2 and the steroid hormone biosynthesis in GCs is more complex than in other cell types. This raised the question of how SIRT2 regulates steroid hormone biosynthesis. However, little was known about the mechanism of SIRT2 on steroid hormone biosynthesis in bovine GCs.

A growing body of recent literature has indicated that the PPARs/LXRα pathways are linked to steroid hormone biosynthesis. For example, progesterone secretion of granulosa cells was inhibited by PPARγ activation in porcine [33] and rat cells [34], which contradicts the results in mature mouse follicles [6] and porcine theca cells [35]. In addition, the estradiol secretion was also regulated by PPARs pathways in the corpus luteum during porcine pregnancy [36] and in ovine Sertoli cells [37]. The regulatory effect of PPARs on steroid synthesis is likely dependent on cell types, tissues, animal species and their functional states. Interestingly, a recent report showed that adipogenesis was suppressed by SIRT2 via the up-regulation of FOXO1's binding to PPARγ [33]. Therefore, a redundancy of functions might also exist between the SIRT2 and PPARs/LXRα pathways in steroid hormone biosynthesis. Fortunately, there was evidence from our works of crosstalk between SIRT2 and PPARs/LXRα in bovine GCs. In this study, SIRT2 knockdown or treatment with inhibitors played positive effects on PPARα, but negative effects on PPARγ or LXRs. Previous studies suggested that SIRT1 (probably including SIRT2) might play distinctive and even conflicting roles on PPARs in different tissues. To be specific, SIRT1 was reported to increase PPARγ expression in brown adipose tissues [38], but decreased PPARγ expression in white adipose tissues [39]. The mechanisms underlying these conflicting findings remain unclear. Thus, we attempted to demonstrate that these distinctive roles of the SIRT2 on PPARs in GCs. Interestingly, we next found that SIRT2 knockdown increased Ac-PPARα but had no effect on Ac-PPARγ. Recent findings also showed that acetylation of lysine sites could compete ubiquitin sites, inhibiting ubiquitin-mediated proteasome degradation pathway, which enhanced the stability of protein and increased protein content [40–42]. Thus, SIRT2 knockdown might increase the stability of PPARα by acetylation, but not the stability of PPARγ. In our study, SIRT2 knockdown might block PPARγ expression by mediating transcription factors (e.g., FOXO1, FOXO3a) or as-yet-unidentified actions in ovarian granular cells. Regrettably, it is difficult to clarify the hypothesis in this study, and future studies will be necessary to assess this hypothesis.

Furthermore, we also found that the PPARs/LXRα pathways were important regulators of steroid hormone biosynthesis in bovine GCs. Progesterone secretion was stimulated by treatment with the PPARγ antagonist T0070907 (2 μM), which was in agreement with previous reports [33,34]. In addition, PPARα had a positive effect on progesterone secretion, which was verified by treatment with the agonist Palmitoylethanolamide (20 μM). However, E2 or T secretion was repressed by treatment with the PPARγ antagonist T0070907 or the

PPARα agonist Palmitoylethanolamide. More importantly, the role of SIRT2 knockdown on P4, E2 and T could be mimicked by PPARα activation or PPARγ inhibition in GCs. Additionally, the production of P4 was inhibited, and the secretion of E2 and T was stimulated by the LXRα activator LXR-623 (5 μM) in ovarian granulosa cells. These data were in agreement with previous studies showing that E2 and T secretion were promoted by LXRα [7,43,44]. In this study, the effects of SIRT2 knockdown, PPARα activation or PPARγ inhibition on steroid hormone biosynthesis were abolished by LXRα activation. The results showed that PPARα and PPARγ had opposing roles on P4, E2 and T secretion. Moreover, the results verified our previous works showing that PPARα and PPARγ conflictingly regulated the expression of LXRα. These data convincingly demonstrate that SIRT2 is dependent on LXRα activity to possess a positive effect on E2 and T secretion, but negatively affects the secretion of P4.

The biosynthesis of P4, E2 and T was controlled by steroidogenic enzymes, including StAR, CYP11 A1, 3βHSD, CYP17, 17βHSD and aromatase [45]. Nevertheless, previous works had implicated the PPARs/LXR pathways as a modulator of StAR or steroidogenic enzymes involved in steroid hormone biosynthesis [37,43,44,46,47]. Our present findings also indicated that SIRT2 regulated steroidogenesis via the PPARs/LXRα pathways. This raised the question of whether SIRT2 depended on the PPARs/LXRα pathways to control steroid hormone synthesis pathway. A recent study showed that the expression of StAR and aromatase was stimulated by treatment with resveratrol, which is a potential activator of Sirtuins [23]. In the present study, there was no effect of SIRT2, PPARs and LXRα on 3βHSD or 17βHSD, which was in agreement with the literature [36]. However, the *StAR* mRNA abundance was suppressed by SIRT2 knockdown. In addition, the mRNA level of *CYP17* and *aromatase*, which is involved in E2 or T biosynthesis, was down regulated by SIRT2 knockdown. Conversely, mRNA abundance of *CYP11 A1*, which is a rate-limiting enzyme for P4 biosynthesis, was stimulated by the SIRT2 knockdown. The similar results for the effects of SIRT2 knockdown on the molecular pathway were obtained by treatment with the PPARα agonist or the PPARγ antagonist. More importantly, the effects of SIRT2 knockdown, PPARα activation or PPARγ inhibition on the steroid hormone synthesis pathway were abolished by LXRα activation. Therefore, this finding suggests that SIRT2 or PPARs are LXRα-dependent for controlling molecular pathway of steroid hormone synthesis in bovine GCs.

In conclusion, our data demonstrate a novel role and mechanism of SIRT2 in regulating P4, E2 and T secretion in bovine GCs (Fig. 8(). We first verified that SIRT2 promoted the expression of *StAR*, *CYP17*, *aromatase*, and suppressed the expression of *CYP11 A1* via PPARs/LXRα pathways. Those findings show that SIRT2 plays critical roles in follicle development and maturation via maintaining steroid hormone homeostasis before mammalian pregnancy.

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