Comprehensive Transcriptome Analysis of Hypothalamus Reveals Genes Associated With Disorders of Sex Development in Pigs

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Research

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Abstract

Background

Disorders of sex development (DSD) is a chronic autoimmune disease characterized by systemic inflammation, pathological osteogenesis, and endocrine abnormality. However, its genetic etiology remains mostly unknown. In addition, little research focuses on the regulation mechanism from the view of transcriptomics in the hypothalamic-pituitary-gonadal axis (HPGA). The hypothalamus is the integrated center of the HPGA mediating neural, hormonal, and environmental stimulus to sex development.

Methods

Three XX-DSD (*SRY*-negative) pig (DSD) and three NF pigs (five months old, 40 kg ± 5 kg) were selected by external genitalia observation and *sex determining region Y gene* (*SRY*) detection. The hypothalamus were sampled for RNA isolation, and the mRNA, lncRNA and miRNA expression profiles were analyzed by sequencing.

Results

A total of 1,258 lncRNAs, 1,086 mRNAs, and 61 microRNAs were found to differentially express in XX-DSD pigs compared with normal female pigs. Many genes in hormone biosynthesis and secretion pathway are significantly up-regulated, and the up-regulation of *GNRH1*, *KISS1* and *AVP* may be the candidate genes leading the abnormal secretion of GnRH. Next, we predicted the lncRNA-miRNA-mRNA co-expression triplets and constructed three competing endogenous RNA (ceRNA) potentially associated with DSD. Functional enrichment suggested *TCONS_00340886*, *TCONS_00000204* and *miR-181a* were related to GnRH secretion.

Conclusions

Our research revealed the first transcriptomic profile in the hypothalamus of XX-DSD pigs and provided new insight in coding and non-coding RNAs that may be associated with DSD in pigs.

Background

DSD are rare conditions characterized by inconsistencies in chromosomal, gonadal, and genital sex development [1]. Humans with DSD suffer from heavy economic and mental pressure. Recent studies have shown that it will increase the risk of gonadal germ cell carcinoma [2]. In domestic animals, DSD decline the product quality, leading to serious economic losses to farmers. Pig intersex is part of animal DSD, called 38, XX disorder of sex development (38, XX-DSD). Typical features of this condition, including 38, XX karyotype, the intersex phenotype of genitalia, and the undeveloped testes or ovotestis. However, the pathogenesis and mechanism of 38, XX-DSD in pigs is unclear. XX-DSD usually accompanies by hormone secretion disorder, and hormone level is one of the criteria for DSD diagnosis. Androstenone produced in testes or ovotestes of XX-DSD pigs can affect meat quality [3]. Progesterone in XX-DSD pigs is 5–90 times higher than that of normal sows [4]. Studying the molecular mechanism of DSD hormone regulation is beneficial for understanding the pathogenesis DSD pig and for improving pork quality.

HPGA is involved in the process of mammalian reproduction from fetal development to puberty to sexual maturity [5]. The differentiation and maturation of reproductive organs are mediated by various reproductive hormones or HPGA [6; 7]. Epigenetic regulation in the HPGA has also gained attention as it is related to DSD, and non-coding RNAs (ncRNAs) are the most studied regulators of gene expression [8]. NcRNAs play important roles in promoting mammalian sexual phenotype [9]. Long non-coding RNAs (lncRNAs) may affect the expression and function of genes regulating sex determination and gonad

development, for example, *forehead box L2* (*FOXL2*) [10], *Dmrt1-related gene* (*DMR*) [11] and *PISRT* (*polled intersex syndrome regulated transcript*) [12]. The role of miRNAs in modulating HPGA is also well documented. For example, miR-124 suppresses Sox9 gene in XX gonad during ovary differentiation [13]. The expression of lin28/let-7 is associated with puberty in the hypothalamus [9], and miR-361-3p is involved in regulating FSH (follicle-stimulating hormone) secretion in a pig hypothalamus cell [14] Moreover, interactions between different protein-coding RNAs (mRNAs) and ncRNAs also play critical roles in various biological processes, such as cancer progression [15], ovarian function [16], and HPAG function [17], via acting as competing endogenous RNAs (ceRNAs) [18; 19]

