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RESEARCH ARTICLE

RNA-Seq Transcriptome Analysis to Unravel the Gene Expression Profile of Ovarian Development in Xiangxi Cattle

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A B S T R A C T

The molecular mechanisms which regulate the developmental processes of ovary and result in increased reproductive efficiency of beef cattle need further investigations for optimum beef production. The study was designed to screen the signaling pathways and to investigate the genes related to development of ovarian tissues of Xiangxi cattle at different age (6, 18 and 30 months) by transcriptome sequencing and bioinformatics analysis. Illumina II high-throughput sequencing technology was used to seq transcriptomes of nine Xiangxi cattles ovarian tissues at different ages. The total number of ovarian genes assembled were compared with reference genome sequences, differentially expressed gene analysis, KEGG and GO enrichment analysis and finally the candidate genes screening were performed. The results showed that about 68.51 Gb clean data was obtained and clean data of each sample reached to the 6.10 Gb. A total of 26243 expressed genes were screened, of which 4866 genes were new and have not been annotated in any database. A total of 594 differentially expressed genes were annotated between 6 and 30-months groups. Similarly, a total of 141 differentially expressed genes were annotated between 18and 30-mths groups. Among these, the members of G-protein coupled receptors family like follicle-stimulating hormone receptor (FSHR) and Insulin-like 3 peptides (INSL3) participate in signaling pathways. These results suggest that FSHR and INSL3 may be related to the reproductive development and reproductive performance of ovarian tissues in Xiangxi cattle and play an important role.

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INTRODUCTION

Xiangxi cattle is one of the famous beef breeds in China, which serves as major source of beef production in the country (Zhang *et al.,* 2015). However, current beef production is not meeting the demands of the growing population of China (Rahman *et al.,* 2019). The main reason of slower growth of beef industry in China is the low fertility rate in indigenous beef cattle. Previously, traditional selection has been used to improve the fertility, but sex-limiting nature and lower reproductive heritability of indigenous breeds were held responsible for slow growth of beef industry (Haskell *et al.,* 2014). However, exploring the candidate genes linked to ovarian development, ovulation and multiplets may drastically improve the genetics of beef animals through gene and marker-assisted selection (Chen *et al.,* 2015). Therefore, improvement in beef production through modern breeding can easily fulfill the beef requirement of growing population of the developing countries in general and specifically for China.

In animal's breeding and reproduction, primary reproductive organs of female animals are ovaries. Hence, understanding of gene expression and regulation in ovaries are most important to enahnce the reporoductive performance of the animals. Previous transcriptomic experiment in ruminant's ovaries explored fertility related characteristics of different breeds and played an important role in the field of developmental biology (Miao *et al.,* 2016; Yuan *et al.,* 2020). However, previous transcriptomic experiments focused on microarray analyses and effectively explored biological pathways at

the molecular level (Eklund *et al.*, 2006). It has been reported in recent studies that, differently expressed genes related to ovarian development (mRNAs), play a significant role (Franchi *et al.*, 2020; Shabbir *et al.*, 2021). These genes undergo significant biochemical changes during the sexual maturity (Hummitzsch *et al.*, 2019). However, the expression profiles of differentially expressed genes especially that related to ovarian development are still unclear. The advent of high-throughput sequencing-based RNA-Seq technology lay a foundation for the application of transcriptomics in animal research (Ji and Sadreyev, 2018). It is one of the main research directions in animal reproduction for analyzing the relationship between mammalian ovarian gene expression and its physiological function by applying transcriptome technology (Sha *et al.*, 2018).

In this study, ovarian tissues from Xiangxi cattles of 6, 18, and 30 months old, were collected, and mRNA transcriptomic analysis was performed. High-throughput transcriptome sequencing analysis was performed using RNA-Seq technology and expression of related genes for ovarian development were screened. It will provide a base for further understanding the environmental adaptability, molecular breeding, and reproductive performance of Xiangxi cattle.

MATERIALS AND METHODS

A total of nine Xiangxi cattles of 6,18 and 30 months of age were slaughtered and ovarian tissues were collected and placed above liquid nitrogen at -70℃ for extraction of total RNA from ovarian tissues.

