Analysis of Expression Profiles of mRNA and lncRNA in Oviduct During Estrus Phase of Sheep (Ovis Aries) with Two Fecundity (FecB Gene) Genotypes

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Research Article

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Abstract

Background:

In sheep, FecB is the essential biomarker of the fertility, previous researches have provided a detailed insight on the regulation involved estrus phase and FecB in the reproductive-related tissues including hypothalamus, pituitary, and ovary. However, as the host of embryo development and connection between the ovary and the uterus, little is known about the interaction between mRNAs and IncRNAs in sheep oviduct. In the present study, RNA-Seq was performed to identify the transcriptomic profiles of mRNAs and IncRNAs in oviduct during estrus phase of sheep with FecB^{BB/++} genotypes.

Results:

In total, 21,863 IncRNAs and 43,674 mRNAs were identified, 57 DE IncRNAs and 637 DE mRNAs were revealed in the comparisons between follicular phase and luteal phase, 26 DE IncRNAs and 421 DE mRNAs were revealed in the comparisons between FecB^{BB} genotype and FecB^{++} genotype. Functional enrichment analysis suggested that GO and KEGG terms related to reproduction such as SAGA complex, ATP-binding cassette (ABC), Nestin, and Hippo signalling pathway. DE-interaction network suggested that LNC_018420 maybe the key regulators related to embryo development in sheep oviduct.

Conclusion:

This was the first study to reveal the transcriptomic profiles of mRNAs and IncRNAs in the oviduct of FecB^{BB/++} sheep at estrus phase using RNA-Seq. Our findings can provide new understanding on the molecular mechanisms of mRNAs and IncRNAs underlying sheep embryo development and also opening new lines of investigation in sheep reproduction.

Background

The increasing needs for livestock industries to maximize economic benefits calls for molecular markers that enable identification of superior animals. In sheep industry, lambing trait is believed to be one of the most economically important traits, the molecular mechanism underlying sheep lambing trait has been a research hotspot since the 1980s[1, 2]. Bone morphogenetic protein receptor 1B (BMPR1B) is the first major candidate gene revealed strongly associated with the fecundity of Booroola sheep and possesses a mutation (A746G), known as Booroola fecundity (FecB), which results in one amino acid substitution (Q to R) increasing the ovulation rate of Booroola ewes[3, 4]. The FecB mutation has an additive effect on ovulation number and litter size, one copy of the FecB mutation can increase the ovulation number by 1.3–1.6 and the litter size by 0.9–1.2, and two copies by 2.73 and 1.1–1.7, respectively[5].

The evolution of transcriptomic technology driven by the high-throughput RNA Sequencing enabled in-depth research towards non-coding RNAs (ncRNA), which cannot translate into a protein and previously referred to as junk RNAs[6, 7]. Studies in ncRNAs have revealed various imperative biological roles in
mammals such as diseases[8], development[9], reproduction[10], etc. Long non-coding RNAs (IncRNA) is a kind of ncRNAs with lengths of more than 200 nt, notably, IncRNAs can act as microRNA (miRNA) sponges to regulate target gene expression in various biological activities[11–13]. Interaction between IncRNA and its target genes has become a hot topic in the search for novel practical targets in physiology[14] and disease[15], however, specific roles of IncRNAs in reproduction are still limited.

Recent observations indicated ewes with variations in FecB gene also potentially to produce singleton[16], hence, the molecular mechanism of FecB remains to be determined and warrants further investigation. Considering the essential roles of the IncRNAs in mammals, non-FecB mutation carrier (FecB++, mutant type, M) Small Tailed Han (STH) sheep and FecB mutation carrier (FecBBB, wild type, w) STH sheep were selected during follicular and luteal phases. RNA-seq was performed for the identification and characterization the transcriptomic profiles of IncRNAs and mRNAs in the oviduct. Furthermore, systematic analysis was conducted to add depth to our understanding of the molecular mechanism of FecB and screening for candidate IncRNAs/mRNAs that affecting sheep fertility.