The hypothalamus is the integration center of the HPGA mediating neural, hormonal, and environmental stimulus to sex development. Gonadotropin-releasing hormone (GnRH), an essential regulatory for HPGA, regulates the gonad development and sex hormone secretion [20]. In our previous study, we also found that the number of organelles in GnRH neurons of the hypothalamus increased and fused in the XX-DSD pigs [21]. However, the molecular mechanism was not clear. Here, whole-transcriptome RNA sequencing (RNAseq) of the hypothalamus in the XX-DSD pigs were performed to uncover the gene regulation mechanism at both the mRNAs, lncRNAs and miRNAs levels. Moreover, ceRNA networks were constructed to identify critical genes associated with DSD.

Methods

Experimental Design And Sample Collection

In this study, we selected the hypothalamus of DSD and normal female (NF) pigs raised at the Zhongshan pig farm (Guangdong, China). After external genitalia observation and *SRY* detection[21], three XX-DSD (*SRY*-negative) pig (DSD) and three NF pigs (five months old, 40 kg ± 5 kg) were selected to slaughter for sample collection. All samples were frozen in liquid nitrogen immediately and stored in a – 80 °C freezer until RNA isolation.

RNA isolation

According to the manufacturer's recommended protocol, total RNA were extracted from six frozen hypothalamus using TRIzol reagent (Invitrogen, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). The quality of extracted RNA was determined using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library construction and sequencing

A total of 3 μg of RNA per sample was used as the material for RNAs library preparation. Ribosomal RNA was removed from each sample using the epicenter Ribo-zero TM rRNA Removal Kit (Epicenter, USA). According to the manufacturer's recommendations, cDNA libraries were prepared using the NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, USA). After the purity and quality check, the libraries were sequenced on an Illumina HiSeq 2500 platform and generated 150 bp paired-end reads. Clean data of RNA-seq were obtained by removing reads containing adapter, reads containing poly-N, and low quality reads from raw data. Then, STAR (v2.5.1b) was used to map the cleaned reads to the reference genome (*Sscrofa* 11.1).

Small non-coding RNA (sncRNA) libraries were generated using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.) following the manufacturer's recommendations. The libraries were purified to recover fractions of 140 to 160 nt (the length of small non-coding RNA plus the 3' and 5' adaptors). The quality was measured with the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. Then the libraries were sequenced on an Illumina HiSeq 2500 platform and 50 bp single-end reads were generated. Several criteria were implemented to generate clean miRNA reads, including removing reads containing poly-N, 5' adapter contaminants, ploy A or T or G or C. Low quality reads from raw data and reads without a 3' adapter or the insert tag were also removed. Furthermore, the cleaned data of miRNA were matched to the reference sequence (*Sscrofa* 11.1) by Bowtie [22].

LncRNA and miRNA Identification

StringTie 1.3.2d [23] was used to the transcriptome based on the clean reads mapped to the reference genome. Following transcriptome assembly, the assembled transcripts were annotated using the gffcompare program. The following four steps were used to identify the novel lncRNAs based on their characteristics: (1) Transcripts only one exon and shorter than 200 nt, were first discarded. (2) Transcripts that overlapped with the annotation exon in the database were discarded. (3) Novel transcripts without coding potential were selected by CPC2 [24], Pfam [25] and CNCI [26]. (4) The novel lncRNAs were named based on HGNC (The HUGO Gene Nomenclature Committee) standards and the location of its coding gene.

MiRBase20.0 (http://www.mirbase.org/) was used to identify known miRNA, and Mirdeep2 [27] was used to obtain the potential miRNA and draw the secondary structures. Moreover, the miREvo [28] and miRDeep2 [27] were integrated to predict novel miRNA by exploring the secondary structure, dicer cleavage site, and minimum free energy of the small RNA tags not annotated in the previous steps.