RNA quantification and qualification: A total amount of 1 μg RNA per sample was used as an input material for the RNA sample preparations. NEB Next Ultra TM Ribonucleic Acid Library Prep Kit for Illumina (NEB, USA) was applied to create seq libraries by following manufacturer's instructions. Polymerase chain reaction was carried out with Phuision High-Fidelitiy DNA polymerase, Index Primer and Universal PCR primers. At last, PCR products were purified (AMPure XP system) and libraries' qualities were assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing: RNA-Seq analysis was performed by using BMK cloud (www.biocloud.net). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Quality control: Initially, in-house perl scripts were performed for the processing of the raw data (raw reeds) of fastq structure. In the next step, adaptor sequence and lower quality sequences reeds were deleted from the processed raw data sets. After processing, raw sequences obtained were further transformed into clean reeds and clean reeds were than mapped to the reference genome sequences. Furthermore, reeds with exact match or at least one mismatch were again analyzed and annotated on the basis of reference genome. To map with reference genome Hisat2 tools software was used.

Gene expression level and quantification were estimated by fragments per kilobase of transcript per million fragments mapped.

Differential expression analysis: DEGs (differential expression analysis) of age groups of Xiangxi cattle was carried out by using the DE seq. The resulting P values were adjusted by using the Benjamini and Hochberg's approach for controlling the false discovery rate. Resultant genes with corrected P-value <0.01 were obtained by DE seq and were designated as differentially expressed. EB seq was used for the expression analysis of Xiangxi cattle ovarian tissue samples. The FDR <0.05 & Folded Changes ≥1.0 were considered as the edge for significant differential expression.

GO and KEGG pathway enrichment analysis: Gene Ontology (GO) enrichment analysis for the DEGs was further carried out by the GO seq R package which relay on the Wallaenius non-central hypergeometric distribution (Yong *et al.*, 2010) and used to adjust the gene length bias in DEGs. KOBAS software was used to assess the statistical enrichment of DEGs in KEGG pathways.

Determination of protein-protein interaction: The sequences of the DEGs were determined by BLAST (blastx) and compared with the genome of related species (the protein-protein interaction of these exist in the STRING database: http://string-db.org/) to get the predicted PPI of these DEGs. Then the PPI of these DEGs were visualized in Cytoscape (Shannon *et al.*, 2003).

Confirmation of DEGs by qPCR: In the current experiment, 16 DEGs out of the total DEGs were arbitrarily chosen for qPCR analysis to compare the expression profiles taken from RNA-Seq, which was similar with our RNA-Seq (Quantification) findings. Therefore, RNA-Seq obtained in this can be effectively used to perform DEGs analysis of mRNA.

RESULTS

Quality detection of Xiangxi cattle's total ovarian RNA and libraries: In the current study, nine Xiangxi cattle's ovarian tissues were used to extract total RNA and the concentration of extracted RNA was ≥10ng / μL, and the purity (260nm / 280nm) was above 2.0. These results showed that the integrity of the RNA was not compromised and thus it can be used for the next experiment. The qPCR method was used to accurately quantify the effective concentration of the library (the effective concentration of the library was> 4nmol / L). The results showed that a high-quality cDNA library was constructed. The results also showed, as presented in Fig. 1, that a high-quality cDNA library was obtained and constructed successfully.

Quality evaluation of Xiangxi Cattles' ovarian tissues transcriptome sequencing: The transcriptome analysis of the ovarian samples of Xiangxi cattle resulted in 68.51 Gb Clean Data. In the obtained data, the clean data of each

ovarian sample of Xiangxi cattle reached 6.10 Gb, and the percentage of Q30 bases was 92.47% and above (Table 1). The clean reeds of each ovarian sample of Xiangxi cattle were sequenced with selected reference genome, which showed efficiency of 94.53% to 95.59% (Table 2).

Table 1: Summary of clean data of each ovarian tissue sample

Samples	Clean reads	Clean bases	GC Content	%≥Q30
6 mths -1	28,209,282	8,405,959,378	49.80%	92.53%
6 mths -2	23,841,062	7,114,980,668	49.41%	92.47%
6 mths -3	27,123,982	8,082,114,280	51.55%	92.55%
18 mths -1	22,843,285	6,803,055,060	50.68%	92.83%
18 mths -2	22,750,192	6,774,880,420	49.67%	92.99%
18 mths -3	20,483,196	6,102,515,852	49.49%	92.65%
30 mths -1	28,085,427	8,365,875,200	49.83%	95.06%
30 mths -2	27,568,070	8,233,338,012	50.36%	94.88%
30 mths -3	28,939,247	8,627,933,306	49.63%	92.52%

