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BMP15 regulates FSHR through TGF-β receptor II and SMAD4 signaling in prepubertal ovary of Rongchang pigs

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ABSTRACT

Bone morphogenetic protein 15 (BMP15) and follicle-stimulating hormone (FSH) both play important roles in mammalian ovary and follicular development. The aim of the present study is to investigate the effects of BMP15 and FSH in the prepubertal ovary of Rongchang pigs considering a possible signaling mechanism involving TβRII/ SMAD4 and FSHR in granulosa cells. For this purpose, we quantified expression levels of *BMP15*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7*, *TGF-β1*, *TGF-β2*, *TGF-β3*, *TGFβRI*, *TGFβRII,* and *FSHR* via qRT-PCR at different ages in prepubertal ovaries and cultured biopsy of 90-day-old ovary in Rongchang pig. Additionally, the protein levels of BMP15, FSHR, SMAD2, SMAD4, TGFβRI, TGFβRII, TGF-β1, TGF-β2 were quantified via Western blot and the localizations of BMP15, FSHR and TGFβRII were observed via immunofluorescence confocal microscope. The results showed that expression levels of *BMP15, TGF-β1, TGFβRII* and *FSHR* increased significantly at day 60 as compared to day 30 and reached peak value at day 90 in prepubertal ovary of Rongchang pigs. We observed that BMP15, TGFβRII and FSHR was highly presented, which TGFβRII and FSHR displayed co-localization in the follicles of the prepubertal ovaries of 90-day-old Rongchang gilts. Treatment with TGFβRI/II inhibitor LY2109761 significantly decreased the expression of TGFβRI, TGFβRII and SMAD4 and TGFβRI inhibitor LY2157299 decreased TGFβRI, but increased the TGFβRII, SMAD4 and FSHR expression levels. Furthermore, the addition of rBMP15 and rFSH group significantly increased the expression of TGFβRII and FSHR proteins ($P < 0.01$), but no significant change in the expression of TGFβRI ($P > 0.05$) was observed by Western blot. In conclusion, BMP15, TGFβRII and FSHR were increased significantly in the prepubertal ovarian follicles of Rongchang pigs and FSHR expression in GCs was regulated by BMP15 and FSH through the TGFβRII.

1. Introduction

Bone morphogenetic protein 15 (BMP15), a member of the transforming growth factor beta (TGF-β) superfamily, is a growth factor secreted by oocytes (Liu et al., 2019). BMP15 has six conserved cysteine that often forms disulfide bonds that connect with two mature peptide regions to form a biologically active dimer (Gasperin et al., 2014; Liu et al., 2019; Moore and Shimasaki, 2005). Then the dimer binds to the TGF-β receptor (TGFβR) on the granulosa cells (GCs) membrane around the oocytes to further phosphorylate intracellular small mothers against decapentaplegic-related (SMAD) proteins, there by constituting the BMP15/TGF-β/SMAD signaling pathway which regulates follicular development, promotes cumulus cell proliferation, and enhances oocyte development (Gasperin et al., 2014; Liu et al., 2019; Pangas et al., 2008; Zhai, 2013). Studies have shown that there are five subtypes of TGF-β, named TGF-β1, TGF-β2, TGF-β3, TGF-β4, and TGF-β5 (Gipson et al., 2020; Yi et al., 2020). Among the TGFβR family, TGFβRI, TGFβRII, and TGFβRIII have been extensively studied (Hata and Chen, 2016; Schmierer and Hill, 2007). SMADs are divided into the receptor-regulated type (i.e., SMAD1–3, SMAD5, and SMAD8), co-regulated type (SMAD4), and inhibitory type (SMAD6 and SMAD7). In a word, the TGFβR and SMAD family plays a very important role in the process of TGF-β signal transduction (Du et al., 2018; Qin et al., 2019; Tzavlaki and Moustakas, 2020).

As a biological macromolecule, follicle-stimulating hormone (FSH) does not penetrate the cell membrane, but mainly binds to the specific

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FSH receptor (FSHR) on the membrane surface of the target GCs and Sertoli cells (De Pascali et al., 2018; Devillers et al., 2019; Zhao, 2017). Interestingly, the proliferation and differentiation of GCs as well as the secretion of GC follicular fluid which induce the production of aromatase and stimulate the synthesis and secretion of estradiol regulate the development, maturation, and release of oocytes (Devillers et al., 2019; Zhao, 2017). However, BMP15 must bind to the receptors BMPR1B (ALK6) and BMPRII on the cell membrane to activate the downstream SMAD signaling pathway (Gipson et al., 2020; Heath et al., 2017). Research has revealed that the expression of BMP15 in human ovarian GCs induces the expression of FSHR and the cytochrome P450 family 19 subfamily A member 1 (CYP19A1), as well as the production of estrogen (Shimizu et al., 2019). Recent data show that FSHR induction by BMP15 may be utilized for controlling follicular growth and development in human (Liu et al., 2019; Qin et al., 2019). BMP15 mutations cause premature ovarian failure (POF) in women (Santos et al., 2019). In addition, studies of polycystic ovary syndrome (PCOS) in women have shown that the expression of BMP15 and growth differentiation factor 9 (GDF9) in preantral follicles of PCOS ovaries is significantly lower than that in normal ovaries, causing abnormality in early follicular development (Lai et al., 2018; Rossetti et al., 2020; Shimizu et al., 2019). The expression of FSHR in GCs is regulated by BMP15 and the deficiency of BMP15 significantly reduces the expression of FSHR (Qin et al., 2019; Shi et al., 2020). However, the molecular mechanism of the interaction between BMP15 and FSHR at different developmental stages before puberty in various female mammals remains unclear.