Identification of significantly differentially expressed mRNA, lncRNAs and miRNAs

HTSeq v0.6.0 [29] was used to count the reads numbers mapped to each gene. And then, the fragments per kilobase million (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis between DSD and NF group was performed using the DESeq2 R package (1.10.1) [30]. The p.adj < 0.05 & |log2 (foldchange)| ≥ 1.5 were set as the threshold for significant differential expression.

The miRDeep2 quantifier module was used to quantify expression and retrieve counts for the known and novel miRNAs. The miRNA expression levels were estimated by the transcript per million (TPM) [31]. Differential expression analyses across the two groups were also run with DESeq2.

Functional analysis of DEMs

Functional analysis was performed by gene ontology (GO), gene-set enrichment analysis (GSEA) and Kyoto encyclopedia of genes and genomes (KEGG) to determine the biological significance of DEMs by the clusterProfiler R package. The GO term or KEGG pathway with a $p < 0.05$ is considered significantly enriched.

Protein-protein interaction (PPI) network construction

The PPI network analysis was performed based on the STRING database (https://string-db.org/). The network was visualized by Cytoscape 3.7.1, and the topological features of the network, including degree and betweenness centrality (BC) were analyzed. Hub genes were obtained by screening the degree and BC of each node in the network. The degree of a node is the number of edges connecting to other nodes. The BC is an indicator that measures the influence of a node spreading information in the network. The high BC represents the critical role of a node in communication and information diffusion [32]. To identify the significant modules in the network, Cytoscape plug-in MCODE (Molecular Complex Detection) was conducted with a score > 2.

CeRNA network construction

The CeRNA network was built by the significantly differentially expressed miRNAs (DEMis), lncRNAs (DELns) and mRNAs (DEMs). First, the targets lncRNAs and mRNA of DEMis were predicted by mi-Randa[33]. Then the Pearson correlation coefficient (PCC) was used to filter the paired the miRNA-lncRNA and miRNA-mRNA (PCC <-0.5, $p < 0.05$). Co-expressed lncRNA–mRNA pairs were identified by the PCC > 0.99 ($p < 0.05$). According to the selected standard mentioned above, both of lncRNA and mRNA in a co-expressed pair must be the target gene of a common miRNA. The qualified miRNA–mRNA–lncRNA was identified as one co-expression competing triplet. Finally, a ceRNA network was drawn by Cytoscape 3.7.1 with all the predicted competing triplets. The degree of a node was analyzed, which is the number of edges connecting to other nodes.

Confirmation and quantification of miRNAs, lncRNAs and mRNAs by qRT-PCR

To validate the sequencing datasets, we performed the Quantitative real-time PCR for DEMis, DELns and DEMs. *U6* was selected as the endogenous reference gene of miRNA, and *glyceraldehyde-3-Phosphate dehydrogenase* (*GAPDH*) was selected as the endogenous reference gene of mRNA and lncRNAs. Total RNA for qRT-PCR was collected as described above. According to the manufacturer's instructions, cDNA from lncRNA or mRNA was synthesized using the M-MLV RTase cDNA Synthesis kit (Takara, Dalian, China).cDNAs of miRNAs were reverse transcribed using One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China). qRTPCR was carried out on the QuantStudio 3 (Applied Biosystems) with SYBR Premix Ex TaqTM II (Takara, Dalian, China) according to the manufacturer's protocol. The primer sequences are listed in Supplementary Table 1. All reactions were tested in triplicates. Differences in threshold cycles between target genes and housekeeping genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Results

