

Whole Transcriptome Analysis: Implication to Estrous Cycle Regulation

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Research

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Abstract

Background

Estrous cycle is one of female characteristics after sexual maturity, including estrus (ES) and diestrus (DS) stages. Estrous cycle is important in female physiology and its disorder may lead to diseases. In the latest years, effects of non-coding RNAs and mRNA on estrous cycle start to arouse much concern, however, a whole transcriptome analysis among non-coding RNAs and mRNA has not been reported.

Results

Here we report a whole transcriptome analysis of goat ovary in estrus and diestrus periods. Estrus synchronization was conducted to induce the estrus phase and on day 32, the goats naturally shifted into diestrus stage. The ovary RNA of estrus and diestrus stages was respectively collected to perform RNA-sequencing. Then the circular RNA; microRNA; long non-coding RNA; mRNA databases of goat ovary were acquired, and the differentially expressions between estrus and diestrus stages were screened to construct circRNA-miRNA-mRNA/lncRNA and lncRNA-miRNA/mRNA networks, thus providing potential pathways that involved in the regulation of estrous cycle. Differentially expressed mRNAs, such as *MMP9*, *TIMP1*, *3BHSD* and *PTGIS*, and differentially expressed microRNAs, such as miR-21-3p, miR-202-3p and miR-223-3p, which play key roles in estrous cycle regulation were extracted from the network.

Conclusions

Our data provided the miRNA, circRNA, lncRNA and mRNA databases of goat ovary and each differentially expressed profile between ES and DS. Networks among differentially expressed miRNAs, circRNAs, lncRNAs and mRNAs were constructed to provide valuable resources for the study of estrous cycle and related diseases.

Background

Estrous cycle is one of female characteristics after sexual maturity, including estrus (ES) and diestrus (DS) stages. Studies show that disorder of estrous cycle participates in disease progression, such as ovarian carcinoma [1], anxiety [2, 3], epilepsy [4]. Recently, it is reported that neuronal chromatin organization related to neuronal function fluctuates with the estrous cycle in the brain [5], and rats in diestrus stage are easier to display memory impairment than those in estrus stage when undergo restraint and social isolation stress [6], indicating an importance of estrous cycle in physiological regulation. Disorder of estrous cycle is a dramatic symptom of polycystic ovary syndrome (PCOS), which disturbs adolescent females in reproductive ages [7]. In the latest years, effects of non-coding RNAs and mRNA on estrous cycle start to arouse much concern and be studied [8-11], however, joint transcriptome analysis among non-coding RNAs and mRNA has not been reported.

Non-coding RNAs consist of microRNA (miRNA), circular RNA (circRNA) and long non-coding RNA (lncRNA). MiRNAs are a class of endogenous non-coding RNAs at a length of about 20 nucleotides, regulating gene expression by promoting mRNA degradation or preventing translation [12]; CircRNAs, derived by non-classical alternative splicing, are another class of endogenous non-coding RNAs that can expropriate miRNAs as a sponge to block miRNAs from binding to target genes [13-15]; lncRNAs are a category of transcripts longer than 200 nucleotides without open reading frames that achieve functions through interacting with DNA, other RNAs, and proteins by base complementation or secondary structure generated by RNA folding [16].

Our study established miRNA, circRNA, lncRNA and mRNA databases of goat ovaries in estrus group (ES) and diestrus group (DS) and analyzed the differentially expressed ones for circRNA-miRNA-mRNA/lncRNA and lncRNA-miRNA/mRNA networks participated in estrous cycle. To make the networks concise, on the one hand, among the differentially expressed mRNAs, *matrix metalloproteinase 9 (MMP9)*, *tissue inhibitors of metalloproteinases (TIMP1)*, *3 β -Hydroxysteroid dehydrogenase (3BHSD)* and *Prostaglandin I₂ Synthase (PTGIS)* were selected out to screen the differentially expressed miRNAs and circRNAs that potentially regulated their expressions, constructing circRNA-miRNA-mRNA networks. MMP9 is one of matrix metalloproteinases participating in extracellular matrix deconstruction, while TIMP1 is one of their endogenous tissue inhibitors [17]; the equilibrium between MMPs and TIMPs is required for extracellular matrix remodeling during ovarian folliculogenesis [17-19]. 3BHSD is an enzyme involved in the synthesis of progesterone and testosterone [20], which plays essential parts in estrous cycle. PTGIS is a monooxygenase that catalyzes steroids synthesis and converts prostaglandin precursor into prostaglandin I₂, taking critical parts in reproductive processes [21, 22]. Among differentially expressed miRNAs, we found seven miRNAs that might target *TIMP1*, *3BHSD* or *PTGIS* but no miRNA that potentially targeted *MMP9*. Additionally, differentially expressed circRNAs that might sponge the seven miRNAs were screened. Finally, the circRNA-miRNA-mRNA network centered on *TIMP1*, *3BHSD* and *PTGIS* was constructed.