The Rongchang pig is one of three excellent local swine breeds, which has been listed as a national class I protected breed resource in China (Yi et al., 2020). Rongchang gilts experience early puberty at an average age of 106.1 ± 18.7 days and an average weight of 26 ± 10.2 kg (Zhu et al., 2017). Our preliminary research showed that *BMP15* gene was highly expressed in the ovaries of Rongchang pigs and BMP15 activates SMAD signaling pathway through TGFβRII (Ma et al., 2016a; Ma et al., 2016b; Yi et al., 2020). On the base of above data, we proposed a hypothesis that BMP15 changes the expression of SMAD4 and FSHR, thereby affecting the sensitivity of FSH to granulosa cells (GCs). Knowledge of the BMP15/TβRII/SMAD4 signaling pathway on the effects of FSH is important for a better understanding of the follicular development in mammals. The aim of this present study is to investigate the relationship of BMP15 and FSHR through TGFβRII/SMAD4 signaling pathway in prepubertal ovary of Rongchang pigs and lay the foundation for clarifying the regulation mechanism between BMP15 and FSHR.

2. Materials and methods

2.1. Animals and tissue preparation

Female Rongchang pigs named Rongchang gilts around 28-day-old were purchased from the Pig Research Institute of Chongqing Academy of Animal Science (Rongchang, Chongqing, China). All animal experiments were approved by the Animal Care Commission of the College of Animal Science, Southwest University. 15 female pigs were housed in laboratory animal room and maintained on a 14/10-h light-dark cycle with food and water ad libitum at the Laboratory Animal Facility of the College of Animal Science, Southwest University. On days 30, 60 and 90, the five Rongchang gilts were sacrificed after anesthesia, and the left five ovaries of each pig were prepared for immunohistochemistry and ovarian tissue culture, the right five ovaries of each pig were prepared for biochemical analyses by qRT-PCR and Western blot.

2.2. Ovarian tissue culture

The left ovaries of each 60-day-old Rongchang gilts were cut into 400 μm thickness tissue slices using Alabama R&D MD6000 Tissue Slicer (TSE, USA) according to owner's instruction manual. Ovary tissue slices were cultured in 12-well cell culture plate (Corning, USA) at 37 °C, 5% CO_2 with 1000 ml DMEM supplied with culture medium A including 5 mM rBMP15 (R&D, MN, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (HyClone, UT, USA), 10 μg/ml insulin, 5 μg/ml transferrin, 20 nM selenite (ITS medium, Sigma Aldrich, St.Louis, MO, USA), 5% fetal calf serum (FCS, Hyclone, Utah, USA). The culture medium B and C were added TGFβRI/II LY2109761(S2704, Selleck, USA) and TGFβRI LY2157299(S2230, Selleck, USA) on the basis of culture medium A, respectively as described previously (Yi et al., 2020). The culture medium was changed every 24 h. Negative controls were established by omitting 5 mM rBMP15 (R&D, MN, USA). The ovary tissue slices were prepared for biochemical analyses by qRT-PCR and Western blot as described previously (Yi et al., 2020).

Table 1
Sequences of oligonucleotide primers for qRT-PCR used in the study.

Gene	Accession no.	Primer sequence (5′-3′) Annealing temperature (°C)	Size (bp)
BMP15	HQ013301.1	F:CGCATCTGAGGTTCTTGG 60 R:CGCATCTGAGGTTCTTGG	232
FSHR	L31966.1	F:CTGCTTTACATCGACCCT 60 R:CGAATCCCATTCTTACTCA	227
SMAD2	NM_001256148.1	F:TTGATGGTCGTCTTCAGGTAT 60 R:TTTCTGGAATGGAGTGGGTAT	276
SMAD3	NM_214137.1	F:GGAGGAGAAGTGGTGCGAGAA 60 R: ATGGTGGCTGTGAAGGTCAGG	226
SMAD4	NM_214072.1	F:ATCTGCTGCTGCTGGAATCGG 60 R:CTTCATCTAGGAGCTGGAGG	164
SMAD7	NM_001244175.1	F:GTGCGTGGTGGCATACTGGGA 60 R: GTGGCTGACTTGATGAAGATG	255
TGF-β1	NM_214015.2	F:GCGGCAACCAAATCTATGA 60 R:GTGGGCACTGAGGCGAAAA	320
TGF-β2	L08375.1	F:TGCCCTCCTACGGACTTGA 60 R:GCTGAGAACCCTGCTATGCT	229
TGF-β3	NM_214198.1	F: TCTTTCTTGCTGGCTCTGA 60 R:AACACTTGCCTTGTATGACG	306
TGFβRI	NM_001038639.1	F:GGTTCCGTGAGGCAGAGATT 60 R:ATGTGAAGATGGGCAAGTCC	229
TGFβRII	L08375.1	F:TGCCCTCCTACGGACTTGA 60 R:GCTGAGAACCCTGCTATGCT	239
GAPDH	AF017079.1	F:TCTTTCTTGCTGGCTCTGA 60 R:AACACTTGCCTTGTATGACG	198

2.3. Inhibition analysis of TGFβR

The ovary tissue slices were cultured and then were treated with 30 μm TGFβRI/II LY2109761, 10 μm TGFβRI LY2157299, 0.01 IU/ml human FSH-Fc/Fc heterodimer (FSH) (KN015, Suzhou, China), and rBMP15 + FSH respectively as described previously (Yi et al., 2020; Tian et al., 2019). The ovary tissue slices were prepared for biochemical analyses by qRT-PCR and Western blot as described previously (Yi et al., 2020).

