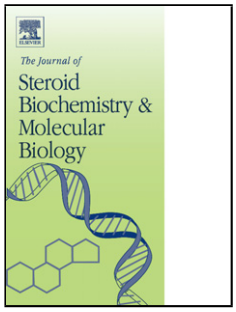

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PII: S0960-0760(17)30155-3
DOI: http://dx.doi.org/doi:10.1016/j.jsbmb.2017.06.004
Reference: SBMB 4957

To appear in: *Journal of Steroid Biochemistry & Molecular Biology*

Received date: 12-12-2016
Revised date: 5-6-2017
Accepted date: 6-6-2017

Please cite this article as: Naisheng Lu, Mengjiao Li, Hulong Lei, Xueyuan Jiang, Weilong Tu, Yang Lu, Dong Xia, Butyric acid regulates progesterone and estradiol secretion via cAMP signaling pathway in porcine granulosa cells, Journal of Steroid Biochemistry and Molecular Biologyhttp://dx.doi.org/10.1016/j.jsbmb.2017.06.004

Butyric acid regulates progesterone and estradiol secretion via cAMP signaling pathway in porcine granulosa cells

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Running title: BA effect on PGCs.

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Highlights

- Butyric acid alters the secretion of progesterone and estradiol in granulosa cells.
- Butyric acid effects the mRNA expression of GPR41/43 in porcine granulosa cells.
- Butyric acid adjusts the steroidogenic genes expression via cAMP signaling

pathway.

Abstract

Butyric acid (BA), one of the short chain fatty acids (SCFAs), has positive actions on the metabolism, inflammation, *etc.*. However, whether it influences the reproductive physiology and if so the detail mechanism involved has not yet been determined. In this study, the porcine granulosa cells (PGCs) were treated with gradient concentrations of BA. After 24 h culture, 0.05 mM BA significantly stimulated the progesterone (P_4) secretion ($P < 0.05$), 5 mM and 10 mM BA significantly inhibited the P_4 secretion ($P < 0.05$). Simultaneously, BA up-regulated the estradiol (E_2) secretion in a dose dependent manner, 5 mM and 10 mM BA significantly promoted the E_2 level ($P < 0.05$). In addition, 10 mM BA significantly promoted the G-protein-coupled receptor 41/43 mRNA ($P < 0.05$). Interestingly, 5 mM BA treatment significantly down-regulated cyclic adenosine monophosphate (cAMP) content ($P < 0.05$), steroidogenic acute regulatory (StAR), steroidogenic factor 1 (SF1), P450scc in the mRNA and/or protein level ($P < 0.05$), and these actions were reversed by cAMP activator forskolin (FK). Moreover, the co-treatment of 5 mM BA and bupivacaine (BPC, the cAMP inhibitor) significantly accumulated the inhibition action of BPC on cAMP, the secretion of P_4, and the abundance of StAR mRNA ($P < 0.05$), inhibited the up-regulation of 5 mM BA on the E_2 secretion ($P < 0.05$). Further, the Global Proteome and KEGG pathway analysis found that 5 mM BA significantly up-regulated the I3LM80 proteins ($P < 0.05$), which is involved in the steroid biosynthesis signaling pathway. 5 mM BA

significantly decreased the F2Z5G3 protein level ($P < 0.05$), and the cAMP signaling pathway. In conclusion, present findings for the first time demonstrated that BA could regulate the P_4 and E_2 hormone synthesis in PGCs via the cAMP signaling pathway.

Keywords: Butyric acid; Porcine granulosa cells; Progesterone; Estradiol; cAMP signaling pathway

1. Introduction

Dietary fiber (DF) is known as having beneficial effects on the reproductive performance of pigs, it improves the oocyte maturity, embryo survival and fetal development in gilts [1], and increases litter size and even their offspring birthweight in sows [2-4]. However in mono-gastric animal, such as human and pigs, DF could not be digested by endogenous enzymes in the gastrointestinal tract, but available as the substrate for bacterial fermentation [5, 6]. High intake of fibrous diet results high production of short chain fatty acids (SCFAs), including acetic, propionic, and butyric acid (BA) in hint gut, which are the major class of bacterial metabolites [7, 8]. Researches have proved that BA have positive effects on regulating metabolism homeostasis, immunity of human and animals. But till now, there are no details about effects of BA on animal's reproductive physiology. Whether BA exerts the beneficial effect of DF on pig fertility remains unknown.

It was reported that, BA could inhibit histone deacetylation, influence cell proliferation, apoptosis [9], induce neuropeptide Y (NPY) release and promote the functional homeostasis of colonic mucosa and the enteric immune system [10, 11]

through the G-protein-coupled receptor 41 (GPR41) mediation. BA works as an energy source rescue colonocytes from both the deficit in mitochondrial respiration and from autophagy [12]. And, Yoo *et al.* (2015) indicated that BA treatment ameliorates cell proliferation and differentiation in the mouse dentate gyrus and colon T-regulatory cells [13]. Moreover, Sun *et al.* (2015) demonstrated that BA takes the effects of anti-oxidant and anti-apoptotic in hepatic ischemia/reperfusion injured mice through brain-derived neurotrophic factor (BDNF)-PI3K/Akt pathway [14]. But in neuroblastoma cells [15] and macrophages [16], BA can activate the cyclic adenosine monophosphate (cAMP) signaling. All above indicate that, the effects of BA on animal are conducted via multiple signaling mechanisms and may differ in different organs, tissues and cells, and display different functions.

It is known that the steroid hormones progesterone (P_4) and 17β-estradiol (E_2) are essential for proper female sexual characters and normal reproductive function. In the ovary, steroid production is regulated by the action of gonadotropins and the activation of cAMP-dependent protein kinase and other protein kinases. The cAMP signaling is involved in most aspects of differentiation and maturation of the granulosa cells in the ovarian follicle, as well as the expression level and activity of steroidogenic enzymes of the granulosa cell. Therefore, for the primary understanding whether BA exert the beneficial effect of DF on the fertility of female pigs, we tested the effects of BA on the expression of steroidogenic genes, and the release of P_4 and E_2 by cultured porcine granulosa cells (PGCs).

2. Material and Methods

All experiments were conducted in accordance with 'the instructive notions with respect to caring for laboratory animals' issued by the Ministry of Science and Technology of the People's Republic of China.

2.1. Porcine ovaries and primary culture of granulosa cells

Porcine ovaries at the early and mid-follicular phase of the estrous cycle were obtained from healthy gilts without visible reproductive abnormalities at the local slaughterhouse of Shanghai. Ovaries were kept in 37 °C saline solution with 1% antibiotic solution (100,000 units/L of penicillin sodium and 100 mg/L of streptomycin sulfate), and then washed in D-Hanks solution with three times. Follicles (3~6 mm diameter) were dissected from ovaries, aspirated, and hemisected. The granulosa cell layer was removed by a plastic inoculation loop as previously described [17]. The disposable sterile syringes were used to draw the granulosa cells along the sidewall of follicles, after then the cells were centrifuged at 1500 rpm for 5 min in 37 °C D-Hanks with centrifuge tubes. Next, the cells were seeded in 24-well cell plates (Corning, USA) at a density of 1.0×10^6 cells/well [18, 19] with DMEM/F12 medium (GIBCO, Grand Island, NY, USA) that was supplemented with 10% FBS (GIBCO, Grand Island, NY, USA), 100,000 units/L of penicillin sodium and 100 mg/L of streptomycin sulfate (GIBCO, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h.

In both series of experiments, cells from one ovary isolation were mixed and used as one replication. Each culture we took at least 6 animals. And for confirmation the

result, we repeated three times culture, thus for each experiment, we took at least 18 animals.

2.2. Granulosa cells treatment

To elucidate whether BA has toxic effect on PGCs, we did cell apoptosis assay. PGCs were incubated in 24-well cell plates (1.0×10^6 cells/well for 1 mL). Cells were then treated with BA at different concentrations (0 mM, 5 mM and 10 mM) for 24 h, and then both adherent and floating cells were harvested by trypsinisation, washed once with PBS. Resuspended cells were incubated with 100 μL of PBS containing 1% BSA and 100 μL of the Muse™ Annexin-V & Dead Cell reagent (Muse apoptosis kit, No. MCH100105) at room temperature for 20 min in the dark. Subsequently, apoptosis was measured immediately using the Muse™ Cell Analyzer (Merck-Millipore, Germany). And the result showed that 5 mM and 10 mM BA did not influence the live, early apoptotic, late apoptotic, dead and total apoptotic of PGCs (Fig. 1 A, B and C). Thus we took 5 mM and 10 mM as non-toxic doses in following trial.