Differential expression analysis of Xiangxi cattle ovarian tissue samples

Screening for differential expression: After differential expression analysis using DESeq2_EBSeq, the differentially expressed gene set among three age groups were obtained; after the FDR (P <0.05) correction and the parameter setting of the absolute value of FC≥1.0, comparison results of Xiangxi cattle at different ages were obtained. Volcano plots are presented in Fig. 2 which provide quick view of gene expression levels and statistically significant differences among the three age groups. The results showed that there were 594 differentially expressed genes between 6and 30-mths old cattles, of which 332 were up-regulated and 262 were down-regulated. Similarly, the 18and 30-mths old cattles had 141 differentially expressed gene, of which 93 were up-regulated and 48 down-regulated. Venn diagrams of the differentially expressed genes of 6-mths vs 30-mths and 18-mths vs 30-mths were elaborated as required (Fig. 2). The Venn diagram shows that a total of 59 genes between 6-mths vs 30-mths and between 18-mths vs 30-mths are common differentially expressed genes.

Functional annotation and enrichment analysis of differential gene: A total of 165 differential genes were annotated between 6 and 30-mths age groups, of which 118 were annotated in the GO database and 126 were annotated in the KEGG database. A total of 266 differentially expressed genes were annotated between 18and 30-mths age groups, of which 195 were annotated in the GO database and 170 were annotated in the KEGG database.

Protein-protein interaction (PPI) network analysis of DEGs: To explore further understanding about the DEGs biological relevance, protein-protein interaction (PPI) network analyses of DEGs was performed by using STRING database. The protein-protein interaction network was observed by using Network X package in Python. Protein-protein interaction network analyses of DEGs between groups of 6-mths vs. 30-mths are presented in Fig. 3A and 18-mths vs. 30-mths are presented in Fig. 3B which were performed on DEGs with FDR ≤ 0.05 and |Log2FC| ≥ 1. The results of PPI represent that DEGs, FSHR and INSL3 play key roles in maintaining the tight connection of the whole network.

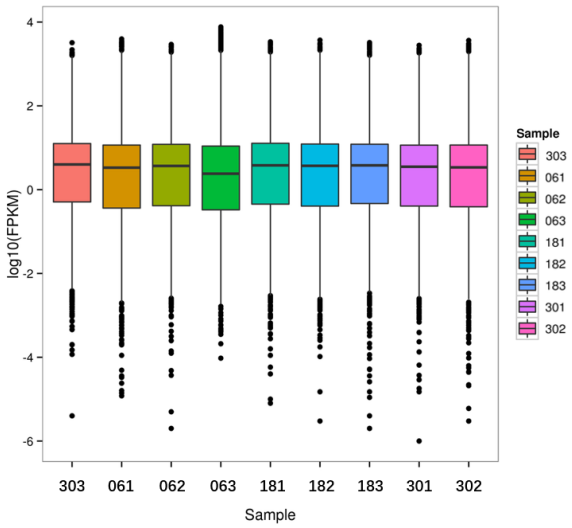

Fig. 1: The abscissa is ovarian samples, ordinate is Lg(FPKM+1) in the gene expression level distribution box plot, and shows the maximum, upper quartile, median, lower quartile and minimum.

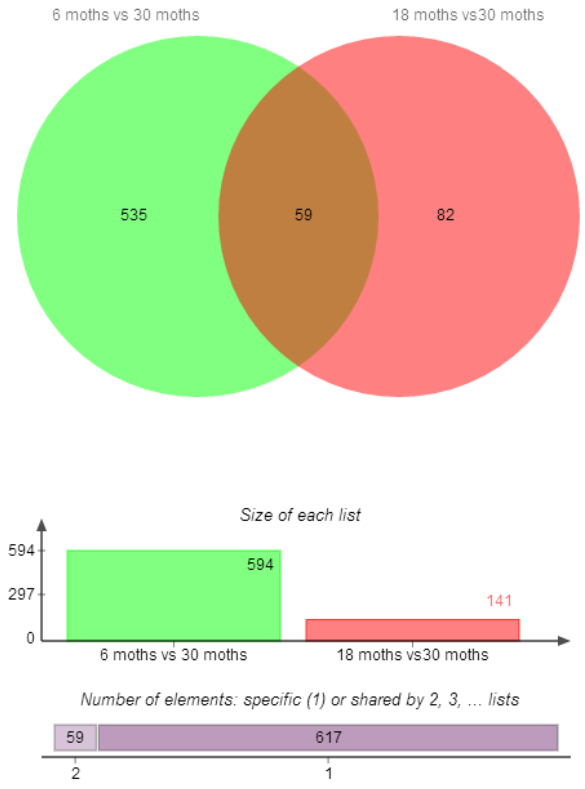

Fig. 2: Vune map of all different expressed gene in ovarian tissues of different age groups.