2.4. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

The total RNA of difference ovarian tissues and cultured biopsy was extracted with Trizol reagent (Invitrogen, CA, USA), respectively and 0.2 μg total RNA was reverse transcribed using a First-Strand Synthesis kit (Vazyme, Nanjing, China) and then qRT-PCR was fulfilled on a QuantStudio 3 Quantitative RT-PCR machine (ABI, MA, USA), using qPCR SYBR Green Master Mix (Vazyme, NJ, USA) and the primers listed in Table 1. After normalization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the relative mRNA expression was determined using the comparative cycle threshold method. Three replicates were collected for each group. Relative mRNA expressions of the genes were detected by the $2^{-\Delta\Delta Ct}$ method.

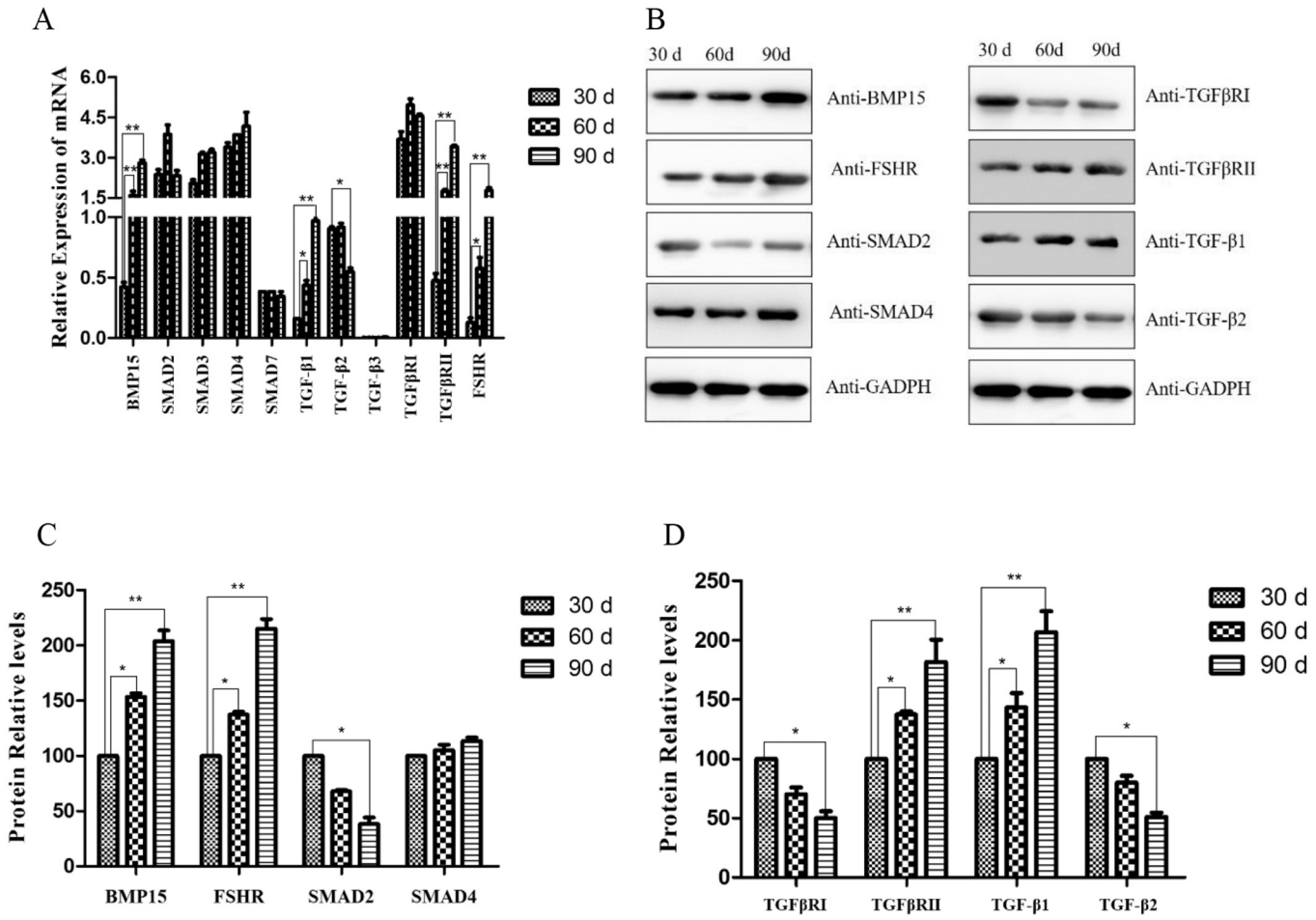

Fig. 1. Expression difference analysis between *BMP15* and SMADs signal related genes of after ovary in prepubertal ovary Rongchang pigs.
A. Relative Comparison of different gene mRNA expression of different genes with in 30 days, 60 days and 90 days old rongchang pigs B, C, D. Western blot and gray analysis of BMP15, FSHR, SMAD2, SMAD4, TGFβRI, TGFβRII, TGF-β1 and TGF-β2. **show very significant difference at $P < 0.01$, * show significant difference at $P < 0.05$.

