The chromatin mapping of H3K4me3 and H3K27me3 modification in ovaries during porcine puberty initiation

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Research

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Abstract

Background

The biological structure and function of mammalian ovaries undergo important developmental changes during pre-puberty. Posttranslational modified histones dynamically regulate ovarian development, which also can respond to the onset of puberty with heritable changes in gene expression that are associated with nucleosome modifications. This research used chromatin immunoprecipitation followed by sequencing (ChIP-seq) technology and bioinformatic analysis to confirm the histone-DNA interaction in pre-puberty ovarian follicles (PreOV) and in-puberty ovarian follicles (InOV). Then, employed qRT-PCR, and cell proliferation and apoptosis testing to further investigate the function of H3K4me3 and H3K27me3 in gene transcription and cell growth.

Results

With the onset of puberty, ChIP-Seq found 946 higher- enrichment and 916 lower- enrichment genes were significantly differentially modified with H3K4me3, while 1954 higher- enrichment and 882 lower- enrichment genes were significantly differentially modified with H3K27me3 in InOV vs. PreOV. Then interestingly, 88 significantly genes were associated with significantly differentially H3K4me3-enriched, H3K4me3-depleted, H3K27me3-enriched, and H3K27me3-depleted modifications, which also were related to female germ development pathway, bone mineralization and fatty acid transport, including *FOXO3*, *RUNX1*, *NOBOX*, *DMRT1*, *MYC*, *LEPTIN*, *ACVR2*, and *BMPR1B*. Furthermore, compare medium-sized antral with Graaf follicles, *FOXO3*, *RUNX1* and *BMPR1B* were low expressed in medium-sized antral follicles, while *NOBOX* and *LEPTIN* were low expressed in Graaf follicles. Finally, H3K4me3 exhibited to promote proliferation and anti-apoptosis, whereas H3K27me3 was more to inhibited proliferation and induced cell apoptosis in porcine granulosa cells (pGCs).

Conclusions

The genome-wide modified profile of H3K4me3 and H3K27me3 showed that H3K27me3 modification was more active than H3K4me3 modification during the onset of puberty in porcine ovaries. H3K4me3 and H3K27me3 acted as activator and repressor to regulate gene transcription that are related to porcine puberty initiation process. Genes including *FOXO3*, *BMPR1B*, *LEPTIN*, *RUNX1*, involved in networks of female gamete generation pathway associating with ovarian follicle development, ovulation, ovulation cycle process and female gonad development, which showed the further direction to investigate the function of H3K4me3/H3K27me3-targets in puberty initiation.

Background

Puberty is a critical physiological process in female pigs and involves the acquisition of reproduce capacity. The onset of puberty is marked by the first estrus period and ovulation in response to boar stimulation [1]. In the pre-puberty, a period of follicle growth, atresia and degeneration, then the cycle repeats. After the onset of puberty, the functional development of the hypothalamic-pituitary-gonadal (HPG) axis improves, which ensure the higher gonadotropin-releasing hormone (GnRH) levels and prompt follicle maturation and ovulation [2]. The factors influencing sow puberty include epigenetics, developmental programming, common diseases such as polycystic ovarian syndrome (PCOS), the cumulative effects of environmental factors, nutritional levels and boar stimulation [3]. However, internal and permanent changes in DNA dynamically regulate gene expression, and the programming, transcription and translation of the genetic machinery related to puberty remain incompletely elucidated. The onset of puberty is strongly determined by genetics [4], this study focused on the effect of epigenetics on sow puberty.

Epigenetics refers to a heritable phenomenon changes in gene expression without altering the DNA sequence; rather, the phenotype is changed without affecting genotype. There are many epigenetic phenomena, such as DNA methylation, histone posttranslational modification, genomic imprinting, maternal effects, gene silencing, and RNA editing [5–8]. Histone posttranslational modification including histone methylation [9], acetylation [10], ubiquitylation [11] and phosphorylation [12], is essential for gene transcription because it forms the chromatin on-off switch by affecting histone-histone, histone-DNA and histone-chaperone interactions [13]. Histone 3 lysine 4 tri-methylation (H3K4me3) and Histone 3 lysine 27 tri-methylation (H3K27me3) are typical chromatin marks of activating and repressing gene transcription respectively. In the promoter region, H3K4me3 loosens chromatin structure and is thus a positive regulator responsible for recruiting nucleosome remodeling enzymes and histone acetylases [14–16] to activate gene transcription, while H3K27me3 is the transcriptional inhibitory marks responsible for promoting a compact chromatin structure [17, 18]. Epigenetic modifications fluctuate greatly from embryo implantation to early estrus in young females [19]. Mellon finds that a repressive histone modification H3K9me2 can elevate GnRH gene transcription [20]. H3K4me3 and H3K27me3 regulate development-related transcription factors (TFs) by forming bivalent domains (BDs), which can activate or inhibit the kisspeptin gene expression, and then affect the puberty initiation of female rats [21]. Generally, histone methylations are closely related to sexual maturity of rat, we speculate that H3K4me3 and H3K27me3 may be involved in the puberty initiation of sows.