BA (A stock solution of 1 M in DMEM/F12 medium). After reaching confluence, approximately 70%, the granulosa cells were treated with a series doses of BA (0, 0.05, 0.5, 5 or 10 mM) in DMEM/F12+1% Insulin-Transferrin-Selenium (ITS) for 24 h in 24-well cell plates. Then, the cell supernatant was collected into centrifuge tubes for detecting P_4 and E_2, and the cells were used to extract the total RNA.

Activator and inhibitor. To test and verify the mechanisms of BA on hormone secretion, the cAMP activator forskolin (FK) 10 μM (Sigma, USA) and its inhibitor

bupivacaine (BPC) 2.3 μM (Selleck, USA) were used to detect its effects on P_4 and E_2 secretion, cAMP content and related genes expression after treatment 24 h. Here, the selected BA concentration is 5 mM.

2.3. Hormone assay

P_4, and E_2 content measurements were performed on medium samples. For P_4 and E_2 secretion assays, the EIA kits (Cayman, Germany: P_4, Item No. 582601, Intra-assay CV < 7.3%, Inter-assay CV < 16.4%; E_2, Item No. 582251, Intra-assay CV < 12.3%, Inter-assay CV < 5.5%) were used to detect the cell supernatant (24 h) with ELISA method.

2.4. cAMP assay

After 24 h stimulation, the cells were washed twice with D-Hanks, and lysed using 100 μL 0.1 M HCl buffer containing 1 mM PMSF. The content of cAMP in the cell lysate was detected using EIA Kit (Cayman, Germany, Item No. 581001, Intra-assay CV < 11.5%, Inter-assay CV < 8.1%) according to the manufacturer's protocol with ELISA method. Total protein (TP) of the cell lysate was detected using a commercial kit (Thermo, USA) based on bicinchoninic acid (BCA) method [20]. The cAMP content of each well of cells was then normalized by the TP. Each sample included at least 3 replicates for experiment.

2.5. RNA extraction and Real-time quantitative PCR

Total RNAs were extracted from cells after treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After

treated with DNase I (Takara Bio Inc, Shiga, Japan), 2 μg of total RNA was reverse transcribed to cDNA in a final 20 μL by the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and random primers oligo-dT18 according to the manufacturer's instructions. Beta-actin (β-actin) was used as candidate housekeeping genes. SYBR Green Real-time PCR Master Mix reagents (Toyobo Co., Ltd., Osaka, Japan) and both sense and antisense primers (200 nM for each gene) were used for real-time quantitative PCR. PCR reactions were performed in ABI7500 instrument (Applied Biosystems, Foster, CA, USA). Each sample included at least 3 replicates for experiment. The selected primer pairs of target genes were listed in the Table 1.

2.6. The Western Blot assay

After reaching confluence, PGCs were treated with presence or absence 5 mM BA in 24-well cell plates. After 24 h culture, cells were lysed in RIPA lysis buffer containing 1 mM PMSF. The total protein concentration was determined using BCA protein assays. After separation on 10% SDS-PAGE gels, the proteins were transferred to PVDF membranes, and then blocked with 5% (w/v) non-fat dry milk (B&D, USA) in TBS containing tween 20 (TBST) for 2 h at room temperature. Subsequently, the PVDF membranes were incubated with the indicated antibodies, including rabbit anti-β-actin (1:1000, Bioss, bs-0061R), rabbit anti-Steroidogenic acute regulatory (StAR) (1:500, sc-25806), rabbit anti-Steroidogenic factor 1 (SF1) (1:500, sc-28740), goat anti-Cholesterol side-chain cleavage enzyme (P450scc) (1:500, sc-18040) and goat anti-3β-Hydroxysteroid dehydrogenase (3β-HSD) (1:500, sc-30820), all from Santa Cruz Biotechnology, Santa Cruz, CA. Primary antibodies incubation were performed at 4 °C

overnight and followed by incubations with the appropriate secondary antibody (1:2000, CWBIO) for 1 h at room temperature. Protein expression was measured with Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology, China) and normalized to β-actin expression.

2.7. The proteomic and pathway assay

Experiments for proteomic analysis were performed by PTM Biolab, Inc. (Hangzhou, China). Briefly, after 24 h culture in 24-well cell plates, the protein sample was extracted and digested with trypsin, then labeled with TMT reagent kit with incubation for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation. The sample was then fractionated into fractions by high pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Next, the sample was then analyzed by LC-MS/MS, which the resulting MS/MS data were processed using Mascot search engine (v.2.3.0), and tandem mass spectra were searched against Uniprot Sus scrofa database (34,117 sequences). FDR was adjusted to < 1% and peptide ion score was set > 20. In this project, proteins expression of quantitative ratio over 1.3 was considered as up-regulation while quantitative ratio less than 1/1.3 (0.77) was considered as down-regulation (T test, $P < 0.05$).

Further, the bioinformatics methods, including Gene Ontology (GO)/protein annotation (Http://www.ebi.ac.uk/GOA/), KEGG pathway annotation, subcellular localization, were used to analysis and discover the target proteins/pathways.

2.8. Statistical analysis

All the data are expressed as means ± standard error of the mean (SEM). Significant differences between the control and the treatment were determined by Independent-Samples T test. One-way analysis of variance (ANOVA) was used to test the significant differences in cAMP signal pathway (SPSS 18.0, Chicago, IL). Differences were considered significant when $P < 0.05$.

3. Results

3.1. BA alters steroid hormone synthesis by influence the expression of steroidogenic genes

P_4 and E_2 are important roles in the regulation of the estrous and menstrual female reproductive cycles. Here, PGCs were treated with 0 mM, 0.05 mM, 0.5 mM, 5 mM and 10 mM BA respectively for 24 h, the result showed that 5 mM and 10 mM BA significantly down-regulated the PGCs P_4 secretion ($P = 0.000$), but 0.05 mM BA up-regulated P_4 secretion by 25.2% ($P = 0.002$) (Fig. 2A). Interestingly, BA increased the E_2 secretion of the PGCs in a dose-dependent manner, 5 mM and 10 mM BA significantly increased the level of E_2 by 1.17 fold ($P = 0.002$) and 1.28 fold ($P = 0.000$) respectively (Fig. 2B), however low doses of BA (0.05-0.5 mM) did not achieve obvious stimulation. Based on the above results, 5 mM BA is the minimum effective dose for both P_4 and E_2 secretion, hereby 5 mM BA was taken as treatment dose for further understanding the mechanism of the regulation of BA on PGCs steroid secretion.

The synthesis of steroid hormones, including P_4 and E_2, are conducted by steroidogenic genes, includes StAR, SF1, P450scc and 3β-HSD. Here, the expression profile of StAR, SF1, P450scc and 3β-HSD mRNA (Fig. 4B) indicated that, 5 mM BA significantly inhibited the mRNA expression of StAR (P = 0.000), SF1 (P = 0.019) and P450scc (P = 0.000) respectively, but significantly promoted 3β-HSD mRNA expression (P = 0.000, Fig. 4B). Accordingly, the protein expression profile of steroidogenic genes exhibited the similar pattern, 5 mM BA significantly decreased the SF1 and P450scc protein level (P = 0.015, P = 0.016, Fig. 4C), however, no significant changes of StAR, and 3β-HSD were found by the treatment of 5 mM BA (P > 0.05).

3.2. BA alters steroidogenesis via cAMP signaling pathway

To investigate whether the effect of BA on PGCs mediated via GPRs, we also detected the GPR41 and GPR43 mRNA expression in response to different doses of BA. In the figure 3A, we observed that 10 mM BA significantly elevated GPR41 mRNA (P = 0.000), but the 0.05 mM, 0.5 mM and 5 mM BA did not achieve significant effect. It is interesting that the GPR43, another member of GPRs family, was also strongly enhanced by 10 mM BA (P = 0.000), moreover, there have a dose-dependent effects with the BA of 0.5 mM, 5 mM and 10 mM (Fig. 3B).

As shown in figure 5A, 5 mM BA significantly decreased the cAMP content (P = 0.000), which is the second messenger for steroidogenesis. FK works as the activator of cAMP, 10 μM FK significantly elevated the cAMP protein level (P = 0.000), and the mRNA expression level of StAR (P = 0.000), SF1 (P = 0.000), P450scc (P = 0.000),

and 3β-HSD (P = 0.000) (Fig. 5D, E, F and G), stimulated the secretion of P_4 and E_2 (P = 0.000, 0.009) (Figure 5B and C). Importantly, 5 mM BA co-treatment with 10 μM FK significantly inhibited the of promotion action of FK on the secretion of P_4 and E_2 (P = 0.000), as well as the mRNA expression of cAMP down-stream genes, including StAR (P = 0.000), SF1 (P = 0.000) and P450scc (P = 0.000) (Fig. 5D, E and F), but did not reverse the effects on the 3β-HSD mRNA level and the cAMP content.