On the other hand, miR-21-3p-miR-202-3p and miR-223-3p were picked out to extract their circRNA sponges and target mRNAs/lncRNAs for their intense relation to women reproduction: miR-21-3p is associated with poor ovarian response to fertilization [23]; miR-202-3p controls female fertility and regulates oogenesis [24]; and miR-223-3p is involved in ovarian cancer invasion [25] and PCOS [26]. The predicted target mRNAs/lncRNAs and circRNA sponges of miR-21-3p-miR-202-3p and miR-223-3p in the differentially expressed database were screened in this study to build the circRNA-miRNA-mRNA/lncRNA network focused on miR-21-3p-miR-202-3p and miR-223-3p, laying a foundation for further exploration on pathways regulating estrous cycle. Furthermore, the structure of lncRNAs was analyzed and the lncRNAs that might be miRNA precursors were screened, which is helpful to figure out the possible path on miRNA formation.

Methods

Animal and Ethics

Two-year-old female Xinong Sannen goats were kept in a breeding basement of Northwest A&F University with enough space, food and water. Estrus synchronization was conducted with PGF2 α , medroxyprogesterone, FSH and PMSG. At the first day, 0.2 mg of PGF2 α was given by intramuscular injection and vaginal medroxyprogesterone suppository was applied. On day 10, 20 IU of FSH was dosed twice in an 11-hour interval by intramuscular injections. On day 11, the vaginal plug was removed, and 200 IU of PMSG and 0.1 mg of PGF2 α were injected intramuscularly. Twelve days post-treatment, teaser goats were used to differentiate estrus from diestrus animals, and on day 32, the goats were in diestrus period. Three random goats in estrus or diestrus were slaughtered respectively, and ovary tissues were frozen in liquid nitrogen immediately. RNAiso Plus (Takara, Japan) was applied to isolate total RNA of tissues in accordance with manufacturer's protocol. All of the surgical procedures were approved by the Animal Care and Use Committee of the Northwest A&F University and conformed to national guidelines.

miRNA sequencing

Total RNA was separated by size using agarose gel electrophoresis for segments of 18-30 nucleotides to be linked with 3' adaptors. The products were purified further to get segments of 36-44 nucleotides by Urea-PAGE gel, and linked with 5' adaptors to get miRNA samples. Then reverse-transcription PCR was conducted, whose products went through a separation by 3.5% agarose gel to get segments of 140-160 base pairs. The gel extraction product was prepared as miRNA library for miRNA sequencing.

circRNA sequencing

The ribosomal RNA was removed from total RNA and linear RNA was degraded by Rnase R. Then fragmentation buffer was applied to get short fragments of circRNAs, which were used as templates for first-strand cDNA synthesis by random hexamers. Second-strand cDNA was synthesized by dNTPs, RNase H and DNA polymerase I. QiaQuick PCR kit was used to purify the products with EB buffer. After end repair, base-A adding and sequencing adaptor adding, the products was purified again by size with an agarose gel. PCR amplification was conducted to establish circRNA library for circRNA sequencing by Illumina HiSeq 2500.

mRNA and lncRNA sequencing

The mRNA was obtained and broken into short segments at the length of 200-500 nucleotides as templates for cDNA synthesis after ribosomal RNA was removed from total RNA. First-strand cDNA was synthesized by random hexamers and then dNTPs, RNase H and DNA polymerase I were applied to acquire the second strand. PCR amplification was performed after ligate adapter and Uracil-N-glycosylase treatment to establish the library for sequencing. LncRNA was differentiated from mRNA using CNCI and CPC software by the evaluation of coding ability.

Results

Overview of circRNA sequencing

An average of 84,629,465 (DS) and 80,303,146 (ES) clean reads were obtained in two libraries. After the data were quality-controlled and filtered, 99.28% (DS) and 98.95% (ES) of high quality (HQ) clean reads were generated. The mapped rRNA reads were removed from HQ clean reads, and then 20 base pairs at both ends of the unmapped reads was picked as Anchors Reads, which were aligned to caprine genome later. In this experiment, 22,333 novel circRNAs were found from the mapped reads and no existed circRNAs were detected. The information of circRNAs is shown in Table S1, including source gene ID, chromosome and genomic location, length, and annotation type. The distribution of the identified circRNAs on caprine chromosomes was presented in Figure 1A, which showed that most circRNAs were on chromosomes 1, 2, 3, 8, 10 and 11. In general, most circRNAs were 400 nucleotides in length (Figure 1B) and six types of circRNAs were identified, with annot_exon being the most common type (Figure 1C).