In this current study, the main intent was to obtain genome-wide and time-specific modification of H3K4me3 and H3K27me3 on the onset of puberty. Besides, the regulation of H3K4me3 and H3K27me3 in gene transcription was identify. Moreover, cell proliferation and apoptosis were analyzed after interfering with endogenous H3K4me3 or H3K27me3 in porcine granulosa cells. This research would expose the genome-wide H3K4me3-H3K27me3 bivalent modification during the porcine puberty.

Materials And Methods

Pre-puberty and in-puberty porcine ovaries

The experimental groups conformed to a full sibling design. The six gilts came from 3 litters of full siblings and were separated into the pre-puberty ovarian (PreOV) group and the in-puberty ovarian (InOV) group. The three gilts in one group had one sibling gilt in the other group. Gilts in the PreOV group were slaughtered at day 180, before puberty initiation, whereas those in the InOV group were slaughtered on the day of puberty initiation. The follicles in one ovary were separated into three groups: big follicles (BF, > 5 mm), medium follicles (MF, 5 mm $\geq$ MF $\geq$ 3 mm) and small follicles (SF, $\geq$ 3 mm) [22]. The BF from the PreOV and InOV groups were fixed in 1% paraformaldehyde. The follicles from the ovary were stored in liquid nitrogen at -80 °C.

Porcine granulosa cell culture

The ovaries used for pGCs culture were collected from a slaughterhouse in Guangzhou. Ovaries that lacked a corpus luteum but had clustered follicles full of follicular fluid were chosen. The ovaries were stored in phosphate-buffered saline (PBS) (Hyclone, Logan, UT, USA) containing 1% penicillin-streptomycin (Thermo Fisher, Waltham, CF, USA) on ice and were used for pGCs culture under sterile conditions. The pGCs culture method was modified from published procedures [23]. The pGCs were separated from 3- to 5-mm antral follicles with a 1 mL disposable sterile syringe (with a 4.5-gauge needle), washed twice with Dulbecco's Modified Essential Medium (Hyclone, Logan, UT, USA) and cultured in medium supplemented with 10% foetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Thermo Fisher, Waltham, CF, USA) in an incubator with 5% CO₂ at 37 °C [24].

H3K4me3 and H3K27me3 inhibitors and agonists

H3K4me3 levels were decreased by 150 nM antagonist BCI-121 (#SML1817, Sigma, St. Louis, MO, USA) and increased by 2 nM agonist PBIT (#SML1058, Sigma, St. Louis, MO, USA). The antagonist NC and agonist NC for H3K4me3 were 150 nM and 2 nM dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). H3K27me3 levels were decreased by 6 nM antagonist GSK126 (#1346574-57-9, MedChemExpress, NJ, USA) and increased by 2 nM agonist GSK-J4 (#1373423-53-0, MedChemExpress, NJ, USA) [25]. The antagonist NC and agonist NC for H3K27me3 were 6 nM and 2 nM DMSO (Sigma, St. Louis, MO, USA).

ChIP-Seq analysis of H3K4me3 and H3K27me3

The BF from each group were fixed in 1% paraformaldehyde, resuspended in lysis buffer, and disrupted with a Branson 250 Sonifier to obtain DNA in the size range of 200 to 1500 bp. Solubilized chromatin was diluted 10-fold in ChIP buffer; after removal of a control aliquot, the samples were incubated at 4 °C overnight with antibodies against H3K27me3 (#07-449, Millipore, German) and H3K4me3 (#ab8580, Abcam, Cambridge, UK). Immune complexes were precipitated with protein A-sepharose, washed sequentially with low-salt immune complex wash, LiCl immune complex wash, and TE, and then eluted in elution buffer. After cross-link reversal and proteinase K treatment, ChIP and control DNA samples were extracted with phenol-chloroform, precipitated in ethanol, treated with RNase and calf intestinal alkaline phosphatase, and purified with a MinElute Kit (Qiagen, Dusseldorf, German) [26].

The purity and concentration of DNA samples were determined with a Qubit® Fluorometer. DNA samples underwent end-repair, A tailing and adaptor ligation using a TruSeq Nano DNA Sample Prep Kit (#FC-121-4002, Illumina, San Diego, USA), following the manufacturer's instructions. Approximately 200- to 1500-bp fragments were size selected using AMPure XP beads. The final size of the library was confirmed by an Agilent 2100 Bioanalyzer. The samples were diluted to a final concentration of 8 pM, and cluster generation was performed on the Illumina cBot using a HiSeq 3000/4000 PE Cluster Kit (#PE-410-1001, Illumina), following the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 4000 using a HiSeq 3000/4000 SBS Kit (300 cycles) (#FC-410-1003, Illumina), according to the manufacturer's introduction.

Data collection and analysis

Analysis of sequencing data. Sequence reads were generated by the Illumina HiSeq 4000 system, and image analysis and base calling were performed using off-line basecaller software (OLB V1.8.0). After passing the Illumina chastity quality filter, the clean reads were aligned to the pig genome (susScr3) using BOWTIE software (v2.1.0).