In contrast to FK, BPC works as the inhibitor of cAMP, 2.3 μM BPC significantly down-regulated the cAMP level (P = 0.000, Figure 5A) and the mRNA level of StAR and P450scc, inhibited the secretion of P_4 (P = 0.000) (Fig. 5D, E and B), but did not influence the E_2 secretion and the 3β-HSD mRNA level (Fig. 5C and G). The co-treatment of 5 mM BA and 2.3 μM BPC significantly accumulated the inhibition action of BPC on cAMP (P = 0.000, Fig. 5A) and the secretion of P_4 (P = 0.000, Fig. 5B), accumulated the action of BPC on the abundance of StAR mRNA level, inhibited the up-regulation of 5 mM BA on the E_2 secretion (P = 0.002). Whatever, compare to BA, the co-treatment of 5 mM BA and 2.3 μM BPC did not have effects on the StAR, P450scc and 3β-HSD mRNA expression level.

3.3. The proteome and KEGG pathway analysis

In order to explore the potential mechanisms for BA's effects on steroid hormone secretion of PGCs, we used Global Proteome and KEGG pathway annotation in Sus scrofa to analyze metabolic pathways and gene trans-activation networks that are altered by 5 mM BA treatment. As shown in Table 2, 5 mM BA significantly up-

regulated the Steroid biosynthesis signaling pathway (P = 0.002), which is closely related to the P_4 and E_2 synthesis. In addition, 5 mM BA also up-regulated ribosome and amoebiasis signaling pathway (P = 0.022).

In contrast, some metabolic signaling pathways activity were down-regulated by the 5 mM BA, which including the protein digestion absorption (P < 0.001), ECM-receptor interaction (P < 0.001) and PI3K-Akt (P < 0.001), cAMP signaling, calcium signaling, *etc.* (Table 1).

Using TMT labeling and HPLC fractionation followed by high-resolution LC-MS/MS analysis, quantitative global proteome analysis was performed. We identified a total of 21 up- and 43 down-regulated proteins (Table 3). Afterwards, according to GO annotation information of identified proteins, we observed that the I3L7K2 and I3LM80 proteins, which were up-regulated by 5 mM BA treatment, belongs to the reproductive process of GO terms level 2. Further, we observed that 5 mM BA significantly decreased the protein level of F2Z5G3, which is the one signal molecular of the cAMP signaling pathway, calcium signaling pathway, and membrane of GO terms level 2 (Table 4). These pathways play an important role in the regulation sex hormone secretion, such as P_4, E_2 and testosterone.

4. Discussion

This study for the first time observed that BA could directly regulate the P_4 and E_2 secretion in PGCs. In PGCs, StAR, the rate-limiting factor in hormone-dependent steroidogenesis, transports cholesterol from the outer to the inner mitochondrial

membrane, and makes it available for P450scc utilization [21, 22]. StAR knockout mice lost the ability of steroid synthesis [23]. Mitochondrial enzyme P450scc can catalyze the synthesis of cholesterol to pregnenolone. And the conversion of pregnenolone to P_4 is catalysed by microsomal enzyme 3β-HSD. SF1 is the transcription factor that controls the expression of the steroidogenic genes. Impairment of SF1 expression in granulosa cells resulted in multiple defects, such as abnormal estrous cycle, infertility, and reduction of steroidogenesis in female mice [24]. Researches indicated that, the suppression of P_4 production in granulosa cells was accompanied by the down-regulation of the expression StAR, P450scc and 3β-HSD [25-27]. In consistent with above researches, we observed that 5 mM BA decreased the gene expression of SF1 mRNA and protein, down-regulated StAR and P450scc mRNA expression.

However, the effects of 5 mM BA on the mRNA level of 3β-HSD and E_2 were not consistent with the inhibitory action of BA on StAR and P_4. Sirotkin *et al.* (1995) observed that estrogen can down-regulated the level of cAMP and P_4, but P_4 obviously stimulated the cAMP production in human granulosa cell culture [28]. It indicated a feedback regulation mechanism of steroidogenesis within the granulosa cells. Low dose of BA (0.05 mM) up-regulated P_4 secretion greatly and slightly stimulated E_2 secretion, whereas high dose of BA (5 mM and 10 mM) obviously evaluated the E_2 secretion but significantly decreased the P_4 level. Thus the inhibitory effects of 5 mM BA on the level of cAMP, and the expression of StAR and SF1, and the secretion of P_4 may due to the feedback regulation of high level of E_2. In addition, the protein levels of D0G6X3 (Farnesyl-diphosphate farnesyltransferase 1) and A7L861 (Squalene epoxidase) were

up-regulated by 5 mM BA in the proteomic data (Table 3), and both of them have roles in promoting the cholesterol synthesis [29]. Sun *et al.* (2011) reported that, the expression of 3β-HSD mRNA in PGCs was increased with the size of follicular, larger follicles PGCs expressed higher than small and medium follicles [30]. And, larger follicles PGCs secret high level of E_2, the different size follicles have different steroid hormone level, and the ratio of E_2 to P_4 in follicular fluid is associated with the 3β-HSD expression [30]. In present study, the ration of E_2 to P_4 in the PGCs culture medium had the similar variation pattern as the 3β-HSD mRNA when the PGCs were challenged with BA, FK, BPC, BA + FK or BA + FK. Thus, the synthesis of steroid hormones in PGCs are depend on the activity of the steroidogenesis genes, whereas the steroid hormone level in PGCs culture medium or follicular fluid may influence the expression of steroidogenesis genes. Further, the growing granulosa cell also secretes androgen, the another precursor of E_2 [31, 32], the 3β-HSD response for the conversion of androstenediol to testosterone [33], thus the increased 3β-HSD in present trial may cause more testosterone secretion. CYP19A1 is the rate-limiting gene for regulating the conversion of androgen to E_2 [34]. Whether and how BA effect on the androgen synthesis and the expression of CYP19A1, what roles of them involved in the feedback loop needs to be elucidated in the future.

There is no data indicate the BA concentration in the ovary and follicular fluid of pigs, whereas the plasma BA concentration was about 15~30 μM in sows and varied with the amount of DF they ingested [35]. Usually, the biochemical composition of the follicular fluid are well correlated with the serum composition, including Na^+, K^+, Cl^-,

glucose, lactate, β-hydroxybutyrate, urea, total protein, triglycerides, non-esterified fatty acids (NEFA) and total cholesterol [36, 37]. In present *in vitro* trial of PGCs, we screened out the effective dose of BA on the secretion of P_4 was more than 0.05 mM, and the effective dose for E_2 secretion was more than 5 mM. Wang *et al.* (2012) found that 10 μM BA was the minimum effective dose for the protein kinase A (PKA) activity in Caco-2 cells [38]. Li *et al.* (2014) observed that 1 mM BA stimulated the mRNA of peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (CEBPα) in porcine stromal vascular fraction (SVF) cells [39]. These indicate that the *in vitro* dose of BA may not fully reflect the physiological level and function *in vivo*. *In vivo*, the sex steroid production begins with follicle-stimulating hormone (FSH) from the anterior pituitary, FSH stimulate the granulosa cells to convert androgens to E_2 by aromatase during the follicular phase of the menstrual cycle. Further the BA is not the sole SCFA in the follicular fluid, others such as acetate acid and propionic acid also may attend the regulation of steroid hormones synthesis. For instance, the acetate acid concentrations were 0.17~0.31 mM, 0.34~1.5 mM, 0.19~0.38 mM in ovarian follicular fluids of sheep, pig and cow, respectively [40]. For understanding the importance of the physiological level of BA in follicular, further trial may combine the FSH and other SCFAs.

As we know, GPR41 and GPR43 are the specific receptors of SCFAs. Here, we found that GPR41/43 mRNA were expressed in PGCs, and 10 mM BA significantly improved the GPR41/43 mRNA expressed, influenced the secretion of P_4 and E_2. Previous researches reported that propionic acid could significantly promote the leptin

secretion when Ob-Luc cells with over-expression GPR41, but the stimulation was greatly abolished while with short interfering RNA (siRNA) GPR41 [41]. Butyrate stimulated L cells and C57BL/6 mouse to release peptide YY (PYY), but the PYY secretion was significantly decreased after knockout of GPR41 ($GPR41^{-/-}$) or GPR43 ($GPR43^{-/-}$) [42, 43]. These results demonstrated that SCFAs via the GPR41/43 mediation to play regulatory roles. However, Wang *et al.* (2009, 2012) observed that SCFA could stimulate luciferase gene expression from a cAMP response element (CRE)-containing plasmid in CHO cells independent of their receptors GPR41 and GPR43, so as in Caco-2 cells [38, 44]. Li *et al.* (2014) observed that SCFAs promoted the primary porcine preadipocytes (Stromal vascular fraction of subcutaneous fat, SVF) differentiation was not mediated through the GPR41/43, because they did not observe GPR41 and GPR43 mRNA in SVF and adipocyte [39]. This discrepant indicate the role of GPR 41/43 may differ in different cell type.