Identification of mRNA, lncRNA and miRNA in the DSD hypothalamus

The external genitalia of XX-DSD pig showed atypical female sexual characteristics. The vulva was located below the anus, and the clitoris swelling protruded outside the vulva. After external genitalia observation and molecular identification (Fig. 1), the hypothalamus from DSD and NF groups were sampled for RNA-seq and small RNA-seq analysis. Sequencing of six transcriptome libraries generated a total of 651,894,346 raw paired-end reads, and a total of 630,405,324 clean reads were obtained (Table 1). After mapping the clean reads to the reference genome (*Susrofa* 11.1), we detected 22,409 mRNAs, 474 annotated lncRNAs, and 13,248 novel lncRNAs. Comparison of the genomic characterizations of the lncRNAs with mRNAs showed that lncRNAs had shorter transcripts length and open reading frame (ORF) lengths than mRNAs. A higher percentage (86.73%) of lncRNAs had 2 to 4 exons (Fig. 2A-C).

Table 1
Summary of sequencing data

	Sample	RNA-seq				miRNA-seq			
Groups		Raw_reads	Clean_reads	Mapping rate (%)	Q30 (%)	Raw_reads	Clean_reads	Mapping rate (%)	Q30 (%)
	H1	110,678,932	107,929,138	93.3	91.56	17,640,048	17,037,451	97.67	96.58
DSD	H2	109,396,514	107,296,926	87.97	91.5	22,671,555	22,196,313	97.05	96.87
	H3	95,137,076	910,320,02	91.06	91.01	19,599,180	19,375,616	97.00	97.01
	HC1	95,595,932	921,263,80	91.02	93.07	18,892,330	18,507,737	96.23	97.73
NF	HC2	124,027,710	118,310,522	85.06	92.36	18,285,778	17,932,141	97.56	97.70
	HC3	117,058,182	113,710,356	84.37	91.77	21,421,570	21,121,242	98.27	97.91

Meanwhile, we obtained a total of 118,510,461 clean reads from 116,170,500 raw reads generated in all the miRNA libraries (Table 1). The lengths of most clean reads were 20–24 nt (Fig. 2D). After mapping and annotation, about 70% of clean reads were classified as miRNAs, including 363 known miRNAs and 245 novel miRNAs (Fig. 2E).

Expression profiling of mRNAs, lncRNAs and miRNAs

Expression levels of mRNA and lncRNA were quantified using FPKM, while TPM was used to determine the expression levels of miRNA. A total of 1,086 DEMs were identified between the DSD and NF pigs (Fig. 3A). Most genes from the hormone biosynthesis and secretion pathway, such as *oxytocin/neurophysin I prepropeptide* (*OXT*), *GNRH1*, *agouti related neuropeptide*

(*AGRP*), and *follicle stimulating hormone subunit beta* (*FSHβ*) were significantly up-regulated in DSD pigs. For ncRNAs, 61 miRNAs (37 up-regulated and 24 down-regulated; Fig. 3B) and 1,258 lncRNAs (755 up-regulated and 503 down-regulated; Fig. 3C) were differentially expressed in DSD pigs, respectively.

Functional analysis was performed to determine the biological significance of the DEMs using GO and KEGG method. Our results showed that 595 GO functions ($p < 0.05$) (Supplementary Table 2) were significantly annotated. Interestingly, t DEMs enriched in sex development and hormone signaling were almost up-regulated, such as *doublesex and mab-3 related transcription factor 1* (*DMRT1*), *estrogen receptor 1* (*ESR1*), *FSHβ*, *Wnt family member 5A* (*WNT5A*), *steroidogenic factor 1* (*NR5A1; SF-1*) and *GNRH1*. The expression profiles of DEMs enriched in at least sex development and hormone signaling pathways were listed in Fig. 4A. The top 20 significant pathways were showed in the (Fig. 4B). According to the pathological study before, we focus on the GnRH secretion pathway ($P = 0.0004$). Further, KEGG-GSEA analysis showed that genes involved in the GnRH secretion pathway were up-regulated (Fig. 4C). *Kiss1 metastasis-suppressor* (*KISS1*) gene, the critical factor of mediating the negative feedback of sex steroids upon GnRH release [34], was up-regulated (Fig. 4D).