**Results**

**Overview of Sequencing Results**

On average, 93,948,897 (MF), 93,872,202 (ML), 94,768,814 (wF), and 99,720,231 (wL) raw reads were obtained from the oviduct tissues, the clean reads were 91,719,898 (MF), 90,158,838 (ML), 91,784,720 (wF), and 96,577,435 (wL). The mapping rates of MF, ML, wF, and wL were calculated as 97.62%, 96.05%, 96.85%, and 96.84%, respectively. Details are shown in Additional file 1.

Based on CPC, CNCI, and PFAM, 19,969 novel IncRNAs were identified from the unknown transcripts (Fig. 1A), within which 57.3%, 34.8%, and 7.9% were revealed as intronic IncRNA, lincRNA, and antisense IncRNA, respectively (Fig. 1B). In addition, 1,894 annotated IncRNAs and 43,674 mRNAs were also revealed. Overall, 21,863 IncRNAs and 43,674 mRNAs were served as candidates for following analyses.

As Fig. 1C and 1D shows, the length of the IncRNAs was mainly between 200 bp-3,000 bp (1388 bp on average), the majority of IncRNAs have 2–8 exons (4 on average). Regarding mRNAs, the length was mainly distributed in the range of 100 bp -5,000 bp (3810 on average), the majority of mRNAs have 1–20 exons (13 on average), both of which were markedly greater than that of IncRNAs.

**Transcriptomic Profiling and DE Analysis**

FPKM was performed to estimate the expression level of candidate transcripts (Fig. 2), our results showed that mRNAs had a significantly higher expression level than that of IncRNAs (p < 0.05), and the expression level of candidate transcripts was relatively lower in follicular phase than luteal phase.

Based on FPKM, DEseq2 was performed to identify DE IncRNAs and DE mRNAs. In the comparisons between follicular phase (F) and luteal phase (L), a total of 45 (27 up- and 18 down- regulated), and 12 (4 up- and 8 down- regulated) DE IncRNAs were revealed between MF vs. ML (Fig. 3A) and wF vs. wL
(Fig. 3B), respectively. Regarding mRNAs, a total of 431 (269 up- and 162 down-regulated), and 206 (106 up- and 100 down-regulated) DE mRNAs were revealed between MF vs. ML (Fig. 3C) and wF vs. wL (Fig. 3D), respectively.

In the comparisons between \textit{FecB}^{BB} genotype (M) and \textit{FecB}^{++} genotype (w), a total of 14 (10 up- and 4 down-regulated), and 12 (3 up- and 9 down-regulated) DE lncRNAs were revealed between MF vs. wf (Fig. 4A) and ML vs. wL (Fig. 4B), respectively. Regarding mRNAs, a total of 214 (109 up- and 105 down-regulated) DE mRNAs were revealed between MF vs. wF (Fig. 4C) and ML vs. wL (Fig. 4D), respectively.

The Venn diagrams of the DE lncRNAs and DE mRNAs between different comparison groups are shown in Fig. 5. LNC_006270 and 4 mRNAs (C2CD5, EPB41L1, EML4, and VIRMA) were found DE in all comparisons. Details are provided in Additional file 2.

Furthermore, the heatmaps of DE lncRNAs and mRNAs (Fig. 6) were constructed to reveal the difference between the transcriptomic profiles of MF, ML, wF, and wL. Our results demonstrated that the transcriptomic profiles between follicular phase (F) and luteal phase (L) were remarkably different, however, the difference between \textit{FecB}^{BB} genotype (M) and \textit{FecB}^{++} genotype (w) were not significant.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis**

After obtaining DE lncRNAs and DE mRNAs, GO and KEGG analyses were conducted using the target genes of DE lncRNAs and DE mRNAs. Figure 7 and Fig. 8 shows part of the top enriched GO terms and KEGG pathways, details are provided in Additional file 3.