Typically, when one of GPRs binds to its particular ligand, the receptor either stimulates or inhibits the production of cAMP, and amplifies the signals. In MA-10 mouse Leydig tumor cells, cAMP coordinately stimulated the expression of StAR protein and mRNA, which were consistent with high level of P_4 production [45]. BPC is the inhibitor of the cAMP, it could significantly inhibit the cAMP production, and its downstream genes including P450 gene expression [46]. And here in PGCs, 5 mM BA had the similar action as the BPC, it greatly inhibited cAMP production, and accompanied with the decreasing of its downstream genes expressions including SF1, StAR and P450scc, and the secretion of P_4. And such inhibitory actions were reversed

by the cAMP activator FK. The co-treatment of BA and FK significantly increased the cAMP production, elevated the expression of SF1, StAR, P450scc, and P_4 level. Daems C *et al.* (2015) observed that the FK promoted the P_4 secretion and StAR mRNA level in Leydig cells [47]. These results demonstrated that the BA regulates the steroidogenesis in PGCs via cAMP signaling pathway.

In order to further explore the mechanism about BA affecting the sexual hormone secretion of PGCs, the PGCs Global Proteome and KEGG pathway were profiled. BA treatment resulted 21 up- and 43 down-regulation proteins. In the down-regulated proteins, F2Z5G3 (Calmodulin, CALM) is the one signal molecular of the cAMP signaling pathway and calcium signaling pathway. F2Z5G3 down-regulation may lead to the inhibition StAR expression and P_4 secretion [48, 49]. This further confirmed that BA regulated the PGCs hormone secretion via cAMP signaling pathway.

Except F2Z5G3, BA treatment also decreased the protein level of Q29307 and APOA1 in PGCs. Q29307 works as an ATPase inhibitor [50], down-regulation of Q29307 may result more ATP production, and subsequently increase the ratio of ATP: AMP, inhibit the activity of AMP-activated protein kinase (AMPK), and affect fatty acid metabolism [51, 52]. APOA1 is the major protein component of high density lipoprotein (HDL) in plasma. It can promote the fat efflux, including cholesterol, the substance for steroid hormone synthesis. Contrary to the inhibitory action on the Q29307 and APOA1, BA treatment up-regulated 6 lipids biosynthesis proteins (DOG6X3, A7L861, I3LM80, I3L7K2, A5YV76 and F8SM59) in PGCs. This suggested that the BA on the PGCs may involve in energy and lipid metabolism.

In addition, BA treatment up-regulated ribosome and amoebiasis signaling pathway, down-regulated some metabolic signaling pathways activity, which including the protein digestion absorption, ECM-receptor interaction and PI3K-Akt. Further, according to GO annotation information of identified proteins, we observed that the I3L7K2 and I3LM80 proteins, which were up-regulated by 5 mM BA treatment, belongs to the reproductive process of GO terms level 2. These results are consistent with previous researches, that SCFAs may serve as the energy source to attend metabolism [53], it also may work as signaling substance to regulate hormone secretion, anti-inflammatory reaction and apoptosis [11]. This implicated that, the effects of BA on PGCs contain multi-signaling pathway and functions, but the individual mechanism and inter or cross talking actions remain to be elucidated.

In summary, the present study for the first time demonstrated that BA regulated the P_4 and E_2 synthesis in PGCs via the cAMP signaling pathway, which may be mediated through GPR41/43. These findings raised a possibility that SCFAs, main microbial metabolites of DF fermentation, may regulate the animal reproduction through the cAMP signaling pathway, however, the details mechanism requires further study.

Conflicts of interest

The authors declare to have no conflict of interest.

Acknowledgments

Authors thank the Dr. Defu Zhang for helpful cells isolation. This work was supported by Shanghai Key Project for Agro Scientific Research 2014 (7-2-6), National

Key Research and Development Program of China (2016YFD0500502), and Shanghai Youth Talent Project for Agro System 2016 (1-33).**Reference**

[1] E.M. Ferguson, J. Slevin, M.G. Hunter, S.A. Edwards, C.J. Ashworth, Beneficial effects of a high fibre diet on oocyte maturity and embryo survival in gilts, Reproduction, 133 (2) (2007) 433-439.

[2] A.C. Weaver, J.M. Kelly, K.L. Kind, K.L. Gatford, D.J. Kennaway, P.J. Herde, W.H. van Wettere, Oocyte maturation and embryo survival in nulliparous female pigs (gilts) is improved by feeding a lupin-based high-fibre diet, Reprod. Fertil. Dev., 25 (8) (2013) 1216-1223.

[3] E.M. Ferguson, J. Slevin, S.A. Edwards, M.G. Hunter, C.J. Ashworth, Effect of alterations in the quantity and composition of the pre-mating diet on embryo survival and foetal growth in the pig, Anim. Reprod. Sci., 96 (1-2) (2006) 89-103.

[4] T.L. Veum, J.D. Crenshaw, T.D. Crenshaw, G.L. Cromwell, R.A. Easter, R.C. Ewan, J.L. Nelssen, E.R. Miller, J.E. Pettigrew, M.R. Ellersieck, N. North Central Region-42 Committee On Swine, The addition of ground wheat straw as a fiber source in the gestation diet of sows and the effect on sow and litter performance for three successive parities, J. Anim. Sci., 87 (3) (2009) 1003-1012.

[5] R. Jha, J.D. Berrocoso, Review: Dietary fiber utilization and its effects on physiological functions and gut health of swine, Animal, 9 (9) (2015) 1441-1452.

[6] J.H. Cummings, A.M. Stephen, Carbohydrate terminology and classification, Eur. J. Clin. Nutr., 61 Suppl 1 (2007) S5-18.

[7] A. Koh, F. De Vadder, P. Kovatcheva-Datchary, F. Backhed, From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites, Cell, 165 (6) (2016) 1332-1345.

[8] J.M. Wong, R. de Souza, C.W. Kendall, A. Emam, D.J. Jenkins, Colonic health: fermentation and short chain fatty acids, J. Clin. Gastroenterol., 40 (3) (2006) 235-243.

[9] J. Wu, Z. Zhou, Y. Hu, S. Dong, Butyrate-induced GPR41 activation inhibits histone acetylation and cell growth, J. Genet. Genomics, 39 (8) (2012) 375-384.

[10] L. Klampfer, J. Huang, T. Sasazuki, S. Shirasawa, L. Augenlicht, Oncogenic Ras promotes butyrate-induced apoptosis through inhibition of gelsolin expression, J. Biol. Chem., 279 (35) (2004) 36680-36688.

[11] A. Farzi, F. Reichmann, P. Holzer, The homeostatic role of neuropeptide Y in immune function and its impact on mood and behaviour, Acta Physiol. (Oxf), 213 (3) (2015) 603-627.

[12] G. den Besten, K. van Eunen, A.K. Groen, K. Venema, D.J. Reijngoud, B.M. Bakker, The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism, J. Lipid Res., 54 (9) (2013) 2325-2340.

[13] D.Y. Yoo, D.W. Kim, M.J. Kim, J.H. Choi, H.Y. Jung, S.M. Nam, J.W. Kim, Y.S. Yoon, S.Y. Choi, I.K. Hwang, Sodium butyrate, a histone deacetylase Inhibitor, ameliorates SIRT2-induced memory impairment, reduction of cell proliferation, and neuroblast differentiation in the dentate gyrus, Neurol. Res., 37 (1) (2015) 69-76.

[14] J. Sun, F. Wang, H. Li, H. Zhang, J. Jin, W. Chen, M. Pang, J. Yu, Y. He, J. Liu, C. Liu, Neuroprotective Effect of Sodium Butyrate against Cerebral Ischemia/Reperfusion Injury in Mice, Biomed Res. Int., 2015 (2015) 395895.

[15] B. Yusta, J. Ortiz-Caro, A. Pascual, A. Aranda, Comparison of the effects of forskolin and dibutyryl cyclic AMP in neuroblastoma cells: evidence that some of the actions of dibutyryl cyclic AMP are mediated by butyrate, J. Neurochem., 51 (6) (1988) 1808-1818.

[16] L.T. Sunkara, X. Zeng, A.R. Curtis, G. Zhang, Cyclic AMP synergizes with butyrate in promoting beta-defensin 9 expression in chickens, Mol. Immunol., 57 (2) (2014) 171-180.