2.5. Western blot

The ovaries and cultured biopsy were lysed in ice-cold protein lysis buffer (RIPA, Beyotime, Huangzhou, China) supplied with protease inhibitor (Beyotime, Huangzhou, China). The lysates were centrifuged at 20000 ×*g* for 10 min at 4 °C to remove tissue debris. Lysate supernatant was boiled in 5× protein loading buffer for 10 min. Each equivalent protein (20 μg) was separated by 12% SDS-polyacrylamide gel and electro-transferred to polyvinylidene difluoride membranes (CST, MA, USA). The membranes were blocked for 2 h with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies overnight at 4 °C. The primary antibodies against the purposed protein were BMP15 (1:1000, Abcam, Cambridge, UK) , SMAD4 (1:1000, Abcam, Cambridge, UK), TGF-β1(1:1000, Abcam, Cambridge, UK) , TGF-β2 (1:500, Abcam, Cambridge, UK), TGFβRI (1:800, Abcam, Cambridge, UK) , TGFβRII (1:1000, Abcam, Cambridge, UK), FSH-R (1:500, Abcam, Cambridge, UK), rBMP15(1:1000, R&D, MN, USA) and GADPH (1:2000, Abcam, Cambridge, UK). GADPH was used as the reference control. Afterward, the membranes were incubated with the suitable horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz, CA, USA), the protein bands were detected using a FUSION FX-XT imaging system (VILBE, Paris, France) and quantified by Image Fusion-capt/evolution software.

2.6. Immunofluorescence

The Rongchang gilt ovaries were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin wax, and sectioned at 6 μm. Then they were deparaffinized with xylene, rehydrated through a graded series of alcohol, and subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). After deparaffinization, rehydration, and antigen retrieval, the sections were washed three times in phosphate-buffered saline (PBS) and blocked, followed by incubating with a primary antibody (1:100 dilution of a mouse anti-BMP15 antibody; abcam) in blocking buffer overnight at 4 °C. Staining was visualized with a Cy3-conjugated goat anti-rabbit IgG (Beyotime, Hangzhou, China) or a FITC-labeled Goat Anti-Mouse IgG (H + L) (Beyotime, Hangzhou, China) diluted at 1:200 for 50 min and incubated with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Beyotime, China) for 10 min to stain the nucleus. After the final wash in PBS, fluorescent images were detected by a fluorescent microscope (LEICA Dmi8, Germany). Negative controls were established by omitting primary antibodies.

2.7. Statistical analysis

GraphPad Prism 6 software was used for statistical analyses. One-way ANOVA and the Newman-Keuls test were used for testing the difference more than two groups. Student's test was used for comparing between two groups. The data is presented as the mean ± SEM of at least 3 independent experiments. Statistical significance was considered as