**GO and KEGG analysis using DE lncRNAs and mRNAs identified in follicular phase (F) vs. luteal phase (L)**

In the comparisons between MF and ML, cis-target genes of identified DE lncRNAs were found significantly enriched in 156 GO terms, top enriched GO terms in biological process (BP), cellular component (CC), and molecular function (MF), were organelle organization (GO:0006996), extracellular region (GO:0005576), and hydrolyzing O-glycosyl compounds (GO:0004553), respectively. Trans-target genes of DE lncRNAs were found significantly enriched in 156 GO terms, top enriched GO terms in BP, CC, and MF, were nitrate assimilation (GO:0042128), internal side of internal side of plasma membrane (GO:0009898), and binding (GO:0005488), respectively. DE mRNAs were found significantly enriched in 125 GO terms, top enriched GO terms in BP, CC, and MF, were cellular component organization or biogenesis (GO:0071840), extracellular region (GO:0005576), and binding (GO:0005488), respectively. Regarding KEGG enrichment analysis, cis-target genes of DE lncRNAs, trans-target genes of DE lncRNAs, and DE mRNAs, were found significantly enriched in 7, 19, and 14, KEGG pathways, respectively. Within
which, several important pathways related to reproduction were enriched, such as Hippo signaling pathway (oas04390), cAMP signaling pathway (oas04024), and circadian rhythm (oas04710), etc.

In the comparisons between wF and wL, cis-target genes of identified DE lncRNAs were found significantly enriched in 44 GO terms, top enriched GO terms in BP, CC, and MF, were response to chemical stimulus (GO:0042221), ATP-binding cassette (ABC) transporter complex (GO:0043190), and protein binding (GO:0005515), respectively. Trans-target genes of DE lncRNAs were found significantly enriched in 68 GO terms, top enriched GO terms in BP, CC, and MF, were G-protein coupled receptor signaling pathway (GO:0007186), peroxisome (GO:0005777), and transmembrane signaling receptor activity (GO:0004888), respectively. DE mRNAs were found significantly enriched in 111 GO terms, top enriched GO terms in BP, CC, and MF, were cellular response to stress (GO:0033554), photosystem II (GO:0009523), and ion binding (GO:0043167), respectively. Regarding KEGG enrichment analysis, cis-target genes of DE lncRNAs, trans-target genes of DE lncRNAs, and DE mRNAs, were found significantly enriched in 4, 3, and 6, KEGG pathways, respectively. Within which, several important pathways related to reproduction were enriched, such as PPAR signaling pathway (oas03320), MAPK signaling pathway (oas04010), and ECM-receptor interaction (oas04512), etc.

**GO and KEGG analysis using DE lncRNAs and mRNAs identified in FecB**<sup>BB</sup> genotype (M) vs. **FecB**<sup>++</sup> genotype (w)

In the comparisons between MF and wF, cis-target genes of identified DE lncRNAs were found significantly enriched in 77 GO terms, top enriched GO terms in BP, CC, and MF, were nitrogen compound metabolic process (GO:0006807), small-subunit processome (GO:0032040), and DNA binding (GO:0003677), respectively. Trans-target genes of DE lncRNAs were found significantly enriched in 425 GO terms, top enriched GO terms in BP, CC, and MF, were cellular process (GO:0009987), Golgi apparatus part (GO:0044431), and transferase activity, transferring phosphorus-containing groups (GO:0016772), respectively. DE mRNAs were found significantly enriched in 135 GO terms, top enriched GO terms in BP, CC, and MF, were organic substance metabolic process (GO:0071704), adherens junction (GO:0005912), and binding (GO:0005488), respectively. Regarding KEGG enrichment analysis, cis-target genes of DE lncRNAs, trans-target genes of DE lncRNAs, and DE mRNAs, were found significantly enriched in 4, 10, and 7, KEGG pathways, respectively. Within which, several important pathways related to reproduction were enriched, such as AMPK signaling pathway (oas04152), PI3K-Akt signaling pathway (oas04151), and GnRH signaling pathway (oas04912), etc.