CircRNA source gene analysis and differentially expressed circRNAs analysis

GO terms of circRNA source genes were enriched in three aspects (Table S2): Biological Process (9,566 genes), Cellular Component (3,274 genes) and Molecular Function (8,786 genes). It is found that circRNA source genes mainly involved in cellular process (GO:0009987), single-organism process (GO:0044699), biological regulation (GO:0065007), cell (GO:0005623), cell part (GO:0044464), organelle (GO:0043226), binding (GO:0005488) and catalytic activity (GO:0003824) terms (Figure 2A). The KEGG pathway database was applied to analyze metabolic processes that the source gene participated in, and 287 KEGG pathways were found (Table S3). The top 20 enriched pathways are shown in Figure 2B, including Oocyte meiosis (ko04114), Thyroid hormone signaling pathway (ko04919), Oxytocin signaling pathway (ko04921) and Progesterone-mediated oocyte maturation (ko04914), which are closely related to reproduction traits.

When $|\log_2(\text{Fold Change})| > 1$ and $p < 0.05$, the circRNA would be regarded as differentially expressed circRNA. Overall, 676 differentially expressed circRNAs were discovered in ES group compared to DS group, including 347 upregulated and 329 downregulated ones (Figure 2C). The heatmap of cluster analysis is shown in Figure 2D. Besides, all acquired circRNAs were analyzed to predict targeted relationship with existed miRNAs, and 22,207 circRNAs were found to be combined with 433 miRNAs, creating 447,870 combining opportunities between circRNAs and miRNAs (Table S4).

Sequencing and analysis of miRNA

Low quality reads (quality value less than 20 or containing N bases) were removed to get tag sequences, which were aligned with miRNAs in miRBase database (<http://www.mirbase.org>) to identify existed or known miRNAs. Novel miRNAs were identified with hairpin motif prediction referring to reference sequences. The expression of miRNAs is listed in Table S5. There were 168 differentially expressed miRNAs found between ES and DS groups with 165 of them in ES group had lower expressions than in DS group (Table S6); target genes of differentially expressed miRNAs were predicted and subjected to GO and KEGG enrichments analyses, and the results indicates that predicted target genes involved in 54 GO

terms (Table S7) and 295 pathways (Table S8). The enriched GO terms and top 20 of enriched pathways are shown in Figure 3A and 3B respectively.

Sequencing and analysis of mRNA and lncRNA

Groups DS and ES acquired 87,594,681 and 84,720,001 clean reads respectively, with 99.88% and 99.89% were HQ clean reads respectively. Reads unmapped to rRNA were selected and aligned to caprine genome. In total, there were 30,688 reference isoforms, to which 80.14% (24,593) isoforms were mapped, and 12,470 new isoforms were found.

Coding transcripts of the isoforms were defined as mRNA. A total of 182 differentially expressed mRNAs were identified, of which 117 mRNAs were lower expressed. *TIMP1* [19, 27], *MMP9* [17, 18], *3BHSD* [20, 28] and *PTGIS* [29], which are essential for follicular and ovarian developments, were included. Enrichments were performed and differentially expressed mRNAs were found to function in 43 GO terms and 193 pathways. The result of GO enrichment is shown in Figure 4A, and the top 20 enriched pathways are displayed in Figure 4B.

CNCI and CPC were applied to screen lncRNAs from the isoforms by coding ability. In this study, 4,384 lncRNAs were found, among which 39 lncRNAs were downregulated and 2 lncRNAs were upregulated in ES group. To explore the functions of identified lncRNAs, target genes of the all lncRNAs in cis (Table S9) and trans (Table S10) were predicted. However, no potential target relationship between differentially expressed lncRNAs and mRNAs was found. We then analyzed all lncRNAs to predict lncRNAs that might be precursors of miRNAs (Table S11), where one of the lncRNAs differentially expressed between the ES and DS groups, TCONS_00080902, was found to be a possible precursor of one of the differentially expressed miRNAs, miR-223. The three predicted secondary structures of TCONS_00080902 are shown in Figure 4C.