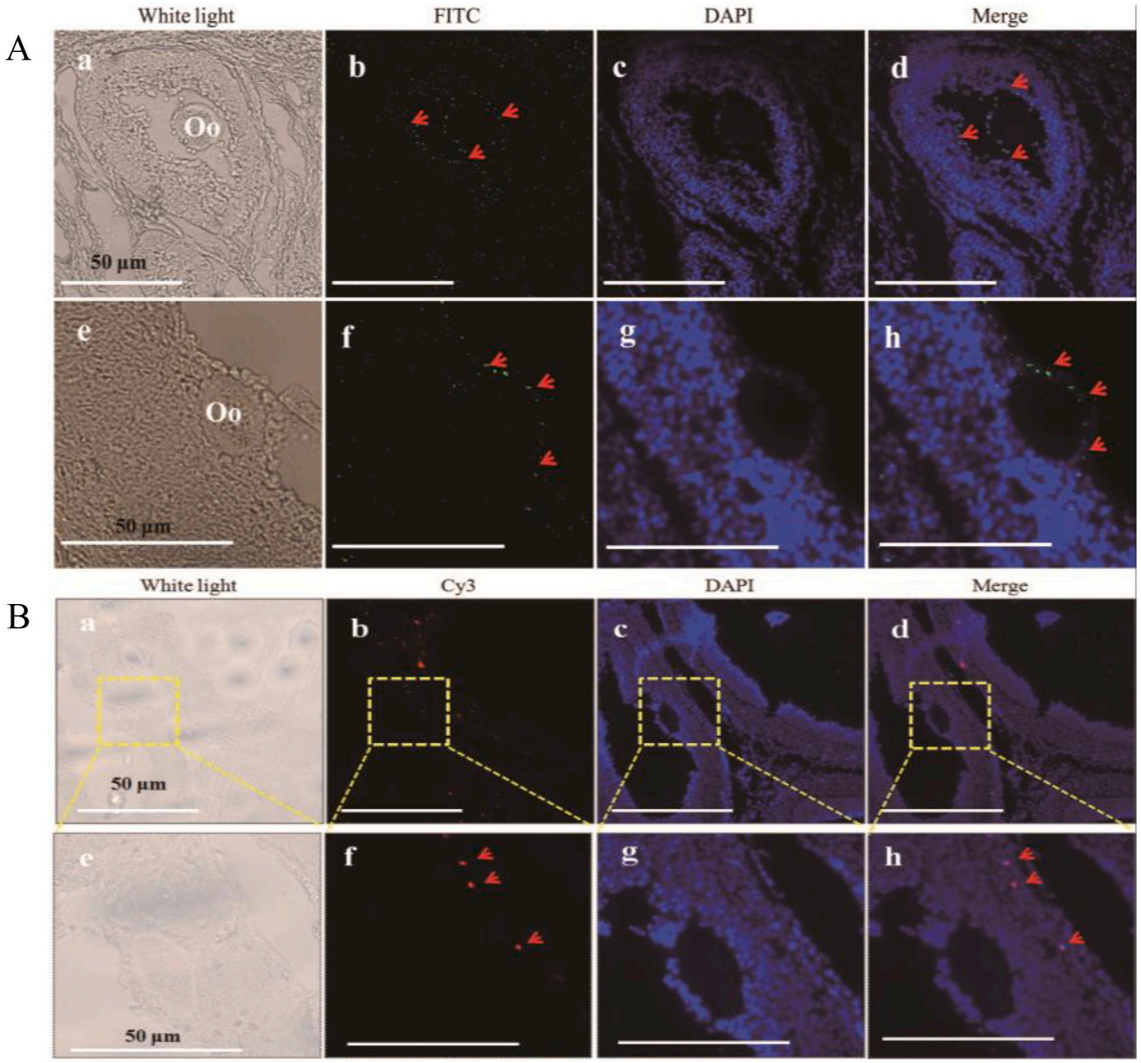

Fig. 2. The distribution analysis of BMP15 and FSHR protein at follicle in prepubertal ovary of Rongchang pigs.
A: a. Follicular tissue of ovary; b. BMP15 protein staining by FITC; c. DAPI; d. Merged image; e. Follicular tissue of ovary; f. BMP15 protein staining by FITC; g. hoechst33258 staining; h. Merged image; The bar represents 50 μm.
B: a. Follicular tissue of ovary; b. FSHR protein staining by cy3; c. hoechst33258 staining; d. Merged image; e. Follicular tissue of ovary; f. FSHR protein staining by cy3; g. hoechst33258 staining; h. Merged images The bar represents 50 μm.

follows: * show significant difference at $P < 0.05$, ** show very significant difference at $P < 0.01$.

3. Results

3.1. Expression of BMP15, FSHR, and TGF-β/SMAD signaling-related genes

To analyze the expression of *BMP15*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7*, *TGF-β1*, *TGF-β2*, *TGF-β3*, *TGFβRI*, *TGFβRII*, and *FSHR* gene among different prepubertal ovaries of Rongchang gilts, total RNA and proteins were isolated from 30, 60 and 90 days old ovary tissues in prepubertal Rongchang gilts and analyzed by qRT-PCR and Western blot, respectively. The results were standardized by reference gene *GADPH*. The result illustrated that, the mRNA expression of *BMP15*, *TGF-β1*, *TGFβRII* and *FSHR* increased significantly at 60 days as compared to 30 days and reached peak value at 90 days ($P < 0.01$, Fig. 1A). Meanwhile the result also showed that, in comparison to 30 days, 60 days and 90 days old, mRNA expressions of *SMAD2*, *SMAD3*, *SMAD4*, and *TGFβRI* gene were abundantly expressed; *SMAD7* gene was a low expressed and *TGF-β3* almost not expressed (Fig. 1A). Simultaneously, the protein levels of BMP15, TGF-β1, TGFβRII and FSHR were also dramatically up-regulated among different prepubertal ovaries of Rongchang gilts ($P < 0.01$, Fig. 1B, C and D), and the SMAD2, TGFβRI, and TGF-β2 were downregulated, but no significant change in the expression of SMAD4 protein (Fig. 1B). In general, BMP15, FSHR, TGFβRII and TGF-β1 were significantly upregulated at 30 days and 60 days ($P < 0.01$, Fig. 1C and D), while TGFβRI, TGF-β2, and SMAD2 were significantly downregulated in the prepubertal ovaries of Rongchang gilts ($P < 0.05$, Fig. 1C and D).

3.2. Distribution of BMP15 and FSHR

To analyze the distribution of BMP15 and FSHR proteins in the follicles of the prepubertal ovaries of 90-day-old Rongchang gilts, we performed immunofluorescence staining on paraffin-embedded ovarian tissues and observed the results using fluorescence microscope. The result showed that the green fluorescent signal of BMP15 in the GCs around the oocytes of 90-day-old Rongchang gilts was present, as shown by the red arrow (Fig. 2A), in addition, a strong red fluorescent signal of FSHR was found in those cells, as shown by the red arrow (Fig. 2B). In general, the result revealed that the BMP15 and FSHR was expressed in

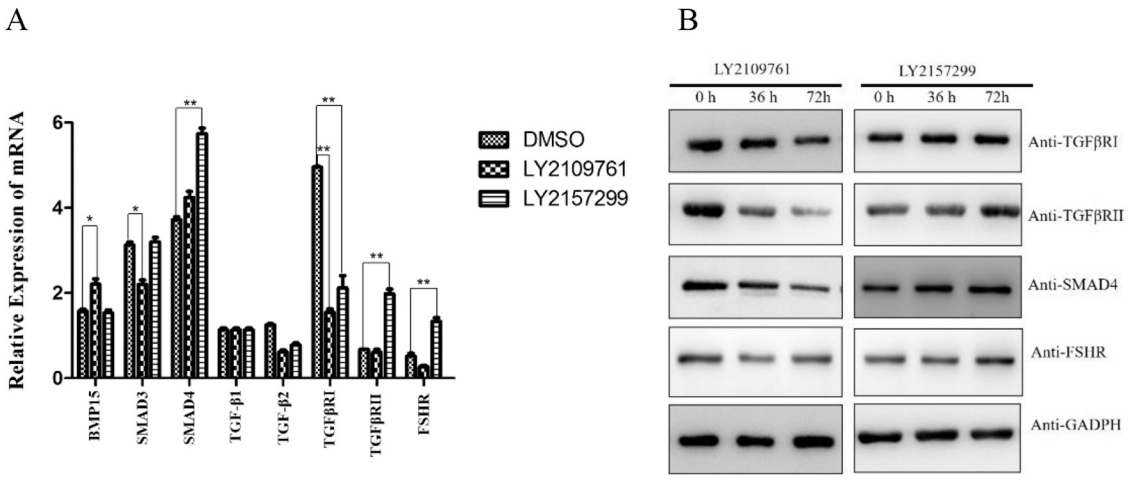

Fig. 3. Expression difference analysis between *BMP15*, *TGFβRII* and SMADs signal related genes under TGFβR inhibition.
A: mRNA expression difference analysis by qRT-PCR. **show very significant difference at $P < 0.01$, * show significant difference at $P < 0.05$. B: protein expression difference analysis by Western blot.

the follicles of the prepubertal ovaries of 90-day-old Rongchang gilts (Fig. 2A and B).