[17] C. Glister, D.S. Tannetta, N.P. Groome, P.G. Knight, Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells, Biol. Reprod., 65 (4) (2001) 1020-1028.

[18] A.V. Sirotkin, M. Meszarosova, Comparison of effects of leptin and ghrelin on porcine ovarian granulosa cells, Domest. Anim. Endocrinol., 39 (1) (2010) 1-9.

[19] A. Kolesarova, M. Capcarova, A.V. Sirotkin, M. Medvedova, J. Kovacik, In vitro assessment of silver effect on porcine ovarian granulosa cells, J. Trace Elem. Med. Biol., 25 (3) (2011) 166-170.

[20] R.C. Goldschmidt, H.K. Kimelberg, Protein analysis of mammalian cells in monolayer culture using the bicinchoninic assay, Anal. Biochem., 177 (1) (1989) 41-45.

[21] B.J. Clark, J. Wells, S.R. King, D.M. Stocco, The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-

10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR), J. Biol. Chem., 269 (45) (1994) 28314-28322.

[22] B.J. Clark, D.M. Stocco, Expression of the steroidogenic acute regulatory (StAR) protein: a novel LH-induced mitochondrial protein required for the acute regulation of steroidogenesis in mouse Leydig tumor cells, Endocr. Res., 21 (1-2) (1995) 243-257.

[23] K.M. Caron, S.C. Soo, W.C. Wetsel, D.M. Stocco, B.J. Clark, K.L. Parker, Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia, Proc. Natl. Acad. Sci. USA, 94 (21) (1997) 11540-11545.

[24] C. Pelusi, Y. Ikeda, M. Zubair, K.L. Parker, Impaired follicle development and infertility in female mice lacking steroidogenic factor 1 in ovarian granulosa cells, Biol. Reprod., 79 (6) (2008) 1074-1083.

[25] A. Pierre, C. Pisselet, J. Dupont, B. Mandon-Pepin, D. Monniaux, P. Monget, S. Fabre, Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells, J. Mol. Endocrinol., 33 (3) (2004) 805-817.

[26] H.M. Chang, J.C. Cheng, H.F. Huang, F.T. Shi, P.C. Leung, Activin A, B and AB decrease progesterone production by down-regulating StAR in human granulosa cells, Mol. Cell. Endocrinol., 412 (2015) 290-301.

[27] Fadhillah, S. Yoshioka, R. Nishimura, K. Okuda, Hypoxia promotes progesterone synthesis during luteinization in bovine granulosa cells, J. Reprod. Dev., 60 (3) (2014) 194-201.

[28] A.V. Sirotkin, J. Mlyncek, J. Lavrincik, J. Bulla, L. Hetenyi, The ability of steroid hormones to control cAMP and cGMP production by human granulosa cells in culture, Cell. Signal., 7 (1) (1995) 61-65.

[29] L.H. Reddy, P. Couvreur, Squalene: A natural triterpene for use in disease management and therapy, Adv. Drug Deliv. Rev., 61 (15) (2009) 1412-1426.

[30] J.Z. Yanling Sun, Zhiguang Ping, Lina Fan, Chunqiang Wang, Wanhong Li, Chen Lu, Lianwen Zheng and Xu Zhou, Expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in normal and cystic follicles in sows, Afr. J. Biotechnol., 10 (32) (2011) 6184-6189.

[31] V.G. Garzo, J.H. Dorrington, Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin, Am. J. Obstet. Gynecol., 148 (5) (1984) 657-662.

[32] D. Ye, M. Li, Y. Zhang, X. Wang, H. Liu, W. Wu, W. Ma, K. Quan, E.H. Ng, X. Wu, M. Lai, H. Ma, Cryptotanshinone Regulates Androgen Synthesis through the ERK/c-Fos/CYP17 Pathway in Porcine Granulosa Cells, Evid. Based Complement. Alternat. Med., 2017 (2017) 5985703.

[33] Y. Lachance, V. Luu-The, C. Labrie, J. Simard, M. Dumont, Y. de Launoit, S. Guerin, G. Leblanc, F. Labrie, Characterization of human 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase gene and its expression in mammalian cells, J. Biol. Chem., 267 (5) (1992) 3551.

[34] T.A. Duffy, M.E. Picha, E.T. Won, R.J. Borski, A.E. McElroy, D.O. Conover, Ontogenesis of gonadal aromatase gene expression in atlantic silverside (Menidia menidia) populations with genetic and temperature-dependent sex determination, J. Exp. Zool. A Ecol. Genet. Physiol., 313 (7) (2010) 421-431.

[35] H.Q. Sun, C.Q. Tan, H.K. Wei, Y. Zou, G. Long, J.T. Ao, H.X. Xue, S.W. Jiang, J. Peng, Effects of different amounts of konjac flour inclusion in gestation diets on physio-chemical properties of diets, postprandial satiety in pregnant sows, lactation feed intake of sows and piglet performance, Anim. Reprod. Sci., 152 (2015) 55-64.

[36] J.L. Leroy, T. Vanholder, J.R. Delanghe, G. Opsomer, A. Van Soom, P.E. Bols, J. Dewulf, A. de Kruif, Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum, Theriogenology, 62 (6) (2004) 1131-1143.

[37] J.L. Leroy, T. Vanholder, J.R. Delanghe, G. Opsomer, A. Van Soom, P.E. Bols, A. de Kruif, Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows, Anim. Reprod. Sci., 80 (3-4) (2004) 201-211.

[38] A. Wang, H. Si, D. Liu, H. Jiang, Butyrate activates the cAMP-protein kinase A-cAMP response element-binding protein signaling pathway in Caco-2 cells, J. Nutr., 142 (1) (2012) 1-6.

[39] G. Li, W. Yao, H. Jiang, Short-chain fatty acids enhance adipocyte differentiation in the stromal vascular fraction of porcine adipose tissue, J. Nutr., 144 (12) (2014) 1887-1895.

[40] R.G. Gosden, I.H. Sadler, D. Reed, R.H. Hunter, Characterization of ovarian follicular fluids of sheep, pigs and cows using proton nuclear magnetic resonance spectroscopy, Experientia, 46 (10) (1990) 1012-1015.

[41] Y. Xiong, N. Miyamoto, K. Shibata, M.A. Valasek, T. Motoike, R.M. Kedzierski, M. Yanagisawa, Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41, Proc. Natl. Acad. Sci. USA, 101 (4) (2004) 1045-1050.

[42] B.S. Samuel, A. Shaito, T. Motoike, F.E. Rey, F. Backhed, J.K. Manchester, R.E. Hammer, S.C. Williams, J. Crowley, M. Yanagisawa, J.I. Gordon, Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41, Proc. Natl. Acad. Sci. USA, 105 (43) (2008) 16767-16772.

[43] H.V. Lin, A. Frassetto, E.J. Kowalik, Jr., A.R. Nawrocki, M.M. Lu, J.R. Kosinski, J.A. Hubert, D. Szeto, X. Yao, G. Forrest, D.J. Marsh, Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms, PloS one, 7 (4) (2012) e35240.

[44] A. Wang, Z. Gu, B. Heid, R.M. Akers, H. Jiang, Identification and characterization of the bovine G protein-coupled receptor GPR41 and GPR43 genes, J. Dairy Sci., 92 (6) (2009) 2696-2705.

[45] B.J. Clark, S.C. Soo, K.M. Caron, Y. Ikeda, K.L. Parker, D.M. Stocco, Hormonal and developmental regulation of the steroidogenic acute regulatory protein, Mol. Endocrinol., 9 (10) (1995) 1346-1355.

[46] T. Li, C. Cao, T. Yang, L. Zhang, L. He, Z. Xi, G. Bian, N. Liu, A G-protein-coupled receptor regulation pathway in cytochrome P450-mediated permethrin-resistance in mosquitoes, Culex quinquefasciatus, Sci. Rep., 5 (2015) 17772.

[47] C. Daems, M. Di-Luoffo, E. Paradis, J.J. Tremblay, MEF2 Cooperates With Forskolin/cAMP and GATA4 to Regulate Star Gene Expression in Mouse MA-10 Leydig Cells, Endocrinology, 156 (7) (2015) 2693-2703.

[48] H.S. Abdou, G. Villeneuve, J.J. Tremblay, The calcium signaling pathway regulates leydig cell steroidogenesis through a transcriptional cascade involving the nuclear receptor NR4A1 and the steroidogenic acute regulatory protein, Endocrinology, 154 (1) (2013) 511-520.