Prediction of circRNA-miRNA-mRNA and miRNA-lncRNA functional regulatory network

This study provides information of all predicted binding possibilities of differentially expressed miRNAs to differentially expressed circRNAs/mRNAs/lncRNAs (Table S12). The circRNA-miRNA-mRNA networks involving *TIMP1*, *3BHSD* and *PTGIS* was explored (Table S13); we searched for their upstream miRNAs in differentially expressed miRNA library; besides, differentially expressed circRNAs that have potential to be the miRNAs sponges were filtered. Then the network centering on *TIMP1*, *3BHSD* and *PTGIS* were constructed (Figure 5A). It can be seen that *TIMP1* participates in HIF-1 signaling pathway (ko04066); *3BHSD* participates in Aldosterone synthesis and secretion (ko04925), Ovarian Steroidogenesis (ko04913) and Steroid hormone biosynthesis (ko00140); and *PTGIS* participates in Arachidonic acid metabolism (ko00590), which are important in follicular and ovarian developments [30-33]. It is gratifying that differentially expressed miRNAs potentially targeting *TIMP1*, *3BHSD* and *PTGIS* were screened, while these miRNAs were potentially sponged by lots of circRNAs; for clear presentation, only parts of prominent circRNAs were selected and shown in Figure 5A. This network provides the possible pathways that *TIMP1*, *3BHSD* and *PTGIS* involved in when DS turned to ES.

Moreover, miR-21b-3p, miR-202-5p and miR-223-3p were selected due to their essential roles in follicular and ovarian developments [34-36] to analyze their target relationship with differentially expressed mRNAs/lncRNAs and sponge relationship with differentially expressed circRNAs (Table S14). CircRNAs that potentially sponge two or more of miR-21b-3p, miR-202-5p and miR-223-3p were shown in Figure 5B.

Discussion

In this study, databases of goat ovary mRNA and non-coding RNAs, including miRNA, circRNA and lncRNA, were acquired, and their expressions were compared between ES and DS groups. Then differentially expressed miRNAs, circRNAs, lncRNAs and mRNAs were screened. Abundant expression of non-coding RNAs and mRNA illustrates the subtle regulation in ovary to keep homeostasis. The circRNA-miRNA-mRNA/lncRNA and lncRNA-miRNA/mRNA networks that might involve in the regulation of estrous cycle were predicted based on the differentially expressions between ES and DS groups. Among them, the circRNA-miRNA-mRNA network that *MMP9*, *TIMP1*, *3BHSD* and *PTGIS* involved in and the circRNA-miRNA-mRNA/lncRNA network that miR-21-3p-miR-202-3p and miR-223-3p participated in were extracted and displayed in Figure 5. The significant regulatory role of *MMP9* [17-19], *TIMP1* [17-19], *3BHSD* [20], *PTGIS* [21, 22], miR-21-3p [23]-miR-202-3p [24] and miR-223-3p [25, 26] had been described in previous studies, therefore, we extracted the networks center on them to show key potential regulation pathways of estrous cycle.

Conclusion

Collectively, our data provided the miRNA, circRNA, lncRNA and mRNA database of goat ovary and each differentially expressed profile between ES and DS, and constructed networks among differentially expressed miRNAs, circRNAs, lncRNAs and mRNAs, shedding light on the regulation of estrous cycle and the treatment of estrous cycle related diseases.

Abbreviations

ES: estrus stage; **DS:** diestrus stage; **PCOS:** polycystic ovary syndrome; **miRNA:** microRNA; **circRNA:** circular RNA; **lncRNA:** long non-coding RNA; **MMP9:** matrix metalloproteinase 9; **TIMP1:** tissue inhibitors of metalloproteinases; **3BHSD:** 3 β -Hydroxysteroid dehydrogenase; **PTGIS:** Prostaglandin I₂ Synthase; **HQ:** high quality

Declarations

Ethics approval and consent to participate

All of the surgical procedures were approved by the Animal Care and Use Committee of the Northwest A&F University and conformed to national guidelines.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare that there are no competing interests.

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Authors' contributions

XPA designed the experiment and collected samples for sequencing; YZ was responsible for data analysis and writing; FL, ZHW and SHY helped with sample collection; BYC applied for funds to support this study.