PPT network construction

The PPI network with 62 DEMs was established based on the candidate GO and KEGG analysis to investigate the relationship of DEMs and their potential role in XX-DSD (Supplementary Fig. 1). The genes with the highest BC ≥ 0.01 [35] and top five percent of highest degree [36; 37] is considered as the hub components. Four hub genes were identified and listed in Table 2, including *pro-neuropeptide Y* (*NPY*), *GNRH1*, *AVP* and *glycoprotein hormones a polypeptide* (*CGA*). We also investigated the modularity feature of the PPI network. Two functional modules, comprising 24 genes, were identified using the MCODE method (Supplementary Table 3). *NPY*, *GNRH1*, *AVP*, and *CGA* were observed to occur in the same cluster, suggesting these genes had similar functions in the DSD hypothalamus.

Table 2
Hub genes in the PPI network

Symbol	Betweenness centrality	Degree	LogFC
NPY	0.174	24	4.888
GNRH1	0.156	24	12.904
AVP	0.083	22	11.098
CGA	0.041	17	9.643

CeRNA network construction and Function analysis

A total of 117 DEMs and 101 DELns were targeted by one or multiple DEMis. We also found 23,300 DELns-DEMs pairs by the PCC method. Three ceRNA networks were constructed containing a total of 625 co-expression competing triplets. One of the ceRNA networks (CeNET1) was composed of 12 miRNA nodes, 70 mRNA nodes and 44lncRNA nodes (Supplementary Fig. 2A). CeNET2 concluded 16 miRNA, 45 mRNA and 55 lncRNA were included (Supplementary Fig. 2B). In the CeNET3, there are only one miRNA, two mRNAs and two lncRNAs (Supplementary Fig. 2B).

To further validate the potential functional implication of CeNET in the DSD hypothalamus, we performed GO and KEGG analysis of DEMs in the CeNET. The top three significant GO classifications were serine family amino acid metabolic process, odontogenesis and the ensheathment of neurons (Supplementary Table 4). The pathway annotations of DEMs involved in the cAMP signaling pathway, GnRH secretion and ErB signaling pathway (Fig. 5A) were selected for the subnetwork construction (Fig. 5B). In the subnetwork, *TCONS_00198346* and *TCONS_00157173* were the hub lncRNA, while *ssc-miR-181a* was the hub miRNA (Supplementary Table 6). Moreover, *TCONS_00198346* and *TCONS_00157173* shared *ssc-miR-181a* with *calcium/calmodulin dependent protein kinase IV* (*CAMK4*), *AP-1 transcription factor subunit* (*FOS*), g*lutamate ionotropic receptor AMPA type subunit 2* (*GRIA2*) and *P21 activated kinase 6* (*PAK6*) gene, which enriched in the cAMP signaling pathway and ErbB signaling pathway.

Validation of differentially expressed miRNAs, mRNAs and lncRNAs

To validate the RNA-seq results, the qRT-PCR and the stem-loop qRT-PCR were performed to measure the expression levels of seven DEMs, four DELns and two DEMis. The expression profiles of these RNAs detected by qRT-PCR were consistent with those obtained by sequencing (R^2 = 0.867), which confirmed the reliability of our sequencing results (Fig. 6A). Moreover, four co-expression competing triplets were selected from the sub-CeNET for qRT-PCR to confirm the expression correlation of miRNA and their targets. The expression profiles of co-expression competing triplets also related (Fig. 6B).

Discussion

DSD is a congenital genetic disease that is not fully understood. DSD occurs in approximately 0.18% of the human population [38] and 0.75-20% of the porcine population [39]. Previous studies have focused on the genetic mechanism of DSD. Candidate genes were identified, including *WNT4* [40], *R-spondin1* (*RSPO1*), and *Wilms tumor-associated gene 1* (*WT1*) [41; 42]. Recently, many researchers have shown that epigenetic participates in various biological processes by regulating gene expression. In DSD patients, higher methylation levels in the CpG box located 3 kbp upstream from the *SRY* [43]. However, the role of ncRNAs during sex development is unclear. Moreover, hormone disorder is one of the main clinical symptoms of DSD. Nevertheless, the regulation mechanism of HPAG, which plays a vital role in the endocrine system, is not well characterized. Therefore, it is critical to study the function of ncRNAs in DSD and investigate their potential implications for DSD diagnosis.