In the comparisons between ML and wL, cis-target genes of identified DE lncRNAs were found significantly enriched in 118 GO terms, top enriched GO terms in BP, CC, and MF, were protein metabolic process (GO:0019538), nucleosome (GO:0000786), and calcium ion binding (GO:0005509), respectively. Trans-target genes of DE lncRNAs were found significantly enriched in 43 GO terms, top enriched GO terms in BP, CC, and MF, were urea transport (GO:0015840), membrane (GO:0016020), and hydrolase activity, acting on ester bonds (GO:0016788), respectively. DE mRNAs were found significantly enriched in
194 GO terms, top enriched GO terms in BP, CC, and MF, were heterocycle metabolic process (GO:0046483), cell part (GO:0044464), and ion binding (GO:0043167), respectively. Regarding KEGG enrichment analysis, cis-target genes of DE IncRNAs, trans-target genes of DE IncRNAs, and DE mRNAs, were found significantly enriched in 4, 4, and 6, KEGG pathways, respectively. Within which, several important pathways related to reproduction were enriched, such as p53 signaling pathway (oas04115), TNF signaling pathway (oas04668), and TGF-beta signaling pathway (oas04350), etc.

**DE-interaction network analysis**

In general, 18, 401 cis-target genes of 19,378 corresponding IncRNAs and 8,305 trans-target genes of 11,307 corresponding IncRNAs were predicted. Based on the results of DE analysis, 33 DE IncRNAs were found trans-target 137 DE mRNAs (Fig. 9). Within which, LNC_018420, LNC_016630, and LNC_013441 were quantified generating the highest number of connections with corresponding DE mRNAs (47, 21, and 20, respectively).

**Sequencing Data Validation**

Comparison of the relative expression level of the candidate IncRNAs and mRNAs selected for data validation between RT-qPCR and RNA-Seq are shown in Fig. 10. Collectively, selected IncRNAs and mRNAs showed similar expression patterns between RNA-Seq and RT-qPCR, indicating the reliability and repeatability of our sequencing data.

**Discussion**

Given that the ovulation rate of sheep can be genetically controlled by several major candidate genes[17, 18], there is an urgent need to identify novel biomarkers of prolicacy. Even though several transcriptomic researches associated with prolicacy on sheep hypothalamus[19, 20], uterus[10], ovary[16], etc. have been previously reported, there is no research focus on oviduct. As the connection between the ovary and the uterus[21], recent researches demonstrated that oviduct is essential in hosts fertilization and pre-implantation development of the embryo[22–24], however, the in-depth molecular mechanisms have not been elucidated. In the present study, an indigenous Chinese sheep with sheep (Ovis aries) breed with excellent lambing performance (year-round estrous, 2.61 lambs per year on average), STH sheep[25, 26] was selected, RNA-Seq was performed on oviduct to investigate the transcriptomic profiles during follicular phase and luteal phase in sheep with two FecB genotypes.

**Transcriptomic profiles**

In the present study, 21,863 IncRNAs and 43,674 mRNAs were identified in sheep oviduct, initially, FPKM was performed to establish the expression level, our results showed that majority of the candidate IncRNAs/mRNAs (>75%) were revealed with low expression level (average FPKM<5), candidate IncRNAs/mRNAs with average FPKM >500 account for about 0.4%. Within which, OVGP1 (Ovis aries oviductal glycoprotein 1) was obtained with highest FPKM. Numerous studies have provided a detailed insight on OVGP1 in enhancing the fertilization rate and embryo development in different species[27-30],
in the present study, the expression level of \textit{OVGP1} was remarkably higher in follicular phase than that in luteal phase, our results demonstrated the positive effects of \textit{OVGP1} on the reproductive efficiency in STH sheep. Regarding lncRNAs, LNC\_006353 was found with highest FPKM, notably, the expression levels of LNC\_006353 were higher in follicular phase than that in luteal phase, we hypothesize that LNC\_006353 may be an important regulator in embryo development and could potentially serve as candidate lncRNAs for researches on sheep oviduct.