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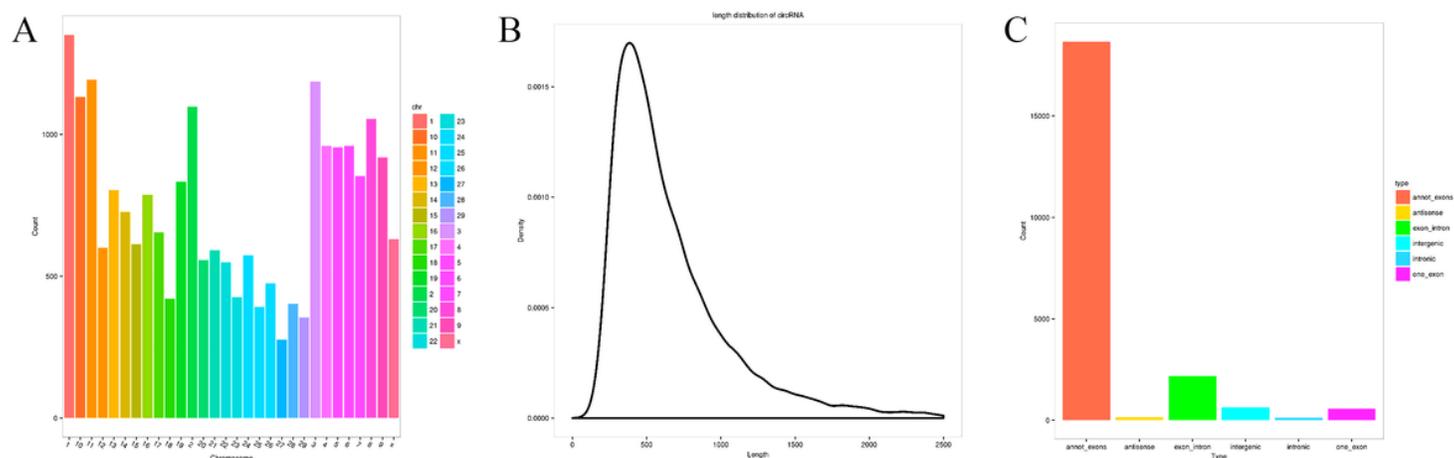
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Figures



The analysis of circRNA source genes. (A) The enriched GO terms of circRNA source genes; (B) The enriched