Confirmation of differential gene expression by qPCR: In the current experiment, 16 DEGs out of the total DEGs were arbitrarily chosen for qPCR analysis to compare the expression profiles taken by RNA-Seq. The results explored that GLP1R, MAP6, DSP, CLMN were upregulated and SNRNP27, DNAJC22, GSTA2, HSD17B1 were down-regulated in groups of 6-mths vs. 30-mths old cattles (Fig. 5A). CLMN, AGER, LECT1, PPAPDC2 were upregulated and INSL3, CHI3L2, PODXL2, APOA4 were down-regulated in groups of 18-mths vs. 30-mths old cattles (Fig. 5B). Therefore, RNA-Seq obtained in this study can be effectively used to perform DEGs analysis of mRNA.

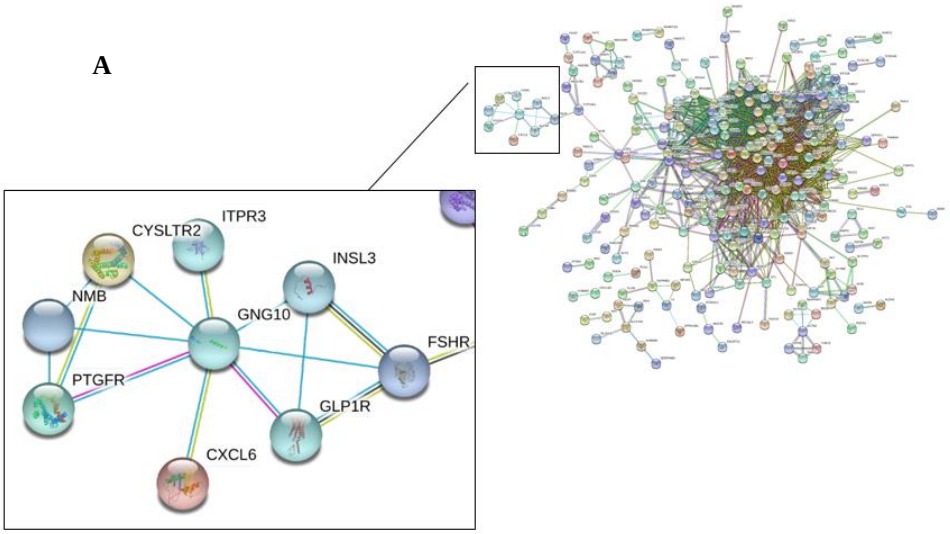

Fig. 3 A: PPI subnetworkl of differentially expressed genes of ovary tissue in 6-mths vs 30-mths.

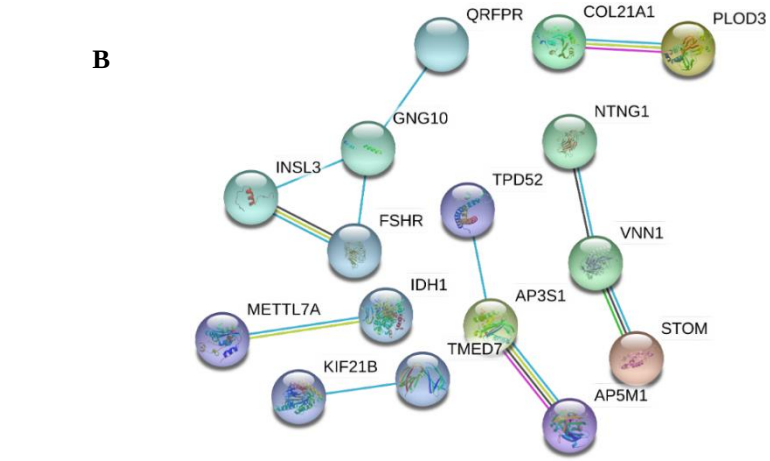

Fig. 3 B: PPI subnetworkl of differentially expressed genes of ovary tissue in 18-mths vs 30-mths.