57 DE lncRNAs and 637 DE mRNAs were revealed in the comparisons between follicular phase and luteal phase, 26 DE lncRNAs and 421 DE IncRNAs were revealed in the comparisons between \textit{FecB}^BB\ genotype and \textit{FecB}^++\ genotype. Our results demonstrated a clear separate between follicular phase and luteal phase, the DE lncRNAs/mRNAs revealed in the comparisons between \textit{FecB}^BB\ genotype and \textit{FecB}^++\ genotype were fewer, which implying a relatively steady transcriptomic state between two \textit{FecB} gene genotypes.

It's worth noting that 4 mRNAs: \textit{C2CD5} (C2 calcium-dependent domain containing 5), \textit{EPB41L1} (erythrocyte membrane protein band 4.1-like 1), \textit{EML4} (echinoderm microtubule associated protein like 4), and \textit{VIRMA} (vir like m6A methyltransferase associated) and LNC\_006270 were found DE in all comparisons. Several researches have been conducted to assess the influence of these genes on metabolic[31-33] and energy balance[34-36], however, little is known about the association between these candidates and reproduction, thus, we hypothesis that these genes may indirectly affect embryo development in sheep oviduct via metabolic progress.

In the comparisons between follicular phase and luteal phase, 18 mRNAs and 3 lncRNAs were obtained DE in both two comparisons. Within which, \textit{FSTL5} and LNC\_016628 were detected having a greater expression levels in follicular phase than luteal phase. \textit{FSTL5} belongs to Follistatin-like protein family, a kind of glycoprotein involved in various pathological and physiological processes[37], and played an important role in mammal pregnancy[38, 39]. Moreover, FSTL was isolated in combination with TGF-\(\beta\) (transforming growth factor beta) super family[40], key regulators in reproduction[41], which indicated that \textit{FSTL5} may also function as a connection between embryo development and oviduct. Regarding DE lncRNAs, LNC\_016628 was detected highly expressed in follicular phase specifically, and predicted target \textit{MARK3}, an receptor of gonadotropin releasing hormone (GnRH)[42], hence, we hypothesized that a regulatory relationship between LNC\_016628, \textit{MARK3}, GnRH, and embryo development may exist. Collectively, our results suggested that \textit{FSTL5} and LNC\_016628 may serve as potential biomarkers in embryo development, the specific mechanisms still require further careful characterization.

\textit{FecB} has been widely studied in the course of ovary and be proved to be key regulator gene in sheep reproduction[43]. In each estrus cycle, \textit{FecB} mutation caused partial inactivation of the TGF\(\beta\)/BMP signaling pathway and leading to increased ovulation rates[44]. In the comparisons between \textit{FecB}^BB\ genotype and \textit{FecB}^++\ genotype, the number of DE mRNAs/lncRNAs were relatively lower than that observed in comparisons between follicular phase and luteal phase, implying that the \textit{FecB} mutation have little influence on transcription stability at mRNA/lncRNA levels in sheep oviduct probably. Contrary
to present results, our previous transcriptomic research in sheep hypothalamus detected a remarkably
greater number of DE mRNAs and miRNAs in the comparisons between two FecB genotypes[20], the
difference in experimental tissues may be responsible for the results. 6 mRNAs and 1 IncRNA were
obtained DE in both two comparisons, none research have been found focused on the relationships
between these DE candidates and reproduction, thus, whether these genes participate sheep embryo
development remains unclear.