KEGG pathways of circRNA source genes; (C) The number of differentially expressed circRNAs between ES and DS groups. (D) The heatmap of differentially expressed circRNAs.

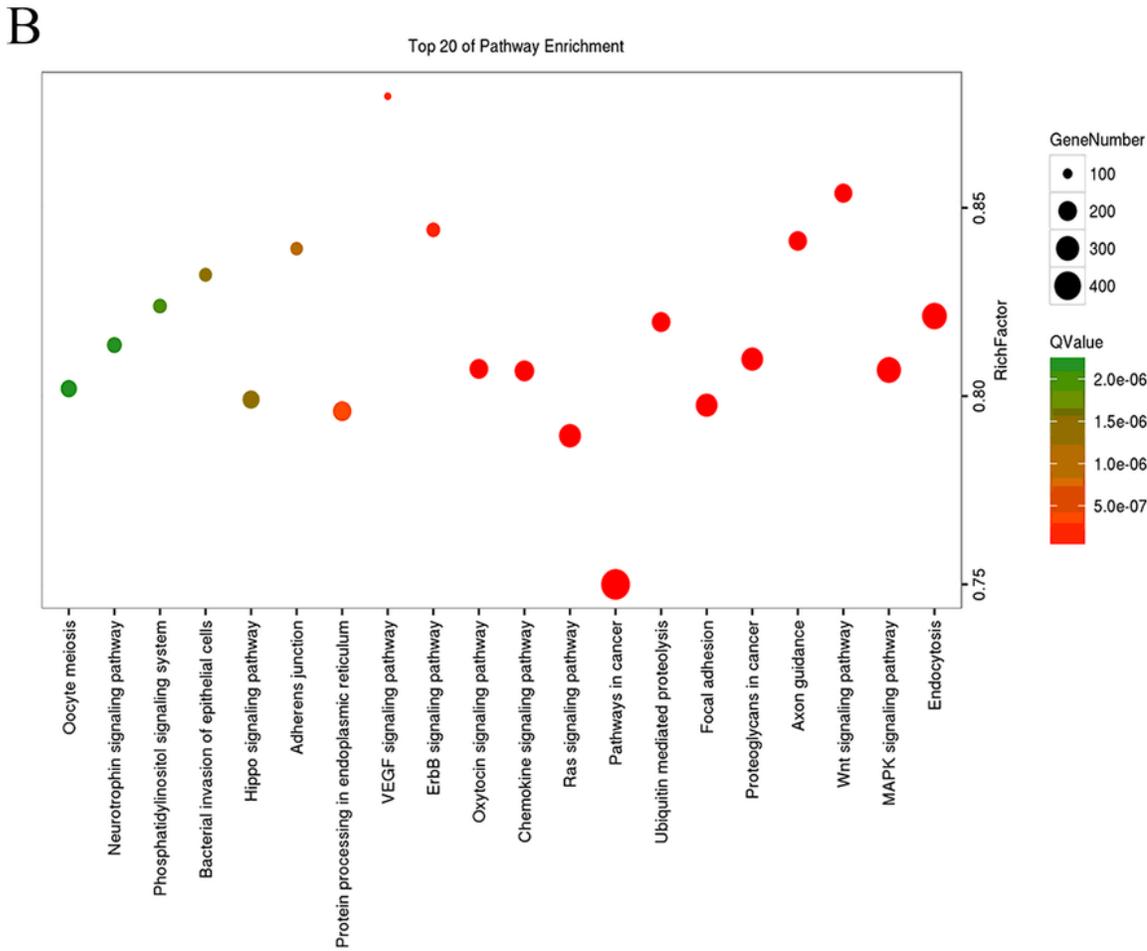
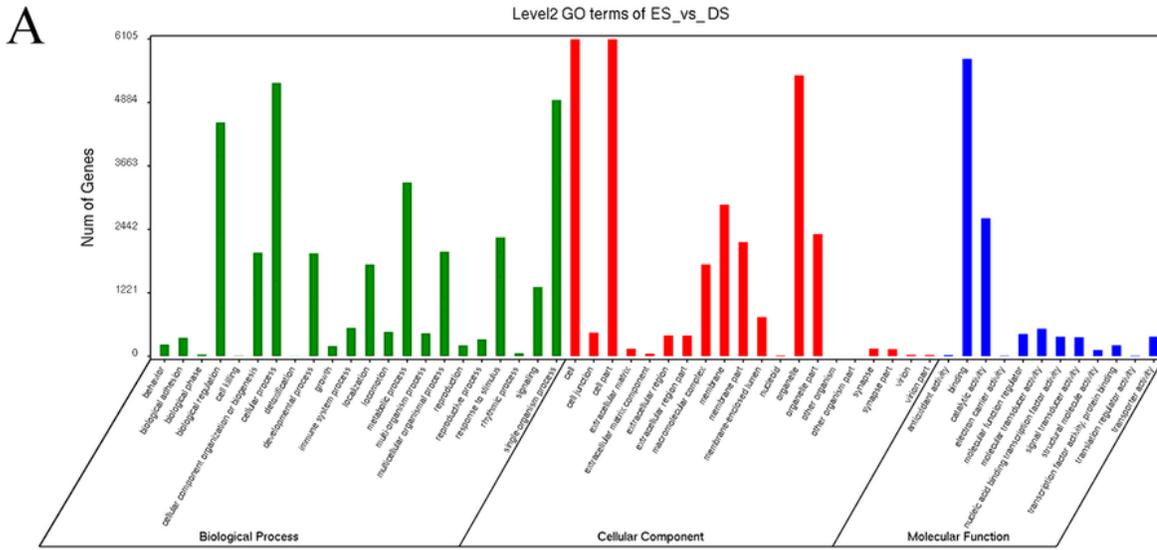