[49] L.J. Martin, N. Boucher, C. Brousseau, J.J. Tremblay, The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca2+/calmodulin-dependent protein kinase I, Mol. Endocrinol., 22 (9) (2008) 2021-2037.

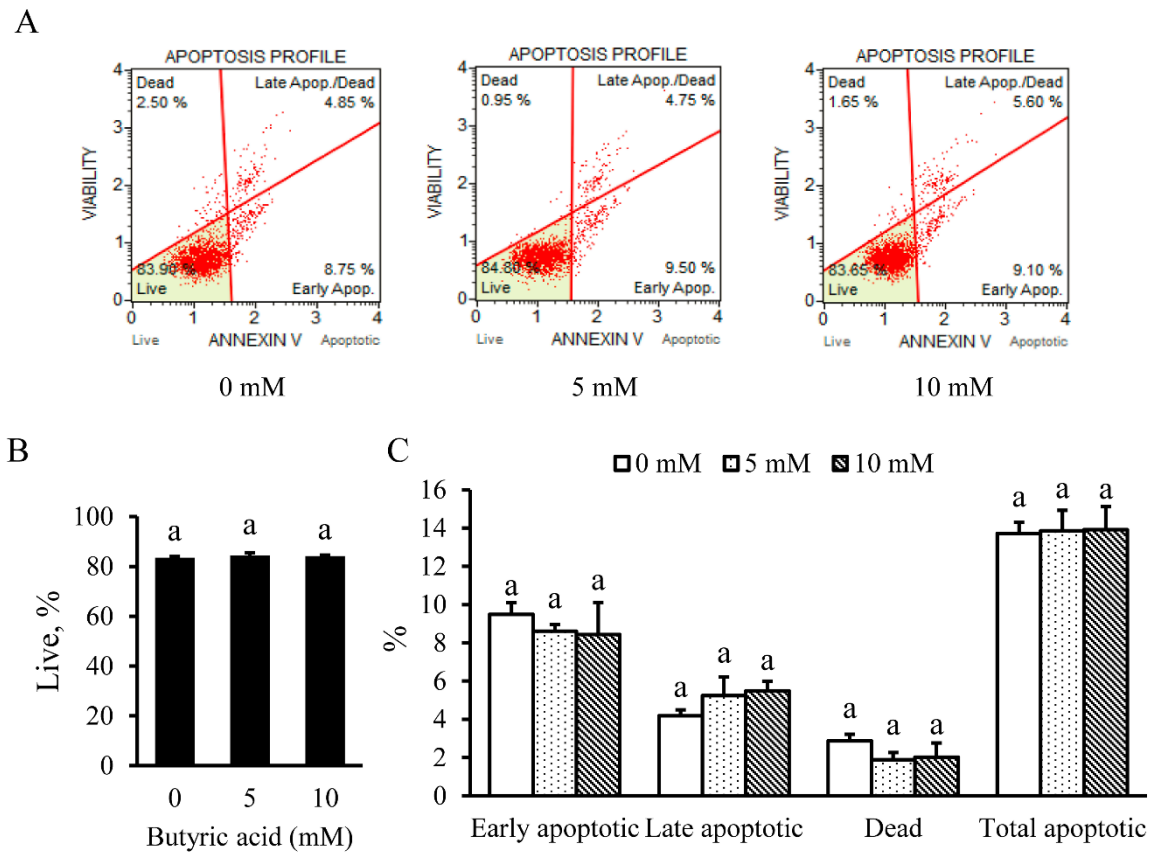
[50] J. Garcia-Bermudez, J.M. Cuezva, The ATPase Inhibitory Factor 1 (IF1): A master regulator of energy metabolism and of cell survival, BBA-Bioenergetics, 1857 (8) (2016) 1167-1182.

[51] T.Y. Liu, X.Q. Xiong, X.S. Ren, M.X. Zhao, C.X. Shi, J.J. Wang, Y.B. Zhou, F. Zhang, Y. Han, X.Y. Gao, Q. Chen, Y.H. Li, Y.M. Kang, G.Q. Zhu, FNDC5 Alleviates Hepatosteatosis by Restoring AMPK/mTOR-Mediated Autophagy, Fatty Acid Oxidation and Lipogenesis in Mice, Diabetes, (2016).

[52] S.J. Kim, T. Tang, M. Abbott, J.A. Viscarra, Y. Wang, H.S. Sul, AMPK Phosphorylates Desnutrin/ATGL and Hormone-Sensitive Lipase To Regulate Lipolysis and Fatty Acid Oxidation within Adipose Tissue, Mol. Cell. Biol., 36 (14) (2016) 1961-1976.

[53] P. Schonfeld, L. Wojtczak, Short- and medium-chain fatty acids in energy metabolism: the cellular perspective, J. Lipid Res., 57 (6) (2016) 943-954.

Figr-1



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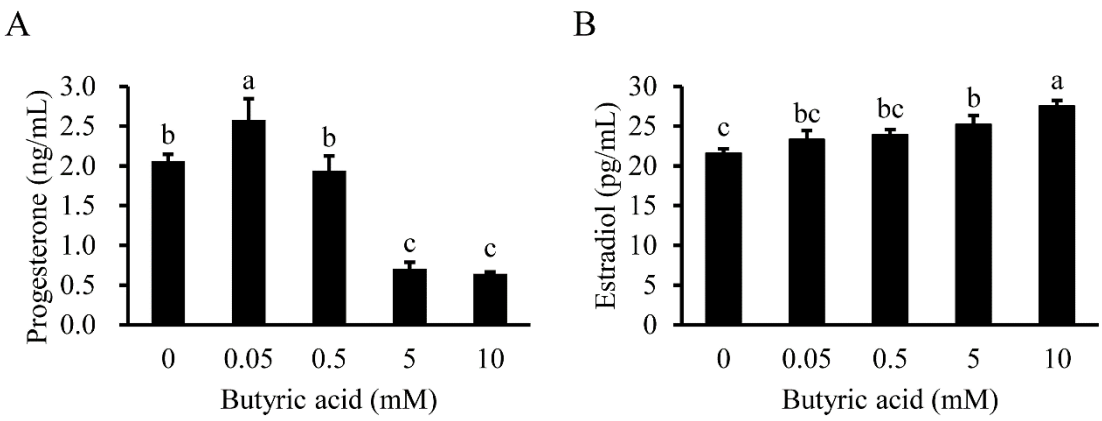
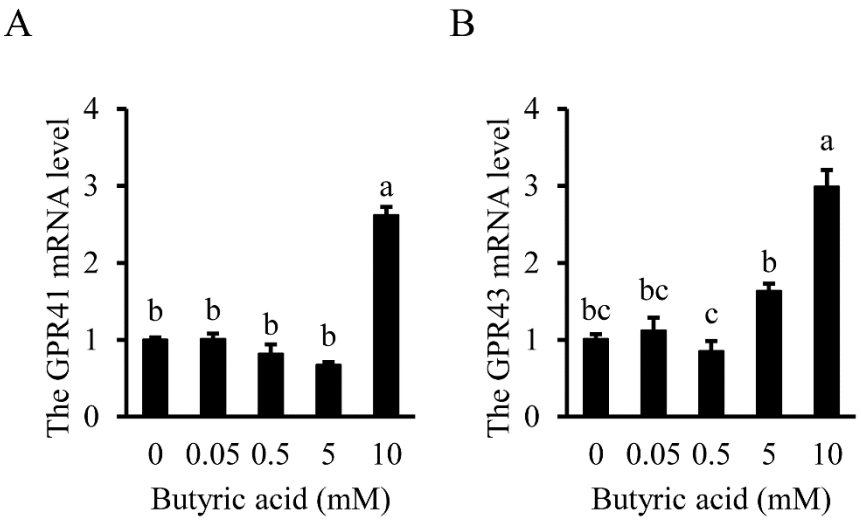
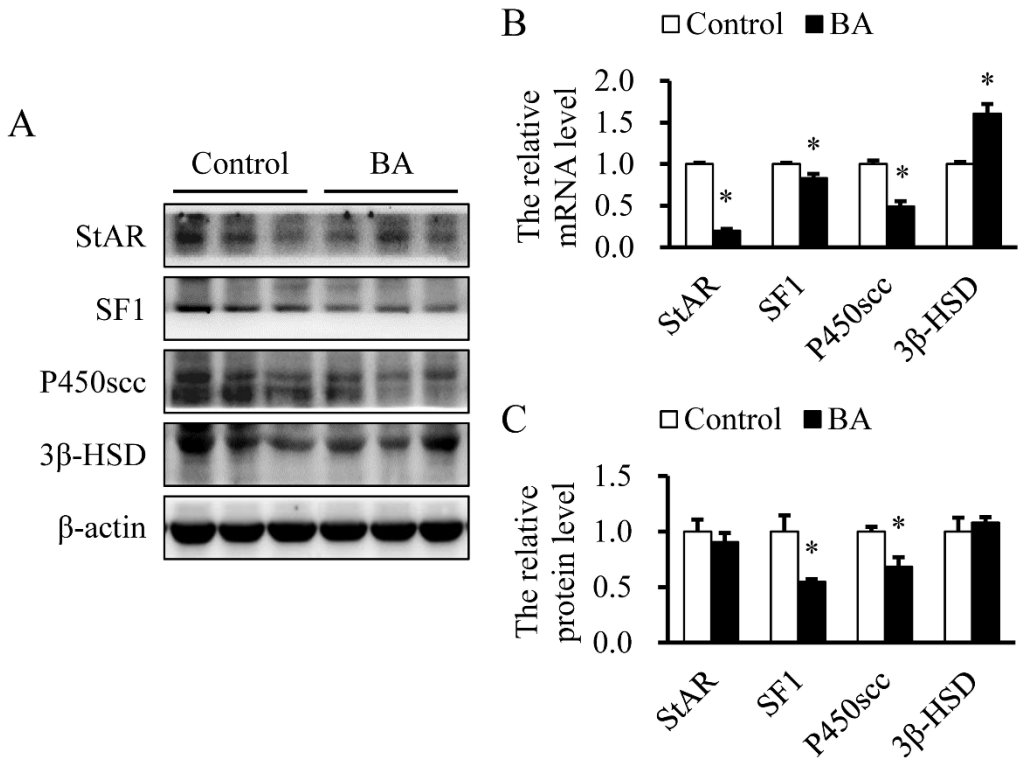