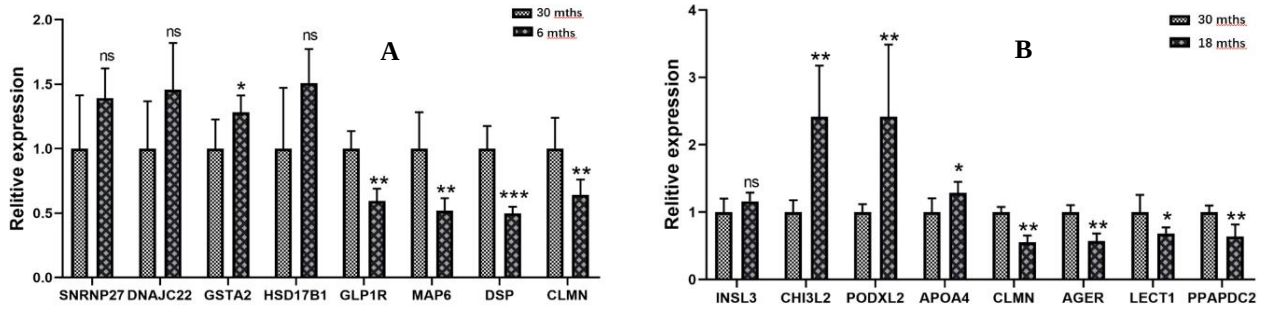

Fig. 4: A and B. Validation of the RNA-Seq (Quantification) results by qPCR. Results represent the mean (±SD) of ovarian tissue of Xiangxi cattle at six, nine and 30 month of age. P≤0.05.

Table 2: Summary of read mapping of ovarian tissues samples

Samples	Total Reads	Mapped Reads	Uniq Mapped Reads	Multiple Map Reads
6 mths -1	56,418,564	53,612,089 (95.03%)	51,787,398 (91.79%)	1,824,691 (3.23%)
6 mths -2	47,682,124	45,241,324 (94.88%)	44,232,447 (92.77%)	1,008,877 (2.12%)
6 mths -3	54,247,964	51,728,378 (95.36%)	50,003,075 (92.18%)	1,725,303 (3.18%)
18 mths -1	45,686,570	43,131,855 (94.41%)	41,933,967 (91.79%)	1,197,888 (2.62%)
18 mths -2	45,500,384	43,493,772 (95.59%)	42,483,252 (93.37%)	1,010,520 (2.22%)
18 mths -3	40,966,392	38,726,893 (94.53%)	37,789,613 (92.25%)	937,280 (2.29%)
30 mths -1	56,170,854	53,616,464 (95.45%)	52,338,247 (93.18%)	1,278,217 (2.28%)
30 mths -2	55,136,140	52,673,589 (95.53%)	51,417,975 (93.26%)	1,255,614 (2.28%)
30 mths -3	57,878,494	55,287,123 (95.52%)	54,163,986 (93.58%)	1,123,137 (1.94%)

DISCUSSION

Cattle fertility is restricted by various reasons, such as low heritability, long breeding cycle and the complex relationship among the growth traits. Therefore, in order to increase reproduction rate and mining special genetic traits in indigenous beef cattle, it is important to explore the molecular mechanism regulating the developmental processes of ovary, particularly focusing on the function of ovary-specific transcription factors and signaling pathways of local cattle breeds.