Figure 3

GO and KEGG enrichment of predicted target genes of differentially expressed miRNA. (A) The enriched GO terms of differentially expressed miRNA target genes; (B) The enriched KEGG pathways of

differentially expressed miRNA target genes.

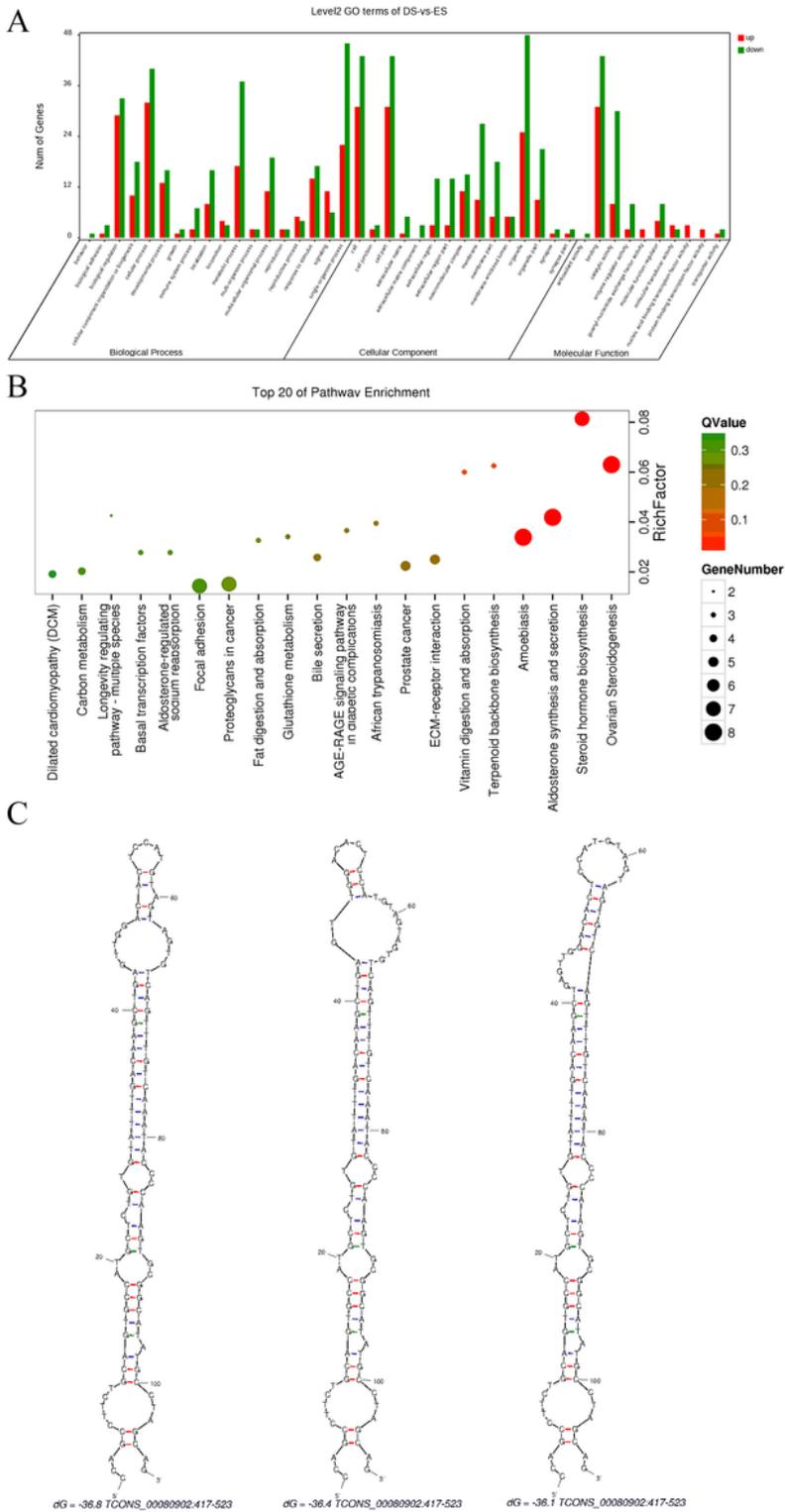
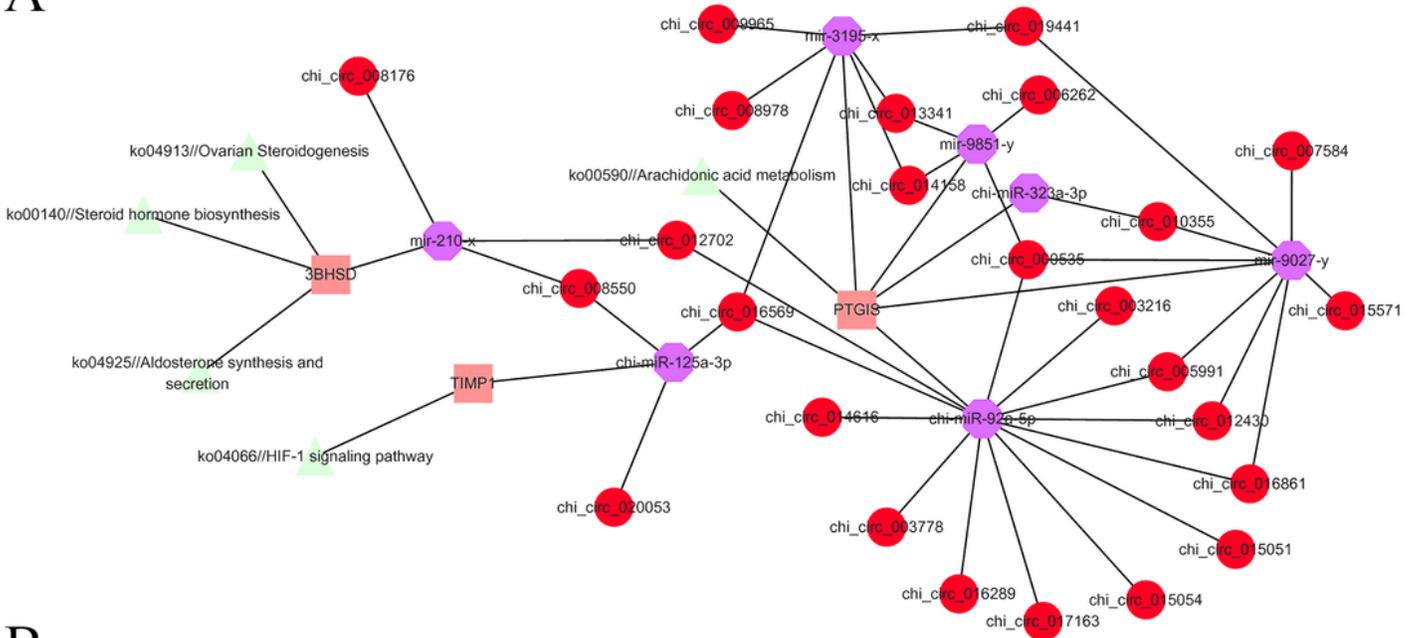