3.3. Expression of BMP15 and TGF-β/SMAD signaling-related genes by treatment of TGFβR inhibitor

The total RNAs were extracted from cultured biopsy of 90-day-old Rongchang gilts cultured in media A, B, and C for 48 h and reverse-transcribed into cDNA templates for qRT-PCR analysis of *BMP15*, *SMAD3*, *SMAD4*, *TGF-β1*, *TGF-β2*, *TGFβRI, TGFβRII,* and *FSHR*, using *GAPDH* as the internal reference gene. At dose 100 ng/ml treatment by rBMP15 to the ovary tissue slices culture medium, the mRNA expression of *BMP15* was increased ($P < 0.05$) and the *SMAD3* and *TGFβRI* was significantly down-regulated ($P < 0.05$ and $P < 0.01$, respectively) in the TGFβRI/II inhibitor (LY2109761) group compared with the DMSO control group. In addition, the mRNA expressions of *SMAD4*, *TGFβRII,* and *FSHR* were up-regulated approximately 2 folds ($P < 0.01$), 6 folds ($P < 0.01$) and 3 folds ($P < 0.01$), respectively, while the *SMAD3*, *TGF-β1* and *TGF-β2* displayed no significant changes in the TGFβRI inhibitor (LY2157299) group ($P > 0.05$) compared with the DMSO control group (Fig. 3A). Simultaneously, the cultured biopsy of 90-day-old Rongchang gilts were collected after treatment of the TGFβRI/II inhibitor LY2109761 and the TGFβRI inhibitor LY2157299 with media B or C for 0 h, 36 h, or 72 h, then performed Western blot analysis of TGFβRI, TGFβRII, SMAD4 and FSHR proteins using GAPDH as an internal reference. The results illustrated that treatment with TGFβRI/II inhibitor LY2109761 significantly decreased TGFβRI, TGFβRII and SMAD4 and TGFβRI inhibitor LY2157299 also decreased TGFβRI, but obviously increased the TGFβRII, SMAD4 and FSHR (Fig. 3B). In general, the result revealed that when TGFβRI was inhibited, the exogenously added BMP15 protein transmitted signals to SMAD4 through TGFβRII, which regulated the expression of FSHR.

3.4. Localization of FSHR and TGFβRII proteins and analysis of BMP15 responses

To clarify the role of BMP15 in regulating the expression of FSHR through TGFβRII, we performed immunofluorescence staining on paraffin-embedded ovary tissue slices of 90-day-old Rongchang gilts and observed by confocal microscope. The results showed that TGFβRII protein was detectable with a strong red fluorescent signal (Figures 4Ab and g) and FSHR with a strong green fluorescent signal by the red arrow (Figures 4Ac and h) in the GCs of the ovarian follicles of the 90-day-old Rongchang gilts. Meanwhile, the results displayed co-localization of TGFβRII and FSHR proteins in the GCs of the ovarian follicles of 90-day-old Rongchang gilts (Figures 4Ae, g). To investigate whether FSH and BMP15 modulates the BMP15, FSHR, TGFβRI, and TGFβRII proteins expression, the cultured biopsy of 90-day-old Rongchang gilts cultured in media A, B, and C were incubated with rFSH (0.01 IU/ ml) for 48 h was checked by Western blot. With the addition of rBMP15 and rFSH to the cultured biopsy of the 90-day-old Rongchang gilts, the BMP15, FSHR, and TGFβRII proteins were dramatically increased, while the expression of TGFβRI displayed no significant changes, compared with TGFβRI/II inhibitor LY2109761 group and blank control group (Fig. 4B). The protein level of BMP15, FSHR, TGFβRI and TGFβRII quantified by Image Fusion-capt/evolution software suggested that the addition of rBMP15 and rFSH group significantly increased the expression of TGFβRII and FSHR proteins ($P < 0.01$, Fig. 4C and D), but no obvious change the expression of TGFβRI ($P > 0.05$, Fig. 4D).

4. Discussion

The development of the ovary is regulated by endocrine, autocrine, and paracrine processes between follicular germ cells and somatic cells (Li, Q., 2018; Ma et al., 2016a). Meanwhile, Ovarian follicle formed of Oocytes, GCs and membranous cells is the functional unit of ovary (Li, Q., 2018; Ma et al., 2016a). BMP15 is a growth factor secreted by oocytes, constitutes the complicated BMP15/TGF-β/SMAD signaling pathway which promotes the development of oocytes, the proliferation of GCs and cumulus cells, the inhibition of apoptosis and luteinization of GCs, then participates in regulating the production of steroid hormones in GCs, such as progesterone(Filatov et al., 2017; Liu et al., 2019; Roy et al., 2018). In this study, the results revealed that BMP15, TGFβRII and TGF-β1 significantly increased from 30 days to 90 days age and BMP15 was relatively high present in the GCs around the oocytes in 90-day-old prepubertal ovary of Rongchang pigs, indicated that the BMP15 protein acts on the surrounding GCs via a particular molecular channel.