Figr-3

Figr-4



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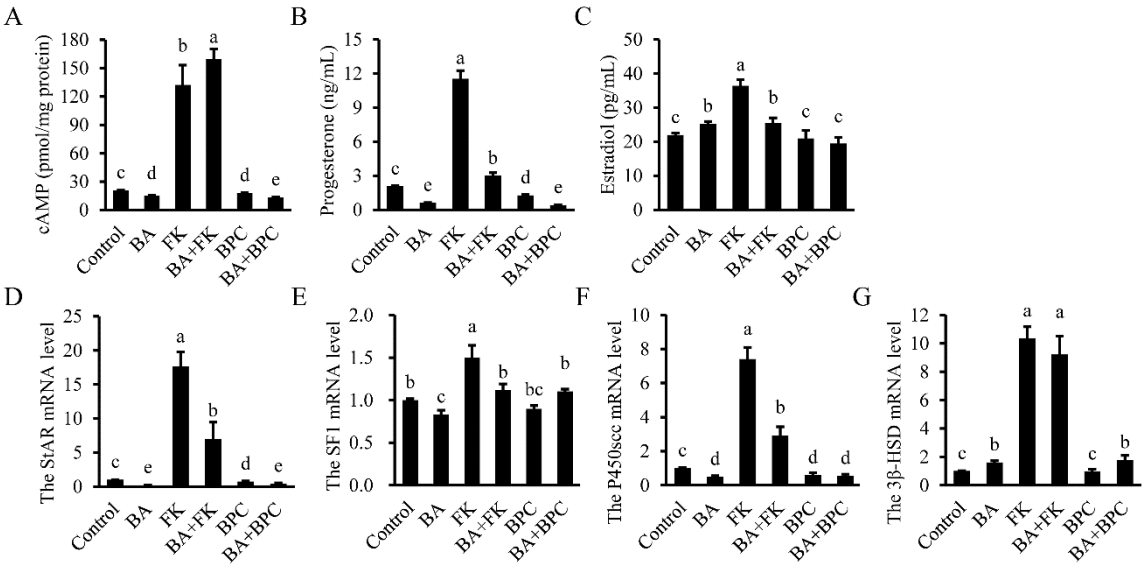

Figr-6**Figure captions**

Fig. 1. The effects of butyric acid (BA) on apoptosis of PGCs. The porcine granulosa cells (PGCs) were seeded into 24-well cell plates for apoptotic. Cells were treated with different concentrations of butyric acid (BA) (0 mM, 5 mM, 10 mM) for 24 h. (A) Flow cytometry analysis of apoptotic PGCs treated by BA at different concentrations. (B) and (C) The statistical results of figure 1A. Data were expressed as the mean ± SEM (n = 6), and the bars with different letters indicate significant difference ($P < 0.05$).

Fig. 2. The effects of butyric acid (BA) on PGCs progesterone and estradiol secretion. The porcine granulosa cells (PGCs) were seeded into 24-well cell plates, after reaching confluence, the cells were treated with different concentrations of butyric acid (BA) (0~10 mM) for 24 h, after then, the cell supernatant was collected for detecting the contents of progesterone (A) and estradiol (B). Data were expressed as the mean ± SEM (n = 6), and the bars with different letters indicate significant difference ($P < 0.05$).

Fig. 3. The effects of butyric acid (BA) on PGCs GPRs mRNA expression. The porcine granulosa cells (PGCs) were seeded into 24-well cell plates with different concentrations of butyric acid (BA) (0~10 mM) for 24 h. After then, the cell were collected for RNA extraction for detection the mRNA level of GPR41 (A), GPR43 (B), respectively. And β-actin was used as a housekeeping gene control. Data were expressed as the mean ± SEM (n = 6), and the bars with different letters indicate significant difference ($P < 0.05$).

Fig. 4. The effects of butyric acid (BA) on PGCs function genes expression. The porcine granulosa cells (PGCs) were seeded into 24-well cell plates with 5 mM butyric acid (BA) for 24 h, after then, the cell were collected for RNA extraction and total protein extraction, respectively, for measure the genes of StAR, SF1, P450scc and 3β-HSD in mRNA level (B) and protein level (C), respectively. (A) The pictures of indicated protein exposure with western blot. And β-actin was used as a housekeeping gene control. The value of control group data was set to 1, and the BA groups have the values of data/control. Data were expressed as the mean ± SEM (n = 6), * $P < 0.05$.

Fig. 5. Butyric acid (BA) inhibits the cAMP signal pathway. The porcine granulosa cells (PGCs) were seeded into 24-well cell plates with 10 μM forskolin (FK) or 5 mM butyric acid (BA) single or combined for 24 h, and also with 2.3 μM bupivacaine (BPC) or 5 mM BA single or combined for 24 h. Then, the cAMP content (A), progesterone content (B) and estradiol content (C) of PGCs were measured, respectively, as well as the mRNA levels of StAR (D), SF1 (E), P450scc (F) and 3β-HSD (G) of PGCs. Data were expressed as the mean ± SEM (n = 6), and the bars with different letters indicate significant difference ($P < 0.05$).

Tble 1. Parameters of oligo-nucleotide primer pairs for the target genes

Target gene	GeneBank accession No.	Primer sequence (5' to 3')	Annealing temperature (°C)	products (bp)
GPR41	JQ_776642.1	F:CTCATCACCAGCTACTGCCG R:AATTCAGGGTGCTGAGGAGC	58	214
GPR43	U_768799.1	F:ACCCATCCACATCCTCCTGC R:GCTGCTGTAGAAGCCGAAACC	60	151
P450scc	NM_214427.1	F: ATGATTCCTGCCAAGACA R: GCCAAAGCCCAAGTTCC	60	147
3β-HSD	AF_232699	F:AGGGTTTCTGGGTCAGAGGATC R: CGTTGACCACGTCGATGATAGAG	60	110
SF1	NM_214179.1	F:CTGCCTCAAGTTCCTCATTCTC R:GGTAGTGGCACAGGGTGTAATC	60	122
StAR	NM_213755.2	F:TTTGTGAGTGTGCGCTGTA R:CCACCTGGGTCTGTGATAG	60	247
β-actin	XM_003124280	F：ATGGATGACGATATTGCTGC R：CCCACGTAGGAGTCCTTCTG	60	164

Table 2. Regulated protein pathway-Sus scrofa (pig) -partial

Items	Mapping	Background	Fold enrichment	Fisher' exact Test P value	Related proteins (Accession)
Up-regulated					
Steroid biosynthesis	2	9	31.88	0.001549	A7L861, I3LM80
Ribosome	3	78	5.52	0.014386	I3LEX0, A1XQU3, F2Z5Q8
Amoebiasis	2	34	8.44	0.021949	F1RLL9, Q5S1U1
Down-regulated					
Protein digestion and absorption	6	17	30.94	1.01E-08	F1RXW0, F1S571, I3LSV6, F1SFA7, F1S021, F1RYI8
ECM-receptor interaction	6	22	23.91	5.87E-08	F1RXW0, F1S571, I3LSV6, F1SFA7, F1S021, F1RYI8
Amoebiasis	6	34	15.47	9.77E-07	F1RXW0, F1S571, I3LSV6, F1SFA7, F1S021, F1RYI8
Focal adhesion	7	59	10.4	1.65E-06	F1RXW0, F1S571, I3LSV6, F1SRV9, F1SFA7, F1S021, F1RYI8

Platelet activation	6	44	11.95	4.80E-06	F1RXW0, F1S571, I3LSV6, F1SFA7, F1S021, F1RYI8
PI3K-Akt signaling	6	65	8.09	4.88E-05	F1RXW0, F1S571, I3LSV6, F1SFA7, F1S021, F1RYI8
cAMP signaling	1	28	3.13	0.276767	F2Z5G3
Calcium signaling	1	29	3.02	0.285165	F2Z5G3

In this project, quantitative ratio over 1.3 was considered as up-regulation or down-regulation.