In our study, we used RNA- and miRNA -sequencing to demonstrate the transcriptional regulation of DSD in the hypothalamus. The hypothalamus contains sexually dimorphic structures responsible for driving sex differences in behavior and physiology, controlling many facets and phases of reproduction [44; 45]. We found that *ssc-miR-9* and *ssc-miR-9-1* were the highest expressed miRNAs in the hypothalamus form both DSD and NF groups, which is in accordance with studies in the human [46] and goat [17]. A total of 1,086 mRNAs, 1,258 lncRNAs and 61 miRNA differentially expressed in the hypothalamus between DSD and NF pigs. Function enrichment analysis revealed that hormone receptor genes, such as *ESR1*, *prostaglandin F receptor* (*PTGFR*), *thyroid stimulating hormone receptor* (*TSHR*) and *progesterone receptor* (*PGR*) were significantly up-regulated, suggesting the endocrine system in the XX-DSD pig was disrupted. Moreover, two male-predominant genes, *DMRT1* and *SF-1*, involved in the development and maintenance of the male sex features were also up-regulated [47]. SF-1 is essential for ventromedial hypothalamus (VMH) development, energy homeostasis [48], female biased brain circuitry and behavior [49]. In SF-1 KO mice, the number of immunopositive PGR cells was reduced in the VMH [49]. We thus hypothesized that the high expression of hormone receptors genes is partially related to the high expression of SF-1.

GnRH is the main factor that integrates the central and peripheral regulatory signals to dictate the gonadotropin release during sex development [50]. In the XX-DSD pigs, the number of organelles in the GnRH neurons cell and nerve fibers around cells was increased, leading to the abnormal increment of GnRH transportation [21]. Similarly, the up-regulation of the GnRH secretion pathway and *GNRH1* gene was also noticed in the present study, which may stimulate *CGA* and *FSHβ* expression [51]. Generally, the secretion of GnRH is regulated by the sex steroid feedback [52]. Sex hormones can inhibit the release of GnRH byγ-aminobutyric acid (GABA) or β-endorphin, but promote the secretion by kisspeptin in the rostroperiventricular continuum of the third ventricle (RP3V) [53; 54]. The AVP in suprachiasmatic nucleus (SCN) neurons can innervate the RP3V kisspeptin neurons, and then orchestrate downstream GnRH secretion [55]. We speculated that the up-regulated of *AVP*/*KISS1*/*GNRH1* signaling transduction in the XX-DSD pigs may affect the up-regulated of GnRH secretion. Furthermore, many studies also claimed that there is coordination between energy states and reproductive functions [56–58]. It has been proposed that leptin induces GnRH releasing by increases the synthesis and secretion of kisspeptin [59], and play a key roles in the hypothalamus to control the reproductive function and energy balance [60]. In our study, the up-regulated expressions of *leptin receptor* (*LEPR*), *KISS1* and *GNRH1* mRNA may also relate to positive energy balance caused by the abnormal elevation of testosterone disorder in DSD pig [21; 61]. In a positive energy balance, leptin has negative effects on the expression of AgRP and NPY [62], which can repress GnRH secretion in a direct or indirect fashion [57; 63; 64]. Controversially, the expressions of *AGRP* and *NPY* also increased in our study. This suggested that the GnRH secretion up-regulation in the XX-DSD was mainly due to the

disruption of the endocrine regulation system, and furthering decelerated the HPAG disorder. Combing the results of PPI network, we consider that *AVP*, *KISS1*, *GNRH1* are the marker genes in the XX-DSD pig hypothalamus.