Peak calling. MACS v1.4 (Model-based Analysis of ChIP-seq) software was used to detect the peaks from ChIP-seq. Statistically significant ChIP-enriched regions (peaks) detected by MACS were identified by comparison of IP to Input or to a Poisson background model using a p-value threshold fold of 10^{-4} . The proportions of different peak lengths were calculated and mapped.

Peak annotation. The peaks in samples were annotated by the nearest gene (the nearest TSS to the centre of the peak region) using the newest UCSC RefSeq database (<http://genome.ucsc.edu/>). The online program BioVenn (<http://www.biovenn.nl/index.php>) was used to visualize the unique and common genes H3K4me3 or H3K27me3 modification in the PreOV and InOV groups, as well as to classify the modification as specific to H3K4me3 or H3K27me3 or as bivalent modification of both histones.

Differentially modified regions. The significantly differentially enriched regions between the two groups (InOV vs. PreOV) were identified by diffReps (cut-off: FC = 1.5, p-value = 0.01). Then, the differentially modified genes were classified as upstream, promoter, exon, intron and intergenic enrichment of H3K4me3 or H3K27me3 as follows: 1) promoter region: 2,000 bp upstream and downstream of the TSS; 2) upstream region: > 2,000 bp but $\leq$ 20,000 bp upstream of the TSS; 3) intron region: defined the same as UCSC RefSeq introns with a slightly different starting genomic coordinate of TSS + 2,000 bp; 4) exon region: defined the same as UCSC RefSeq exons with a slightly different starting genomic coordinate of TSS + 2,000 bp; and 5) intergenic region: other genomic regions not classified as one of the above 4 regions. The enrichment centres of the differentially modified genes were defined based on the classification of the peak. Various combinations of differential H3K4me3 or H3K27me3 modifications were visualized via the online software Bioinformatics & Evolutionary Genomics

(<http://bioinformatics.psb.ugent.be/webtools/Venn/>). The correlation of fold change and peak length for each differentially expressed gene was also included in the scope of investigation.

Pathway analysis. Based on the latest Kyoto Encyclopedia of Genes and Genomes database (KEGG) (<https://www.kegg.jp>), we performed a pathway analysis of the differentially expressed mRNAs. This analysis allows users to determine the biological pathway with significant enrichment of differentially expressed mRNAs. The *P*-value denotes the significance of the pathway; the lower the *P*-value is, the more significant the pathway ($P\text{-value} \leq 0.05$). To better understand the biological function of genes with differentially modified promoters and of 88 genes with the other 4 classifications of differential modification, GO and KEGG integrated analysis was conducted with ClueGO in Cytoscape (version 3.4.0) referring to the human GO and KEGG database [27, 28]. Because the database of porcine genes is small, the identified genes were aligned with the human genome, and the human gene ID was used. Both GO terms and the KEGG pathways were filtered using a *P*-value < 0.05 as the selection criteria.

The biological function of H3K4me3 and H3K27me3 in porcine granulosa cells

Granulosa cell proliferation assay. pGCs grown in 48-well plates were exposed to BCI-121, PBIT, GSK126, GSK-J4 and DMSO, and pGCs proliferation was analysed with EdU kit (#C10310-1, RiboBio, Guangzhou, China) according to the manufacturer's instructions and the literature [24]. In brief, 100 μ L of 50 μ M EdU solution was added to each well for 2 h. Then, the culture medium was discarded, 100 μ L of cell fixing solution (80% acetone) was added to each well for 30 min at room temperature, and the wells were washed twice with PBS. Next, 100 μ L of penetrant (0.5% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS) was added to permeabilize the cells, which were then washed once with PBS prior to the addition of 100 μ L of 1 $\times$ Apollo Staining Solution for 30 min at room temperature in the dark. Subsequently, the staining solution was discarded, 100 μ L of DAPI was added to each well for 30 min at room temperature in the dark, PBS was added, and pictures were taken under a microscope.

Granulosa cell apoptosis assay. pGCs grown in 6-well plates were exposed to BCI-121, PBIT, GSK126, GSK-J4 and DMSO. The cells were digested by trypsin, washed twice with ice-cold PBS and resuspended in 500 μ L of binding buffer. Next, 1.25 μ L of Annexin V-FITC (Sigma, St. Louis, MO, USA) was added, and the samples were incubated in the dark for 15 min at room temperature, followed by centrifugation at 1,000 $\times$ g for 5 min at room temperature to remove the supernatant. The cells were gently resuspended with 0.5 mL of pre-cooled 1 $\times$ solution, and 10 μ L of propidium iodide (PI; 50 μ g/mL) (MedChemExpress, NJ, USA) was added. Finally, the cells were analysed in a flow cytometer (Becton Dickinson Co., San Jose, CA, USA) using the FITC signal detector (FL1) and phycoerythrin emission signal detector (FL2). All experiments were performed at least three times. Cells in the lower right quadrant are annexin-positive/PI-negative early apoptotic cells, and cells in the upper right quadrant are annexin-positive/PI-positive late apoptotic cells [24].