Functional Analysis

In the comparisons between follicular phase and luteal phase, most of the enriched GO terms of DE
candidates were closely related to reproduction process, such as SAGA complex and ATP-binding
cassette (ABC). Regulation of gene expression during embryo development requires cooperation between
histone-modifying enzymes and transcriptional factors, such as Gcn5 and PCAF, which were proved
integrated into a multi-subunit complex called SAGA[45]. ABC transporters shuttle a wide variety of
substrates and part in embryonic development in both direct or indirect way[46-48]. Furthermore, Zou et
al. found that DE genes were also were significantly enriched in ABC transporters in the comprehensive
transcriptomic research in ovarian follicles of uniparous and multiple goats[49]. Our findings indicated
that SAGA complex and ABC transporters may also serve as key factors in sheep embryo development.
Regarding KEGG analysis, Hippo signalling pathway was enriched in both two comparisons. Hippo
signalling pathway is known as a key driver in the preimplantation embryo development[50-52]. Several
researches have revealed that differential Hippo activity is highly correlated with inner cell mass and the
trophectoderm, the first two segregate during early embryo development in mammals[53, 54].
Furthermore, key transcripts of Hippo signalling pathway were detected during different stages in
preimplantation embryo development which highlight the diverse roles of Hippo signalling pathway in
embryo development[55, 56]. Collectively, it’s possible to speculated that Hippo signalling pathway could
influence reproduction by regulating embryo development in sheep oviduct.

In the comparisons between FecB BB genotype and FecB ++ genotype, cellular process involved in
reproduction (GO:0048610) and reproduction (GO:0000003) draws our attention as its close relationship
with embryonic development. Notably, NES (nestin) was found enriched in these GO terms, as the key
regulator in the neural response, most nestin-positive cells were found strongly positive for vimentin[57,
58], a differentiation marker in preimplantation embryos[59, 60]. Hence, the results from our study
indicated that FecB mutant may be involved in nervous system in sheep oviduct. Results of the KEGG
showed that majority of the enriched pathways were close related to metabolic pathways. Consistently,
several metabolic pathways were also found enriched in the RNA-Seq research in sheep hypothalamus
with different genotype[20]. However, TGFβ/BMP signaling pathway was not found enriched in both
comparisons. Considering the molecular regulation of FecB mutant, we hypothesis that FecB mutant may
probably function as regulator in the basic regulation of metabolic processes of non-ovary tissues in
sheep.

DE-interaction network
In the present study, DE-interaction network contains 33 DE lncRNAs with 137 target DE mRNAs was constructed, the topmost connected regulators were LNC_018420 (47) and LNC_016630 (21). Among the target genes of DE lncRNAs, some were also enriched in the GO term and KEGG pathway we mentioned above which related embryo development, such as ZNFs, and ADAMTs. ZNF (zinc finger proteins) constitute the largest superfamily of transcript regulators in mammals, numerous of ZNF genes are identified as imperative biomarkers in the development and differentiation of the nervous system[61, 62], which also proved to be interacted with multiple regulations in the embryonic development[63]. ADAMTs belongs to a group of proteins that have platelet-associated activity, recent research in rats demonstrated that ADAMTs may possibly affect the endometrial receptivity and resulting in implantation failure of the embryo[64]. Our finding suggests that these top lncRNAs with highest connections, especially LNC_018420, maybe the key regulators in the embryo development, their up- or down-regulation may exert different functions on sheep reproduction. Further studies need to be performed deeply to verify this.

**Conclusion**

Approximately 80 DE lncRNAs and 1,000 DE mRNAs were identified in the present research, further analysis indicated that OVGPI, FSTL5, LNC_006353, LNC_006270 may serve as candidates with potential function in the reproductive activities of oviduct. Several critical GO terms, pathways, and DE lncRNAs-DE mRNAs pairs were also revealed. The results of our research can provide a basis for understanding the transcriptomic mechanisms of mRNAs and lncRNAs in sheep oviduct between different FecB genotypes during follicular and luteal phases.