It is known that TGF-β signaling molecule acts as a ligand to activate two different serine/threonine kinase receptors on cell membrane, thereby activating intracellular SMADs, regulating gene expression, and participating in the regulation of follicular development (Moore and Shimasaki, 2005; Shimizu et al., 2019). Studies have shown that the expression of BMP15 begins in primary follicular oocytes in sheep and human ovaries and primordial follicles in mice (Ghoreishi et al., 2019; Roy et al., 2018). *BMP15* is the primary gene that controls the number of ovulation and the traits of fecundity in cattle and sheep (Chen et al., 2017; Ghoreishi et al., 2019). Furthermore, the conditional knockout of *SMAD1*, *SMAD5*, and *SMAD8* in mice results in ovulation disorders and

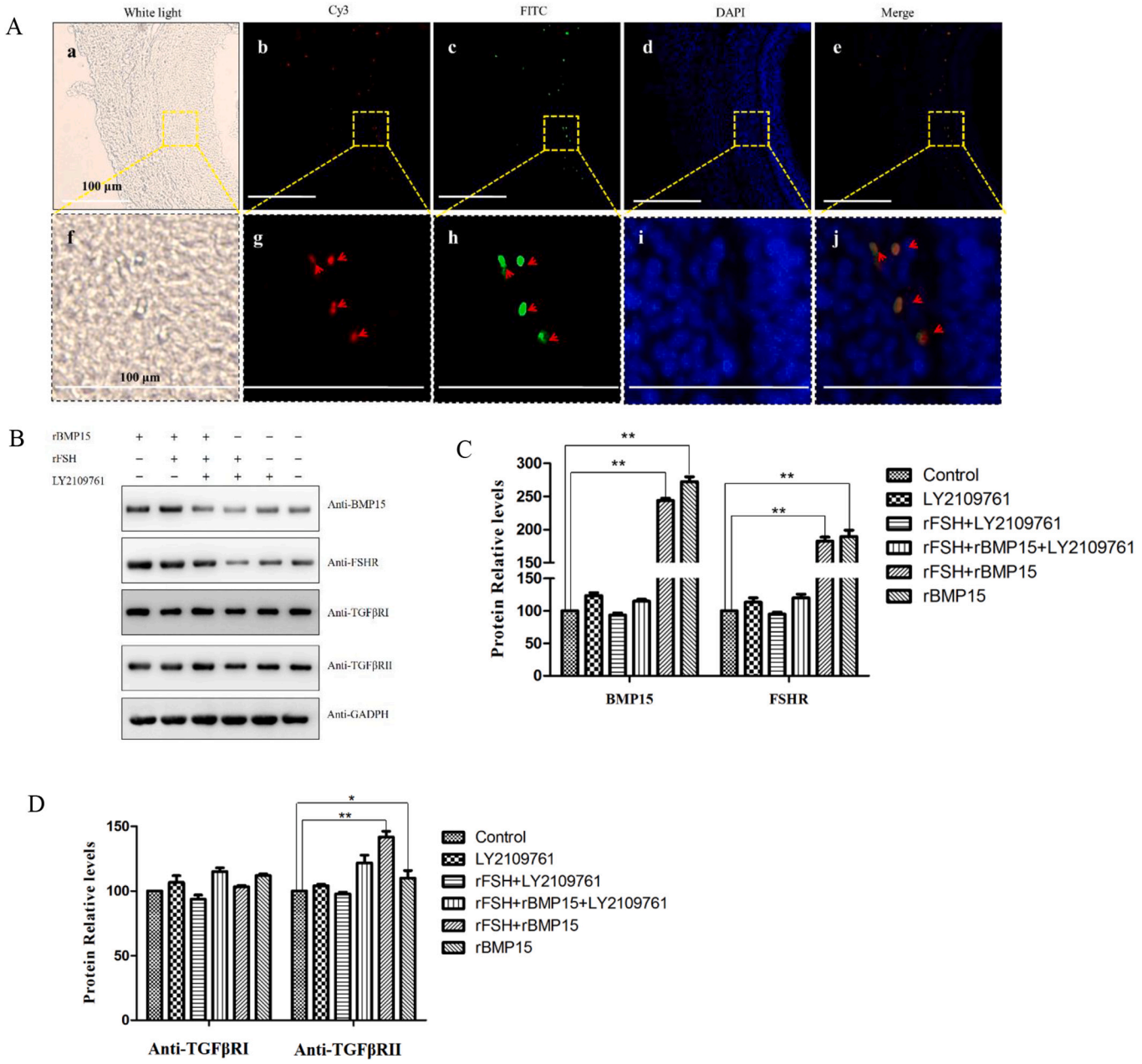

Fig. 4. The localization analysis of TGFβRII and FSHR protein distribution at follicle in prepubertal ovary of Rongchang pigs.
A: a. Follicular tissue of ovary; b. TGFβRII protein staining by cy3; c. BMP15 protein staining by FITC; d. DAPI; e. Merged image; f. Follicular tissue of ovary; g. TGFβRII protein staining by cy3; h. BMP15 protein staining by FITC; i. hoechst33258 staining; j. Merged image; The bar represents 100 μm. B, C, D. Western blot and gray analysis of BMP15, FSHR, TGFβRI and TGFβRII. **show very significant difference at $P < 0.01$, * show significant difference at $P < 0.05$.

infertility (Pangas et al., 2008). The present study reported that expressions of SMAD2, TGF-β2, and TGFβRI showed minor effects compared to BMP15, TGF-β1, TGFβRII, and SMAD4 which are consistent with BMP15/TGF-β/SMAD4 signaling pathway in Rongchang pigs. Obviously, the co-regulated SMAD4 in pigs may perform a more important biological function. However, the SMAD2/3 activates the downstream effector molecules in mice and rats and the SMAD1/5/8 activates downstream effector molecules in humans and sheep, indicate that the diversities of BMP15-activated SMAD signaling pathway in different species (Moore and Shimasaki, 2005; Yi et al., 2020; Zhai, 2013).