H3K4me3/H3K27me3-enriched gene analysis by qRT-PCR

Different grade porcine follicles were used to measure gene expression. According to the size and characteristics, the follicles were classified as MF (3–5 mm), BF (> 5 mm) or Graafian follicles (GF, with an ovulation hole). qRT-PCR analysis was performed for the top three genes with differential histone modification and genes with the 4 different types of H3K4me3 and H3K27me3 modifications, such as *ENY2*, *SPATA24*, *NOBOX*, *MYC*, *LEPTIN*, and *BMPR1B*. qRT-PCR primers are listed in Table S1. The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Results

H3K4me3 and H3K27me ChIP-Seq

The clean reads of H3K4me3 and H3K27me3 ChIP-Seq were aligned to the susScr3 reference genome to obtain the reads matched to the porcine genome. Peak calling (Table S2) found out most of the H3K4me3-modified peaks were 2 kb in length (Figs. 1A, B), and a large proportion of the H3K27me3-modified peaks were 1 kb in length (Figs. 1C, D).

The genome-wide profile of H3K4me3 and H3K27me3 modification is shown in Fig. 2 after peak annotation, and more details in Fig S1 and S2. The numbers of enriched genes on each chromosome are shown in Fig S3. Chromosomes 1 had the most peaks of H3K4me3 and H3K27me3 modification both in the PreOV and InOV groups.

In the PreOV and InOV groups, 3515 and 3551 genes had H3K4me3 modifications, and 1469 and 2576 had H3K27me3 modifications. Furthermore, there were 260 H3K4me3-modified genes unique to the PreOV group and 296 unique to the InOV group, and 3255 genes were modified in both phases (Fig. 3A). The number of phase-specific H3K27me3-modified genes increased from 187 in the PreOV group to 1294 in the InOV group, and 1282 genes were common to these two groups (Fig. 3B).

The specific and bivalent H3K4me3 and H3K27me3 modification analysis showed the H3K4me3- or H3K27me3-specific modification of 2097 and 52 genes in the PreOV group (Fig. 4A) and of 1104 and 129 genes in the InOV group (Fig. 4B). There were 1418 and 2447 genes with bivalent modification in the PreOV and InOV groups, among which 172 and 1201 genes had specific bivalent modifications in PreOV and InOV, and 1246 genes had common bivalent modifications in the two stages (Fig. 4C). The results presented almost double number of genes with bivalent modification of H3K4me3 and H3K27me3 in InOV vs. PreOV, was the results of recruiting H3K27me3 onto the genes with specific H3K4me3 modification. In other word, some active genes in PreOV should be repressed in InOV to comply with relative biological processes in follicles during puberty initiation.

Differentially enriched genes in PreOV vs InOV

In total, H3K4me3 was significantly enriched at 946 genes and depleted at 916 genes upon comparison of InOV vs. PreOV. In addition, 1954 and 882 genes were significantly enriched (InOV vs. PreOV) or depleted (InOV vs. PreOV) in terms of H3K27me3 (Table S3). Differential peaks annotation showed that

most of differentially peaks tittivated intergenic modification of H3K4me3 or H3K27me3 in PreOV and InOV, and the fewest peaks were promoter modification of H3K4me3 or H3K27me3 (Fig. 5).

The H3K4me3 and H3K27me3 modification in gene DNA sequences are usually in multi-sites (Fig. 6). The number of genes with unique differential H3K4me3 enrichment, H3K4me3 depletion, H3K27me3 enrichment and H3K27me3 depletion was 166, 131, 475 and 186, respectively. There were 88 genes even with four significantly differential modified sites. The number of genes with significantly differential H3K27me3 modification was much higher than that in other classifications.

We found that more differentially enriched peaks with a higher enrichment fold change (FC) converged at 1–2 kb in all 4 cases of H3 posttranscriptional modification (Fig. 7A-D). The fold change generally ranged from 2–5 times. The differentially modified genes with a higher fold change caught our attention, such as *ENY2*, *SPATA24*, and *MTRF* in the H3K4me3 enriched group; *LOC100520823*, *CPE* and *miR708* in the H3K4me3 depleted group; and *COPS2*, *USP19* and *MIR9811* in the H3K27me3 depleted group. To focus on 1–2 kb peaks, those longer than 2 kb were ignored, and more genes with shorter peaks and greater fold change were identified in the H3K4me3-enriched, H3K4me3-depleted and H3K27me3-depleted groups (Fig. 7E, F, H). Interestingly, the peaks in the H3K27me3-enriched group differed from those in the other three groups, with a more uniform distribution and a higher fold change focused for longer fragments, such as the top three genes *ACTR2*, *CHBG* and *GPATCH2L* (Fig. 7G).

qRT-PCR analysis of the top three differentially modified genes in mid-size follicles (3–5 mm, MF), big-size follicles (5–6 mm, BF) and maturity follicles (with ovulation hole, GF) showed that the H3K4me3-enriched genes *ENY2* and *SPATA24* were more highly expressed in development follicles (BF and GF) than in MF. *CPE*, which showed H3K4me3-deleted modification, had the highest expression in GF. The levels of *COPS2*, which showed increased H3K27me3 modification, were reduced in mature follicles. Genes depleted of H3K27me3, such as *ACTR3*, *CHBG*, and *GPATCH2L* showed a gradual increase in expression (Fig. 8A). The expression of genes related to female gamete generation, such as *BMPR1B*, *FOXO3*, and *RUNX1*, increased gradually, but *LEPTIN* and *NOBOX* expression decreased during the growth of follicles (Fig. 8B).