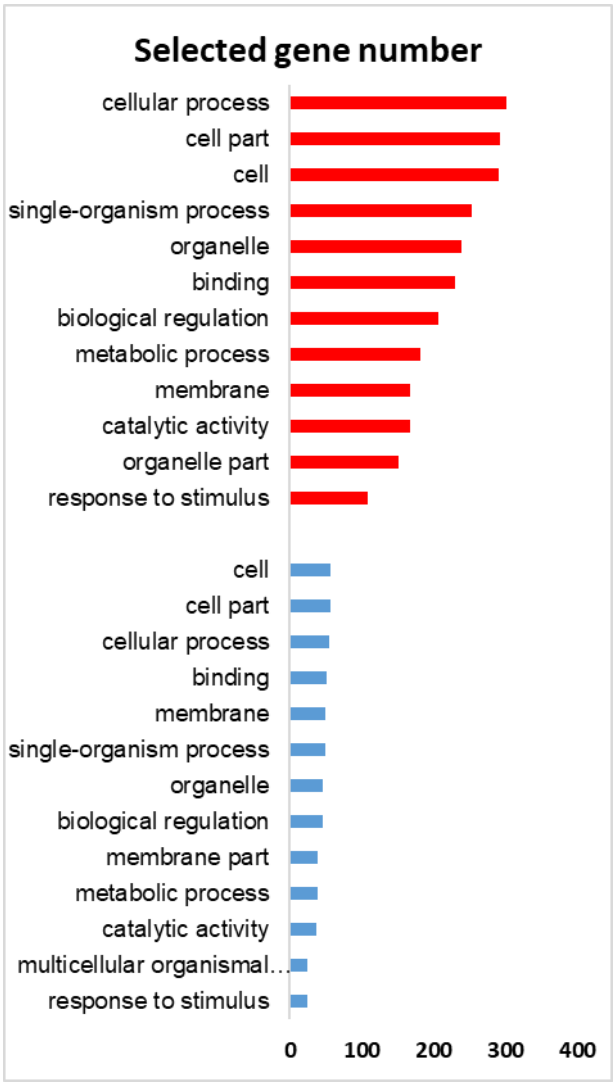

Fig. 5: GO enrichment histogram of expressed genes（TOP 12). Red: The GO enrichment histogram of expressed genes in 6-mths vs 30-mths age group; Black: The GO enrichment histogram of expressed genes in 18-mths vs 30-mths age group.

Studies related to ovarian transcriptome sequencing and animal reproduction have been reported in ducks (Ren *et al.*, 2019), sheep (Sinha *et al.*, 2020), pigs (Yang *et al.*, 2020), and yaks (Lan *et al.*, 2014). In this experiment, half-year-old (ovaries begin to develop), 18 months old (during the early puberty) and 30 months old (ovaries fully matured) were slaughtered to obtain ovarian tissues and RNA-Seq and bioinformatic analysis were performed to explore the molecular markers' information related to reproductive performance of Xiangxi cattle. For the screening of differentially expressing genes in ovarian tissues of Xiangxi cattle three groups of animals were selected having 6, 18 and 30-months of ages. A total of 26243 expressed genes were screened, of which 4866 genes were new and not annotated in the database.

In order to reveal the related functions of expressed genes, GO analysis was performed on the differentially expressed genes obtained by screening. In 6-mths vs 30 mths and 18-mths vs 30 mths comparison groups, the most enriched molecular function of GO were cellular process, cell part, and cell (Fig. 5). In general, the completion of important physiological processes in an organism often requires the participation of multiple genes, each of which performs related functions and undergoes a series of regulations. When studying the occurrence of a phenomenon in the living organism, it is important to clarify the signaling pathways involved in important physiological functions. Therefore, the KEGG signal pathway analysis was performed on the basis of GO analysis. In 6-mths vs 30 months comparison group, the differentially expressed genes were present in large numbers which were related to animal reproductive traits, such as Oocyte meiosis, Glutathione metabolism, p53 signaling pathway and Ovarian steroidogenesis (Fig. 6). Similarly, previous studies have also reported the differentially expressed genes related to animal reproductive traits like Oocyte meiosis (Jaffe and Egbert, 2017), Glutathione metabolism (Menezo *et al.*, 2016), p53 signaling pathway (He *et al.*, 2021) and Ovarian steroidogenesis (Bloom *et al.*, 2016). In 18-mths vs 30 months comparison group, the differentially expressed genes were also found to be many and related to animal reproductive traits like Glutathione metabolism and Arachidonic acid metabolism similar to previously conducted studies, Glutathione metabolism (Menezo *et al.*, 2016) and Arachidonic acid metabolism (Leng *et al.*, 2019) in heifer and mature animals. This shows that during the development and growth processes in Xiangxi cattle, there may be differences in endocrine regulation, which is reflected both in 18 months and 30 months old animals' ovary of Xiangxi cattle.