**Materials And Methods**

**Selection of Experimental Sheep, and Sample Collection**

890 Small Tail Han sheep feed in Yuncheng Breeding Sheep Farm (Yuncheng, Shanxi Province, China) were genotyped in pre-experiment, 6 sheep with the FecB ++ genotype and 6 sheep with the FecB BB genotype were identified and chosen for the following experiment, 3 FecB ++ sheep and 3 FecB BB sheep were slaughtered during follicular phase by euthanasia (KCl (1 mg/kg) intravenous under deep anesthesia using Pentobarbital Sodium (1.5 mg/kg)), and the remaining sheep were slaughtered during luteal phase by euthanasia. Oviduct tissues were collected and snap-frozen in liquid nitrogen and then stored at -80 °C for RNA extraction. Details about experimental sheep treatment, phenotype identification, and initial processing can be found in Zhang et al. [20].

**RNA Extraction, and Sequencing**

Total RNA was extracted from stored oviduct tissues of 12 sheep with TRIzol Reagent. Qubit 3.0 Flurometer and RNA Nano 6000 Assay Kit were used for examining the quality and quantity of isolated RNA, RNA integrity Number (RIN) was checked by Agilent 2100 bioanalyzer as the threshold of RIN ≥ 8.0.
Library construction for sequencing was performed with 5 μg of high-quality RNA. The lncRNA and mRNAs libraries was constructed with extracted RNA using the NEBNext™ Ultra™ RNA Library Prep Kit for Illumina® per the manufacturer's recommendations. LncRNA and mRNA libraries were then sequenced using a HiSeq 2500 platform (pair end, 150 bp), all the sequencing works were conducted by Novogene Technology Co., Ltd. (Beijing, China).

**Read Assembly**

The raw reads in FASTQ format generated by sequencing were subjected to filter for removing low-quality reads based on the following criteria: reads that contained adapters and N (the proportion of bases could not be identified >10%), and low-quality reads (base with sQ ≤ 5 accounts for more than 50% of the entire reads) were removed. Finally, cleaned reads were obtained and aligned to the *Ovis aries* reference genome (Oar_v3.1) using HISAT2[65].

Coding and non-coding RNAs from the transcripts were annotated with CNCI [66], CPC2 [67], and PFAM [68] software. Non-coding RNA candidates with lengths >200 nt, and exon numbers ≥ 2 were identified as candidate lncRNAs. StringTie [69] was applied for candidate mRNAs transcripts assemble.

**Differential Expression Analysis**

The fragments per kilobase per million mapped reads (FPKM) [70] method was used to estimate the expression levels of lncRNA and mRNA transcripts. DESeq2[71] was conducted to identify differential expressed (DE) lncRNA and mRNA transcripts in multiple comparison. lncRNA and mRNA transcripts were identified as significantly DE as the threshold of q value (p values adjusted by Benjamini and Hochberg's False Discovery Rate approach, FDR) <0.05.

**Target Genes Prediction of lncRNAs and DE lncRNA-mRNA Interaction network**

For an in-depth sight on transcriptomic profiles, target genes (cis- and trans-) of DE lncRNAs in multiple comparisons were predicted. Coding genes located 100 kb up-/down- stream of the corresponding lncRNAs were considered as cis-target genes. Besides, Pearson correlation coefficients was calculated between the expression level of multiple coding genes and corresponding lncRNAs. Coding genes were considered as trans-target genes as the threshold of |correlation| ≥ 0.95.

Based on the results of differential expression analysis and target genes prediction, DE lncRNA-mRNA interaction networks containing DE lncRNAs and DE mRNAs in different comparisons were constructed using Cytoscape software [72].

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analyses**

GO and KEGG pathway enrichment analyses were conducted using the DE mRNAs and target genes of the DE lncRNAs based on the results of differential expression analysis and target genes prediction. GO and KEGG enrichment analyses were performed using GOseq R library [73] and KOBAS (KO-Based
Annotation System)[74] programs, respectively. Followed by a Fisher's exact test with FDR multiple test correction, significant enriched GO term or KEGG pathway was determined as the threshold of p < 0.05.