KEGG analyses of differentially enriched genes

The genes with a promoter significantly differentially enriched with H3K4me3, depleted of H3K4me3, enriched with H3K27me3 and depleted of H3K27me3 were involved in 3, 2, 12 and 0 biological pathways, respectively (Table 1). The citrate cycle, ether lipid metabolism, and ECM-receptor interaction were the three pathways involving the genes with a promoter differentially enriched in H3K4me3 in the InOV vs. PreOV comparison. The complement and coagulation cascades and Toll-like receptor signaling pathway were enriched in genes with a promoter differentially depleted of H3K4me3 in the InOV vs. PreOV comparison. Fatty acid elongation, microRNAs in cancer and prostate cancer were the top three most enriched pathways for genes with increased H3K27me3.

Table 1
KEGG pathways for all gene promoters differentially modified by H3K4me3 or H3K27me3

Histone modification	Pathways (Sus scrofa (pig))	P-value	Genes
H3K4me3-enriched genes	ssc00020 ~ Citrate cycle (TCA cycle)	0.0126	<i>ACLY</i>
	ssc00565 ~ Ether lipid metabolism	0.0173	<i>PLA2G7</i>
	ssc04512 ~ ECM-receptor interaction	0.0320	<i>CD47</i>
H3K4me3-depleted genes	ssc04610 ~ Complement and coagulation cascades	0.0328	<i>PLAT</i>
	ssc04620 ~ Toll-like receptor signaling pathway	0.0404	<i>TLR3</i>
H3K27me3-enriched genes	ssc00062 ~ Fatty acid elongation	0.0053	<i>HADHA, HADHB</i>
	ssc05206 ~ MicroRNAs in cancer	0.0053	<i>MIR101-2, MIR143, MIR145, MIR99A, MIRLET7C</i>
	ssc05215 ~ Prostate cancer	0.0073	<i>CREB1, CTNNB1, FOXO1</i>
	ssc04922 ~ Glucagon signaling pathway	0.0092	<i>CREB1, FOXO1, PDHB</i>
	ssc01200 ~ Carbon metabolism	0.01446	<i>GOT2, HADHA, PDHB</i>
	ssc00071 ~ Fatty acid degradation	0.01579	<i>HADHA, HADHB</i>
	ssc01212 ~ Fatty acid metabolism	0.02034	<i>HADHA, HADHB</i>
	ssc00510 ~ N-Glycan biosynthesis	0.02196	<i>DDOST, DPM2</i>
	ssc00280 ~ Valine, leucine and isoleucine degradation	0.02363	<i>HADHA, HADHB</i>
	ssc04141 ~ Protein processing in endoplasmic reticulum	0.03603	<i>BAG1, DDOST, HSPH1</i>
	ssc05164 ~ Influenza A	0.04137	<i>IFIH1, PABPN1, TYK2</i>
	ssc03018 ~ RNA degradation	0.04744	<i>BTG1, DCPS</i>

Integrated analysis of the GO terms and KEGG pathways produced a simpler network of all genes with a promoter differentially modified by H3K4me3 or H3K27me3 (Fig. 9). Three prominent pathways were enriched: the BMP2-WNT4-FOXO1 pathway in human primary endometrial stromal cell differentiation, mitochondrial fatty acid beta-oxidation of unsaturated fatty acids and adenylyl-nucleotide exchange factor activity. The BMP-WNT4-FOXO1 pathway in human primary endometrial stromal cell differentiation was related to the androgen receptor signaling pathway through *DCN*, *FOXO1*, *CTNNB1*, *CREB1*, and *BAG1*. Coincidentally, *BAG1* linked adenylyl-nucleotide exchange factor activity, ATPase regulator activity, and

chaperone-mediated protein folding; this network was formed by *BAG1*, *CRPEL2*, *FKBP3*, and *HSPH1*. The last network of hydro-lyase activity, fatty acid beta-oxidation, fatty acid beta-oxidation and mitochondrial fatty acid beta-oxidation of unsaturated fatty acids was formed by *HADHA*, *HADHB*, *UROS*, *DECR1*, and *ETFA*.

The GO and KEGG confluence analysis of significantly differentially modified genes in terms of H3K4me3 and H3K27me3 modification showed that approximately 16% of the genes (14 of 88), including *FOXO3*, *RUNX1*, *NOBOX*, *DMRT1*, *MYC*, *LEPTIN*, *RPL10*, *BDNF*, *TERT*, *FRZB*, *CD47*, *ACVR2*, *HSP90AB1* and *BMPR1B*, were involved in female gamete development associated with ovarian follicle development, ovulation, ovulation cycle process and female gonad development (Fig. 10). Other enriched pathways joined in female gamete generation pathway according to gene link, such as *BMPR1B* linking between positive regulation of bone and female gamete generation pathway, *LEP* linking between fatty acid transport pathway, and female gamete generation pathway, and *RUNX1* linking between the hematopoietic stem cell differentiation pathway and female gamete generation pathway (Fig. 10).