Figure 4

The analysis of mRNA and lncRNA. (A) The GO enrichment of differentially expressed mRNA; (B) The KEGG pathway enrichment of differentially expressed mRNA; (C) The three predicted secondary structures of TCONS_00080902.

A



B

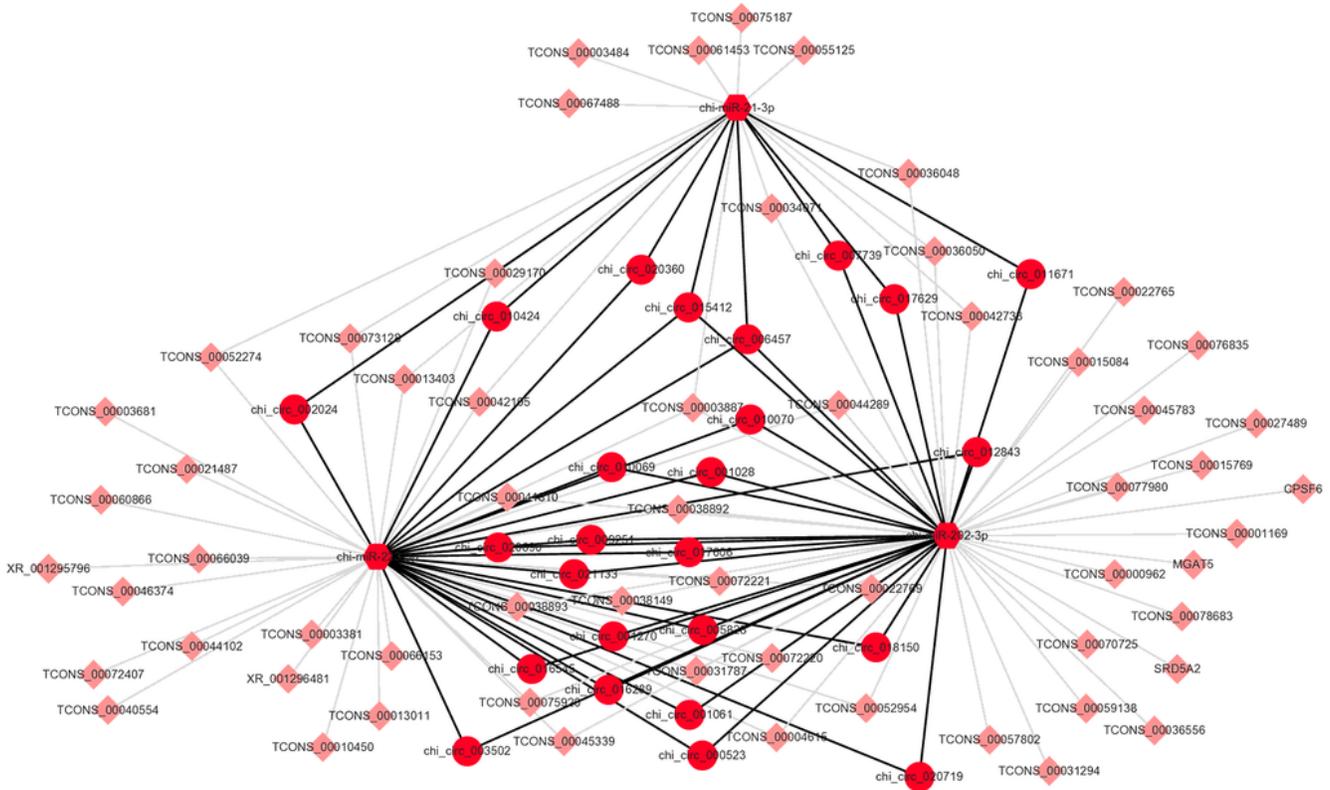


Figure 5

Predicted competing endogenous RNA (ceRNA) networks. (A) Predicted interaction between TIMP1, 3BHSD or PTGIS and differentially expressed miRNAs as well as circRNAs; (B) Predicted target mRNAs/lncRNAs and circRNA sponges of miR-21-3p, miR-202-3p and miR-223-3p.

Supplementary Files

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