Data Validation

Five IncRNAs and five mRNAs were randomly selected to verify the reliability and repeatability of our RNA-Seq data. The primers (Additional file 5) of selected IncRNAs, mRNAs, and reference gene beta-actin were designed using Primer Premier 5 software, and real-time quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate following the SYBR Green I method. \( 2^{\Delta \Delta Ct} \) method [75] was used to calculate the relative expression level. The results were shown as the fold change of relative expression level (mean ± standard error) using GraphPad Prism 6 software. The details about experimental RNA extraction, preparation of first strand of cDNA, and RT-qPCR conditions can be found in Zhang et al.[20]

Abbreviations

mRNA: messenger RNA

IncRNA: Long non-coding RNA

RNA-Seq: RNA Sequencing

STH: Small tail han sheep

F: follicular phase

L: luteal phase

M: Fec\(^B\)\(^{BB}\) genotype, mutant type

w: Fec\(^B\)\(^{++}\) genotype, wild type

FPKM: fragments per kilobase per million mapped reads;

DE: differentially expressed

GO: Gene ontology

BP: biological process

CC: cellular component

MF: molecular function

KEGG: Kyoto Encyclopedia of Genes and Genomes

RT-qPCR: Real-time Quantitative Polymerase Chain Reaction
Declarations

Ethics approval and consent to participate

All experimental procedures in this study were approved by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China (permit number: IAS2019-49). All efforts were taken to minimize pain and discomfort to the animal while conducting these experiments.

Consent for publication

Not applicable.

Availability of data and materials

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA658731.

Competing interests

The authors declare no conflicts of interests.

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Authors’ contributions

WHC, WS and MXC designed this study; MXC contributed to materials and data collection in this study. WHC and ZFL performed the experiments and analyzed the data; WHC wrote the manuscript; WS and MXC revised the final manuscript. All authors approved the final manuscript.

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Figures

Figure 2

FPKM distribution. (A) FPKM distribution of identified IncRNAs and mRNAs. (B) FPKM distribution of different groups.
Figure 3

Differentially expressed (DE) lncRNAs and mRNAs in the comparisons between follicular phase (F) and luteal phase (L). (A) Volcano plot of differentially expressed lncRNAs in MF vs ML, where red and green represent upregulation or downregulation, respectively, same below. (B) Volcano plot of differentially expressed lncRNAs in wF vs wL. (C) Volcano plot of differentially expressed mRNAs in the comparisons between MF vs ML. (D) Volcano plot of differentially expressed mRNAs in the comparisons between wF vs wL.
Figure 7

Top enriched GO and KEGG terms of DE mRNAs/ target genes of DE IncRNAs in the comparisons between follicular phase (F) and luteal phase (L). (A) Top enriched GO terms of cis-target genes of DE IncRNAs in MF vs ML. (B) Top enriched KEGG terms of cis-target genes of DE IncRNAs in MF vs ML. (C) Top enriched GO terms of trans-target genes of DE IncRNAs in MF vs ML. (D) Top enriched KEGG terms of trans-target genes of DE IncRNAs in MF vs ML. (E) Top enriched GO terms of DE mRNAs in MF vs ML. (F)
Top enriched KEGG terms of DE mRNAs in MF vs ML. (G) Top enriched GO terms of cis-target genes of DE lncRNAs in wF vs wL. (H) Top enriched KEGG terms of cis-target genes of DE lncRNAs in wF vs wL. (I) Top enriched GO terms of trans-target genes of DE lncRNAs in wF vs wL. (J) Top enriched KEGG terms of trans-target genes of DE lncRNAs in wF vs wL. (K) Top enriched GO terms of DE mRNAs in wF vs wL. (L) Top enriched KEGG terms of DE mRNAs in wF vs wL.

Figure 9

DE-interaction network, where red and purple represent lncRNAs and mRNAs, respectively.
Figure 10

Comparisons of the results of the RNA-seq and RT-qPCR analyses of DE lncRNAs and mRNAs. (A) MF vs ML. (B) wF vs wL. (C) MF vs wF. (D) ML vs wL.

Supplementary Files

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