The influence of H3K4me3 or H3K27me3 on pGCs

Compared to H3K4me3 antagonist NC, decreasing H3K4me3 significantly inhibited pGCs cell proliferation and induced cell apoptosis. Compared to H3K4me3 agonist NC, increasing H3K27me3 obviously promoted cell proliferation and anti-apoptosis (Fig. 11A, C). Compared to H3K27me3 antagonist NC/ agonist NC, both of decreasing or increasing H3K27me3 significantly restrained cell proliferation (Fig. 11B). however, there were the lowest cell proliferation in H3K27me3 agonist group. Compared to H3K27me3 antagonist NC, reducing H3K27me3 significantly repressed cell apoptosis, and accumulating H3K27me3 markedly induced cell apoptosis (Fig. 11D).

Discussion

H3K4me3 and H3K27me3 were trimethylated at sites of lysine residues 4 and 27 of histone 3, which performed activate and inactivate gene transcription to regulate organism development and growth [29, 30]. It was confirmed that interfering endogenous H3K4me3 or H3K27me3 influenced pGCs proliferation and apoptosis. Granulosa cells are critical components and functional units in ovarian follicles. The health of GCs determines follicle fate [31] and steroid biosynthesis and metabolism [32]. Synergism between granulosa cells and oocytes is the precursor to the initiation of puberty and maintains the normal oestrus cycle and reproduction.

The H3K4me3 and H3K27me3 modified profiles in the porcine follicular genome during the onset of puberty were determined in this study. The results of peak calling of H3K4me3 and H3K27me3 in PreOV and IOV indicated that H3K4me3 and H3K27me3 modifications were dynamic during the transition from pre-puberty to puberty (Fig. 3). It seems like that the dynamical change of H3K27me3 modification was much drastic than H3K4me3 in PreOV and IOV, which comprised of the functional genes with different modification of H3K4me3 and H3K27me3 [15, 18].

The analysis of specific or bivalent modification in the PreOV and InOV groups revealed that H3K4me3 modification remained steady in the two phases but H3K4me3-specific modification was reduced in puberty. Almost double number of genes with bivalent modification of H3K4me3 and H3K27me3 in InOV vs. PreOV, was the results of recruiting H3K27me3 onto the genes with specific H3K4me3 modification. A total of 1246 of 2619 genes with bivalent modification were common to the PreOV and InOV groups (Fig. 4). In other word, some active genes in PreOV should be repressed in InOV under the regulation of H3K4me3 and H3K27me3 to comply with relative biological processes in follicles during puberty initiation.

Differential peaks annotation showed in Fig. 5 that most of differentially peaks showed intergenic modifications of H3K4me3 or H3K27me3 in PreOV and InOV, and the fewest peaks were promoter modification of H3K4me3 or H3K27me3. The research indicated that the most HCNES, defined as bivalent domains including a large H3K27me3-modified region encompassing a smaller H3K4me3-modified region, encode key TFs that regulate embryonic development [29]. It pointed out that promoter modification of H3K4me3 and H3K27me3 related to DNA methylation [33] and chromatin compressing [34] to regulate gene transcription.

The different lengths of the H3K4me3- and H3K27me3-modified peaks were interesting. Figure 7 presented the relationship between differentially enriched peak length and fold change (InOV vs. PreOV). Higher fold changes were observed among H3K4me3-enriched peaks, H3K4me3-depleted peaks and H3K27me3-depleted peaks 1 kb in length, i.e., shorter peaks had a higher fold change in enrichment. However, the fold change in H3K27me3-enriched peaks was dispersed over the 1–2 kb range, which indicates that most genes acquired H3K27me3 modification during the process of puberty. Zhang *et al.* noted that gene transcription and biological function were related to H3K4me3 peak width [35]. The results of qRT-PCR (Fig. 8) for the top three-fold change of H3K4me3-enriched, -deleted genes and H3K27me3-enriched, -deleted genes confirmed the function of H3K4me3-enriched modification was to upregulated gene transcription and H3K27me3-enriched modification was to repressed gene transcription [29].

According to the Venn of H3K4me3 and H3K27me3 differential modification (Fig. 6), it showed the regulation of histone posttranslational modification were in various sites. Not only different kinds of histone methylation [36], but also histone methylation and acetylation cooperated to regulated biology process [37]. The GO and KEGG conjoint analysis of the 88 genes with 4 kinds of differential modification of H3K4me3 and H3K27me3 participated in multifunction pathways relating to puberty. Approximately 16% of the genes (14 of 88), including *FOXO3*, *RUNX1*, *NOBOX*, *DMRT1*, *MYC*, *LEPTIN*, *RPL10*, *BDNF*, *TERT*, *FRZB*, *CD47*, *ACVR2*, *HSP90AB1* and *BMPRI1B*, were involved in female gamete development associated with ovarian follicle development, ovulation, ovulation cycle process and female gonad development (Fig. 10). *FOXO3* is critical for oocyte reserve and female fertility [38, 39]. *RUNX1*, a TF in periovulatory follicles, is induced by an LH surge to transactivate *Ptgs2*, which is essential for ovulation [40]. *Oogenesis homeobox NOBOX* encodes a TF that plays a role in folliculogenesis and the regulation of oocyte-specific genes, among which the target *Rsp2* is essential for follicular development [41].