We noted that the expression levels of some G-protein coupled receptors (GPCRs) varied significantly between age groups. G-protein coupled receptors are made up of the largest superfamily of integral membrane protein receptors. It has been reported that some of the GPCRs are responsible for sensing the plethora of endogenous ligands that play a key role for the functioning of animals' endocrine system (Tse and Wong, 2019). It has also been reported that gonadotropin releasing hormone (GnRH) also contribute in the key functioning of mammalian reproduction by binding to cell surface transmembrane GPCRs and it also activates Gq/11 subfamily of G proteins (Ramakrishnappa *et al.*, 2005).

Previous study has also demonstrated that cellular pathway of follicle-stimulating hormone (FSH) and follicle-stimulating hormone receptor contribute in reproduction of mammals (Papadimitriou *et al.*, 2016). Follicle-stimulating hormone is also known to play significant role in mammalian reproduction and FSH performs its function on binding to its cognate FSHR expressed on the surface of target cells. It has been reported in a recent study that FSHR belongs to the family of GPCRs (Szymańska *et al.*, 2018). Previous study in human presented that FSHR is normally found in cells of the testis and the ovary of human, while it seldom expressed in other bodily tissues (Papadimitriou *et al.*, 2016). The FSHR was expressed in ovarian tissues at six month of age and it has been reported that FSH upregulates cell metabolism and activates MAPK signaling pathway in preantral follicles (Candelaria *et al.*, 2020), representing that FSH was the major contributor which upregulate the cell metabolism in the current study. It has been well established that BMP15 induces FSHR expression in granulosa cells through Smad and non-Smad pathways (Papadimitriou *et al.*, 2016). Therefore, in the

current study, this mechanism of FSHR induction by BMP15 may help in control of follicular growth in ovarian tissue of Xiangxi cattle. A recent study has also presented the functional roles for FSHR in placenta and in uterine myometrium (Shimizu *et al.*, 2019).

Insulin-like 3 peptide (INSL3) is well known member of the insulin-like peptide superfamily. A recent study reported that INSL3 is the only well-known physiological ligand of relaxin family peptide receptor 2 ((RXFP2), a GPCR) (Esteban-Lopez and Agoulnik, 2020)). Insulin-like 3 peptide is primarily produced both in testicular leydig cells and in ovarian theca cells of mammals, which involved in viviparity and internal fertilization and hence in the reproduction. It has also been reported that INSL3 is also produced by theca interna cells of growing antral follicles which is secreted into the bloodstream and is involved in the number and health of the follicles (Ivell and Anand-Ivell, 2018). INSL3 is essentially required for ovarian growth and follicular development and gene expression results confirmed that Insulin-like 3 peptide could be added to genes that control the reproductive characteristics in Xiangxi cattle. Because, previous studies confirm that loss of INSL3 or its receptors in females leads to partial infertility, with reduced follicular number, ovulations and litter size in knockout mice (Ivell and Anand-Ivell, 2018).

Some studies have reported that the expression levels of FSHR and INSL3 may have a certain correlation with the age of mammals. The circulation level of INSL3 is directly related to reproductive lifespan, and it start with puberty and decline at the menopause and hence play a significant role in physiology of different organs, especially the ovaries (Ivell and Anand-Ivell, 2018). Current study results are in agreement with the study of Ivell and Anand-Ivell, (2018) and represent that the amount of FSHR and INSL3 decreased with the age of cows. These results indicated that FSHR and INSL3 were negatively correlated with the growth and development of Xiangxi cattle. This result further suggests optimization of the existing processing programs for simultaneous estrus synchronization and superovulation in indigenous beef cattle.

Conclusions: In this study, Illumina II high-throughput sequencing technology was used to sequence transcriptomes of Xiangxi cattle ovarian tissues at different ages. The preliminary conclusion after analysis was that the differentially expressed genes control the signaling pathways including oocyte meiosis, glutathione metabolism, p53 signaling pathways, arachidonic acid metabolism and ovarian steroidogenesis. This is the first study to demonstrate the ovarian expressions of G-protein coupled receptors family in Xiangxi cattle, and the candidate genes FSHR and INSL3 were related to the reproductive development and reproductive performance of ovarian tissues in Xiangxi cattle.

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Authors contribution: AS and CH designed and conducted the study. WS, YL, and HL managed the cattle, collected, preserved and analyzed the ovarian tissue samples. XL, KY and HL performed RNA extraction and RNA SEQ high-throughput sequencing. BZ, JL and AS performed Pyrosequencing data analyses.

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