Table 3. Regulated protein-Sus scrofa (pig)-partial

Proteins accession	Protein description	KEGG gene	Ration	Subcellular location
Up-regulated				
Lipids biosynthesis				
D0G6X3	**Farnesyl-diphosphate farnesyltransferase 1**	**-**	**1.518**	-
A7L861	Squalene epoxidase	*SQLE*	1.396	plasma membrane
I3LM80	**Uncharacterized protein**	***DHCR24***	**1.326**	**endoplasmic reticulum**
I3L7K2	**Phosphoinositide phospholipase C**	***PLCD***	**1.301**	**nuclear**
A5YV76	Fatty acid synthase	-	1.516	-
F8SM59	Fatty acid synthase (Fragment)	-	1.389	-
Molecule binding				
F1SRM1	Uncharacterized protein (Fragment)	*MYO10*	1.518	nuclear
B2ZI35	F11 receptor	*F11R*	1.502	extracellular
I3LSQ0	Uncharacterized protein	-	1.461	cytosol
F1RFF5	Uncharacterized protein	-	1.446	mitochondria
F2Z5B2	Uncharacterized protein	*TUBB*	1.420	cytosol
Structural molecule				
A1XQU3	60S ribosomal protein L14	*RP-L14e*	1.506	mitochondria
I3LEX0	40S ribosomal protein S9 (Fragment)	*RP-S9e*	1.558	cytosol
F2Z5Q8	Uncharacterized protein	*RP-L35Ae*	1.301	cytosol
F1RLL9	Uncharacterized protein (Fragment)	*COL4A*	1.480	cytosol, nuclear
Stress				
Q5S1U1	Heat shock protein beta-1, HSPB1	*HSPB1*	1.322	mitochondria
F1SEQ7	Uncharacterized protein	-	1.372	extracellular
Down-regulated				
Protein metabolism				
F1RII7	Hemoglobin subunit beta	*HBB*	0.137	cytosol
P18648	**Apolipoprotein A-I**	***APOA1***	**0.769**	**extracellular**
F1SKI0	Myosin-11 (Fragment)	-	0.755	cytosol
F1RYZ0	60S acidic ribosomal protein P2	*RP-LP2*	0.686	mitochondria
F1RNX2	Programmed cell death protein 5	-	0.670	cytosol
Q29307	ATPase inhibitor	-	0.604	mitochondria
Enzyme activity				
I3L650	Uncharacterized protein	*CALD1*	0.730	nuclear
F2Z5G3	**Uncharacterized protein**	***CALM***	**0.734**	**cytosol, nuclear**
I3LI15	Uncharacterized protein (Fragment)	*SETD7*	0.748	cytosol

F1RYI8	Uncharacterized protein	*COL1AS*	0.553	extracellular
I3LSV6	Uncharacterized protein (Fragment)	*COL1AS*	0.534	cytosol
F1SFA7	Uncharacterized protein	*COL1AS*	0.588	extracellular
F1RXW0	Uncharacterized protein (Fragment)	*COL1AS*	0.737	nuclear

In this project, quantitative ratio over 1.3 was considered as up-regulation while quantitative ratio less than 1/1.3 (0.77) was considered as down-regulation.

Table 4. The GO terms of level 2 distribution of regulated expressed protein-Sus scrofa (pig)-partial

GO Terms Level 1	**GO Terms Level 2**	**No. of Protein**	**Protein IDs**
Up-regulated			
Biological process	Metabolic process	10	F1SEQ7, A7L861, F1RLL9, **I3L7K2,** F1RWV6, I3LEX0, A1XQU3, F2Z5Q8, **I3LM80,** I3LSQ0
	Single-organism process	9	F1SEQ7, A7L861, I3LQQ6, **I3L7K2,** F1RFF5, B2ZI35, F2Z5B2, **I3LM80,** I3LSQ0
	Biological regulation	8	F1SEQ7, I3LQQ6, F1RLL9, **I3L7K2,** F1SRM1, I3LEX0, **I3LM80,** I3LSQ0
	Response to stimulus	6	I3LQQ6, F1RLL9, I3L7K2, **I3LM80,** Q5S1U1, I3LSQ0
	Reproductive process	**2**	**I3L7K2, I3LM80**
	Signaling	2	**I3L7K2,** I3LSQ0
Cellular component	Cell	12	F1SEQ7, **I3L7K2,** F1SRM1, Q5S1U1, F1RFF5, I3LEX0, A1XQU3, B2ZI35, F2Z5B2, F2Z5Q8, **I3LM80,** I3LSQ0
	Macromolecular complex	7	I3LEX0, F1RLL9, A1XQU3, F2Z5B2, F1SRM1, F2Z5Q8, I3LSQ0,
	Membrane	5	F1RFF5, A7L861, B2ZI35, F2Z5B2, F1SRM1

Molecular function	Binding	9	A7L861, **I3L7K2**, F1SRM1, I3LEX0, F1RFF5, B2ZI35, F2Z5B2, **I3LM80**, I3LSQ0
	Catalytic activity	7	A7L861, I3LPB5, **I3L7K2**, F1RWV6, F2Z5B2, F1SRM1, **I3LM80**,
	Molecular transducer activity	1	**I3L7K2**
Down-regulated			
Biological process	Single-organism process	18	F1RXW0, **I3L650,** Q29307, F1S8X0, F1SKI0, F1RII7, F1RJL2, F1RQR0, I3LSV6, I3LI15, F1RYI8, F1S021, F1RGX4, F1S571, P18648, F1S0L8, E7CXS1, I3LSM3
	Biological regulation	15	**F2Z5G3,** F1RJL2, F1RQR0, I3LSV6, I3LI15, F1S021, F1RYI8, Q29307, F1SPK8, J9JIL5, P18648, F1S0L8, I3LUD5, E7CXS1, I3LSM3
	Response to stimulus	9	**F2Z5G3,** F1RXW0, I3LSV6, F1S021, F1RYI8, F1S571, P18648, F1S0L8, I3LSM3
Cellular component	Cell	23	F1RSE7, **I3L650,** F1SBT6, Q29307, I3LPH5, F1RYZ0, F1SKI0, I3L5D5, F1RII7, F1SPJ5, **F2Z5G3,** F1RJL2, F1RQR0, I3LSV6, F1SU06, I3LI15, F1SPK8, F1RGX4, P18648, F1S1L1, F1S0L8, I3LUD5, I3LSM3
	Macromolecular complex	20	F1RXW0, F1RSE7, F1SBT6, Q29307, F1RYZ0, F1SKI0, F1RII7, F1SPJ5, **F2Z5G3,** F1RQR0, I3LSV6, F1SU06, F1RYI8, F1S021, F1RGX4, P18648, F1S571, I3LUD5, F1SFA7, I3LSM3
	Organelle	19	F1RSE7, **I3L650,** F1SBT6, Q29307, F1RYZ0, F1SKI0, I3L5D5, F1SPJ5, **F2Z5G3,** F1RJL2, F1RQR0, I3LI15, F1SU06, F1SPK8, F1S1L1, P18648, F1S0L8, I3LUD5, I3LSM3
	Membrane	9	**F2Z5G3, I3L650,** Q29307, F1SPK8, F1S8X0, I3L5D5, F1S0L8, I3LUD5, E7CXS1
Molecular function	Binding	28	F1RSE7, F1RNX2, F1SI69, **I3L650,** F1SBT6, Q29307, F1S8X0, I3LPH5, J9JIL5, F1SKI0, F1SRV9, F1RII7, **F2Z5G3,** F1SPJ5, F1RQR0, F1SU06, I3LI15, F1RYI8, F1S021, F1RGX4, P18648, F1S1L1, F1RVD1, F1RPD7, F1S0L8, I3LUD5, E7CXS1, I3LSM3
	Molecular function regulator	3	**F2Z5G3**, P18648, Q29307