DMRT1 is a testis-specific TF associated with spermatogenesis and male infertility [34], and H3K4me3 and H3K27me3 modification might maximally inhibit the transcription of this gene in ovaries. The results clearly support future studies on all the above genes to investigate their function in the initiation of puberty in porcine ovaries.

The processes of female gamete generation and positive regulation of the bone mineralization pathway were correlation. The multifunctional nature of E2 leads to its involvement in different biological processes. The effect of E2 on the apoE receptor during osteoblast mineralization involves regulating the LDLR family, including LRP5, LRP6, LRP4, and ApoER2 [42]. In addition, BMPR1B is involved in fertility and follicular reserve and is strongly correlated with the FSH receptor in young women but not in old women. BMPR1B has a weak correlation with the LH receptor in young women but a strong correlation in old women [43]. ACVR2 knockdown in vivo inhibited its ability to maintain FSH homeostasis through the HPG axis in male mice [44].

Fatty acid biosynthesis, metabolism, and transport were enriched by 88 differentially expressed genes or differentially enriched promoters. We speculated that pathways related to fatty acid biosynthesis, metabolism and transport are essential for the initiation of puberty. Lipid droplets in steroidogenic tissues, such as ovaries enriched with PLIN1c, PLIN2, and PLIN3, have a cholesteryl ester (CE), which uses free cholesterol (FC) as the preferred cholesterol substrate for steroidogenesis, and HSL is the major neutral cholesterol esterase mediating the conversion of CE to FC [45]. The related genes include *LEPTIN*, *FABP3*, *CROT*, *STARD4*, *PLIN2*, *FRZB*, *SULT1E1*, *KLF5*, *DECR1*, *ETFA*, *HADHA*, and *HADHB*. *LEPTIN* is primarily expressed in fat and acts as a bridge between female gamete generation and the fatty acid transport pathway, which has been identified to be related to puberty [46]. *STARD4* participates in both “Regulation of steroid biosynthesis process” and “Regulation of steroid metabolic process” in granulosa cells and is important for oogenesis, folliculogenesis, ovulation, and pregnancy [32]. *SULT1E1* is an LH-inducible gene [40] and is involved in the inactivation of oestrogen after exogenous GnRH stimulation [47]. Above all, these results broaden the role of fatty acid pathways in the onset of puberty, including the regulation of steroidogenesis, steroid metabolism, and oestrogen activity, and the associated gene expression was regulated at the chromatin level by histone posttranscriptional modification, i.e., H3K4me3 and H3K27me3.

Ovarian GCs are critical components and functional units in ovarian follicles. Synergism between granulosa cells and oocytes is the precursor to the initiation of puberty and maintains the normal oestrus cycle and reproduction. It was confirmed that interfering endogenous H3K4me3 or H3K27me3 influenced pGCs proliferation and apoptosis (Fig. 11). The health of GCs determines follicle fate [31] and steroid biosynthesis and metabolism [32].

Conclusions

The genome-wide modified profile of H3K4me3 and H3K27me3 showed that H3K27me3 modification was more active than H3K4me3 modification during the onset of puberty in porcine ovaries. H3K4me3

and H3K27me3 acted as activator and repressor to regulate gene transcription that are related to porcine puberty initiation process. Genes including *FOXO3*, *BMPR1B*, *LEPTIN*, *RUNX1*, involved in networks of female gamete generation pathway associating with ovarian follicle development, ovulation, ovulation cycle process and female gonad development, which showed the further direction to investigate the function of H3K4me3/H3K27me3-targets in puberty initiation.

Abbreviations

H3K4me3: Histone 3 lysine 4 tri-methylation; **H3K27me3**: Histone 3 lysine 27 tri-methylation; **ChIP-Seq**: chromatin immunoprecipitation followed by sequencing; **pGCs**: porcine granulosa cells; **PreOV**: pre-puberty ovarian follicles; **InOV**: in-puberty ovarian follicles; **HPG** axis: hypothalamic-pituitary-gonadal axis; **SF**: small follicles; **MF**: medium-sized follicles; **BF**: big-sized follicles; **GF**: graafian follicles

Declarations

Ethics approval and consent to participate

The animal experiments were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China) and were approved by the Animal Care and Use Committee of South China Agricultural University, Guangzhou, China (approval number: 2018B116).

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yuyi zhong designed experiment, interpreted data and wrote manuscript. Yingting He is responsible for cell and biological experiments, and paper revision. Xiaolong Yuan analyzed data, and paper revision. Shuqi Diao, and Qingqing as helper in software analysis and graph visualization. Xiaofeng Zhou participated in samples collection and manuscript revision. Zhe Zhang contribute to paper revision. Hao Zhang and Jiaqi Li confirmed experiments designment, paper structure and paper revision. All authors read and approved the final manuscript.