A previous study has shown that FSHR and its intracellular signaling pathways control the development of mammalian follicle and female infertility (Du et al., 2016b). In mice, BMP15 activates BMPR1B (ALK6) and BMPRII on the cell membrane through the TGF-β signaling molecule as a ligand to further activate the downstream SMAD pathway which regulates the expression of FSHR (Cui et al., 2017; Miyazawa et al., 2002; Shimizu et al., 2019). Furthermore, FSHR dramatically decline in porcine ovaries during follicular atresia and the knockout of *FSHR* induces apoptosis in porcine GCs (Tang et al., 2020). SMAD4 is a TGF-signaling molecule by binding to target promoters termed SMAD-binding elements (SBEs), thus promoting signal transmission (Du et al., 2018a; Du et al., 2016b). Using the cultured biopsy as a model for the ovary, this study only validates that BMP15 regulates FSHR through TGFβRII/SMAD4 signaling pathway in prepubertal ovary of Rongchang gilts. The addition of exogenous rBMP15 and rFSH experiment revealed that BMP15 mediated SMAD4 signaling via TGFβRII and regulated the expression of FSHR in GCs when TGFβRI was inhibited, thereby BMP15/

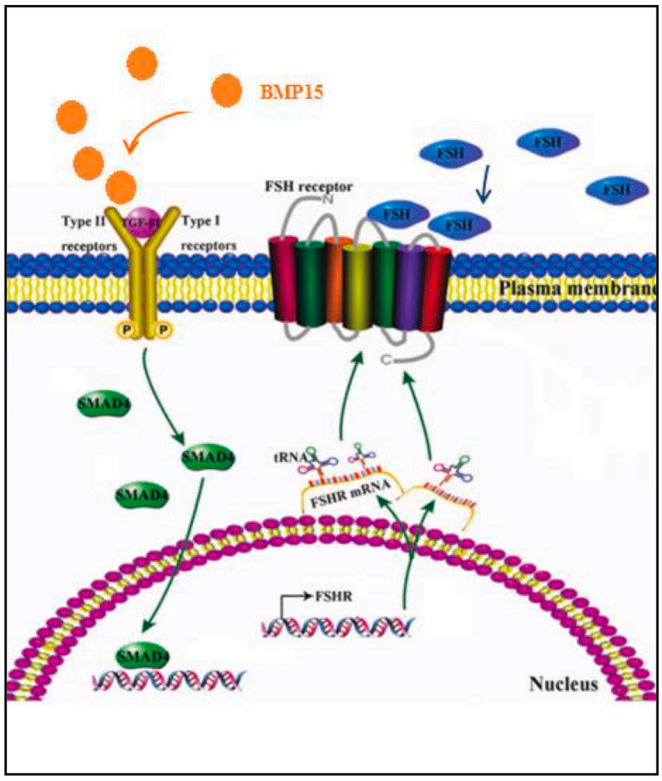

Fig. 5. Hypothetical signaling network of BMP15 and FSH in pig granulosa cells.

TGF-β/SMAD4 were playing an important regulatory role in follicle development of Rongchang pigs. However, the molecular mechanisms involved in the up or downregulation of FSHR during in prepubertal ovary of Rongchang pigs are still unclear.

In mammals, the development of follicles from primordial, primary, and secondary follicles to mature follicles is a continuous process of changes (Alam et al., 2018; Belli and Shimasaki, 2018; Du et al., 2018). The growth of follicles requires FSH and regulatory factors secreted from GCs, membranous cells, and oocytes (Roy et al., 2018; Shimizu et al., 2019; Yin et al., 2019). Moreover, the hypothalamic–pituitary–gonad axis and reproductive system are formed and gradually refined from birth to prepuberty in mammals (Zhao, 2017). During this period, gonadotrophins have little effect on follicle development (Pressing et al., 1992; Zhao, 2017). Before puberty, the development of oocytes mainly depends on the performance of multiple functions by cumulus cells, as well as the secretion of potent factors by oocytes, i.e., oocyte-secreted factors (OSFs) and EGF, to determine the differentiation and function of cumulus cells (Alam et al., 2018; Belli and Shimasaki, 2018). OSFs mainly include GDF9, BMP15, and some fibroblast growth factors (FGFs) (Alam et al., 2018; Belli and Shimasaki, 2018). Consequently, the present results revealed that the specific factor BMP15 secreted by the prepubertal oocytes of Rongchang pigs activates SMAD4 through the TGFβRII in GCs to promote FSHR expression (Fig. 5). In addition, FSH plays a role via FSHR (Fig. 5). The secretion and transportation mechanisms of BMP15 protein in the primordial follicles, primary follicles, secondary follicles, and mature follicles of Rongchang pigs, as well as the detailed mechanism of TGF-β/SMAD4-mediated signal transduction, require further study.

Declaration of Competing Interest

The authors declare that there are no conflict of interests that could have influenced the outcome of this study.

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