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Figures

Figure 1

Different H3K4me3 and H3K27me3 peaks. The H3K4me3 and H3K27me3 peaks identified by MACS were classified into 6 groups by length: <1 kb, 1-2 kb, 2-3 kb, 3-5 kb. (A, B), H3K4me3 peaks in the PreOV and InOV groups. (C, D), H3K27me3 peaks in the PreOV and InOV groups.

Figure 2

Genome scale of H3K4me3 and H3K27me3 modification in PreOV and InOV. The circle inside showed the H3K4me3 and H3K27me3 modified sites in PreOV, and the circle outs indicated the H3K4me3 and H3K27me3 modified sites in InOV. Moreover, the black lines were H3K4me3 modification, and the red lines were H3K27me3 modification.

Figure 3

Venn diagram of H3K4me3 and H3K27me3 modification in the PreOV and InOV groups. (A), Common and specific genes with H3K4me3 modification in PreOV and InOV. (B), Common and specific genes with H3K27me3 modification in PreOV and InOV. Pre-K4 and In-K4 indicate genes with H3K4me3 modification in the PreOV and InOV groups, respectively. Pre-K27 and In-K27 indicate genes with H3K27me3 modification in the PreOV and InOV groups, respectively.

Figure 4

Venn diagram of specific and bivalent H3K4me3 and H3K27me3 modifications in the PreOV and InOV groups. (A), Genes with specific and bivalent H3K4me3 and H3K27me3 modification in the PreOV group. (B), Genes with specific and bivalent H3K4me3 and H3K27me3 modification in the InOV group. (C), Specific and common genes with bivalent modifications in the PreOV and InOV groups. The red circle represents the H3K4me3-modified genes in the PreOV or InOV group. Pre-K4 and In-K4 indicate genes with H3K4me3 modification in the PreOV and InOV groups, respectively. Pre-K27 and In-K27 indicate genes with H3K27me3 modification in the PreOV and InOV groups, respectively.

Figure 5

Annotation of H3K4me3 or H3K27me3 on genomic DNA. (A, B), the percentage of H3K4me3 binding in the upstream, promoter, exon, intron and intergenic regions in the PreOV and InOV groups. (C, D), The percentage of each binding location for H3K27me3 in the PreOV and InOV groups.

Figure 6

Venn diagram of differential H3K4me3 and H3K27me3 modification in the PreOV vs. InOV groups. K4 up (blue), K4 down (red), K27 (green) and K27 (yellow) indicate significant differential H3K4me3 enrichment, H3K4me3 depletion, H3K27me3 enrichment and H3K27me3 depletion.

Figure 7

The relationship of peak length and fold change in H3K4me3 and H3K27me3 modification. (A-D), Fold change for significantly differentially enriched peaks 1-25 kb in length in the H3K4me3-enriched, H3K4me3-depleted, H3K27me3-enriched and H3K27me3-depleted groups. (E-H), Fold change for peaks 1-2 kb in length in the H3K4me3-enriched, H3K4me3-depleted, H3K27me3-enriched and H3K27me3-depleted groups.

Figure 8

qRT-PCR analysis of genes with significant differentially H3K4me3 or H3K27me3, or both modifications. Some of genes were the top three differentially modified genes in the H3K4me3-enriched, H3K4me3-depleted, H3K27me3-enriched and H3K27me3-depleted groups, including ENY2, SPATA24, CPE, COPS2, ACTR3, CHBG, GPATCH2L. Some of genes were associated with the 4 classifications of differential H3K4me3 and H3K27me3 modification in follicles of different grades. MF, BF and GF indicated 3-5 mm in diameter medium follicles, 5-8 mm big follicles and > 8 mm graafian follicles, respectively.

Figure 9

Network analysis of GO terms and KEGG pathways enriched in genes with differential H3K4me3 or H3K27me3 modification in promoter region. The three coloured nodes correspond to three pathways: BMP2-WNT4-FOXO1 pathway in human primary endometrial stromal cell differentiation, adenyl-nucleotide exchange factor activity and mitochondrial fatty acid beta-oxidation of unsaturated fatty acids. The 7 GO terms in purple were enriched in the genes in red.

Figure 11

Cell proliferation and apoptosis function of H3K4me3 and H3K27me3 (A, C), pGCs cell proliferation as detected by EdU. (B, D), pGCs cell apoptosis as detected by flow cytometry with annexin V/PI dual-staining. H3K4me3 antagonist, antagonist NC, agonist, and agonist NC were 150 nM BCI-121, 150 nM DMSO, 2 nM PBIT, and 2 nM DMSO. H3K27me3 antagonist, antagonist NC, agonist, and agonist NC were 6 nM GSK-126, 6 nM DMSO, 2 nM GSK-J4, and 2 nM DMSO.

Supplementary Files

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- [TableS2Datasofpeakcalling.xlsx](#)
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- [FigureS2ChromosomalmapofInOV.svg](#)
- [FigureS1ChromosomalmapofPreOV.svg](#)