ceRNA networks were constructed to identify candidate coding and non-coding RNAs associated with pig XX-DSD. By integrating the RNA-seq and miRNA-seq data, three ceRNA networks were established. DEMs in the ceRNA networks enriched in the cAMP signaling pathway, GnRH secretion and ErB signaling pathway were selected to construct a sub-CeNET. The GnRH signaling pathway continues to signal through the cAMP signaling pathway. The ErB signaling pathway [65] and the cAMP signaling pathway [66] positively regulates GnRH secretion. *Ssc-miR-181a* is the hub miRNA in the sub-CeNET, and it is reported that *miR-181a* was participated the ovarian follicle development [67]. Here *ssc-miR-181a* was predicted to target *PAK6*. PKA6 interacts with multiple binding partners including sex-steroid receptors [68], and co-expressed and inhibit AR signaling [69; 70]. Decreased expression of *ssc-miR-181a* may account for the higher PKA6 protein content in XX-DSD pig, leading disruption of the sex-steroid feedback of GnRH secretion. *Ssc-miR-181a* also targeted *CAMK4*, *FOS* and *GRIA2*, suggesting that *ssc-miR-181a* may participate in the cAMP signaling pathway.

A growing number of studies have indicated that lncRNAs regulating gene expression to function in a variety of reproduction processes at transcriptional, post-transcriptional and epigenetic levels [71; 72]. For example, lncRNAs near the master-switch gene *SXL* (Sex-lethal) promoter regulates sex determination [73], while *OSKAR* and *TXS* involve in gametogenesis in the Drosophila [74; 75]. In sheep, *LNC_001056*, *LNC_000322* and *LNC_000207* associated with hormone secretion and pituitary gland development [76]. Moreover, lncRNAs act as ceRNAs by miRNA competition with mRNAs. However, there is no study about the potential role of lncRNAs involved in DSD pig hypothalamus. Here, we found 38 DELns in the sub-ceRNA network, and *TCONS_00198346* and *TCONS_00157173* were hub lncRNAs. Both of them can target *ssc-miR-181a* to regulate the *PKA6, CAMK4*, *FOS* and *GRIA2* gene expression, suggesting these two lncRNAs may be involved in GnRH secretion regulation in XX-DSD pigs. Interestingly, key genes in the GnRH secretion pathway, including *AVP*, *KISS1* and *GNRH*, were not part of the ceRNA network, and further experiment will be conducted to explore the regulation of these genes.

Conclusions

DSD is a severe threat in the pig breeding. Whole-transcriptome RNAseq identified 1,086 EMs in the hypothalamus of XX-DSD pigs, and *AVP*, *KISS1* and *GNRH* are considered to be the candidate genes for the XX-DSD phenotype in pig. We also found 61 DEMis and 1,258 DELns in the hypothalamus. 625 lncRNA-miRNA-mRNA co-expression competing triplets were identified and used to construct the ceRNA networks. *TCONS_00198346*, *TCONS_00157173* and *ssc-miR-181a* may involve in the regulation of the GnRH release in the XX-DSD pigs.

Declarations

Availability of data and materials

All the raw sequencing data were deposited in the BIG DataCenter (http://bigd.big.ac.cn/) with GSA accession No. CRA002945.

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Contributions

The contributions of the authors were as follows: Shuwen Tan, Yi Zhou, Haiquan Zhao, Jinhua Wu and Ying Yang conducted theanimal experimentation and the laboratory work. Shuwen Tan and Yalan Yang performed data analysis. Shuwen Tanwrotethe manuscript. Hui Yu provided experimental animal. Huabin Zhao performed the scientific input and proof reading. Hua Li designed the experiment, oversaw the development of the study.The author(s) read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The protocol for ourexperiments was reviewed and approved by the Institutional Animal Care and Use Committeesof Foshan Universityand Wuhan University.

Consent for publication

All authors provide consent to publish this paper.

Competing interests

The authors declare that they have no competing interests.

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Figures

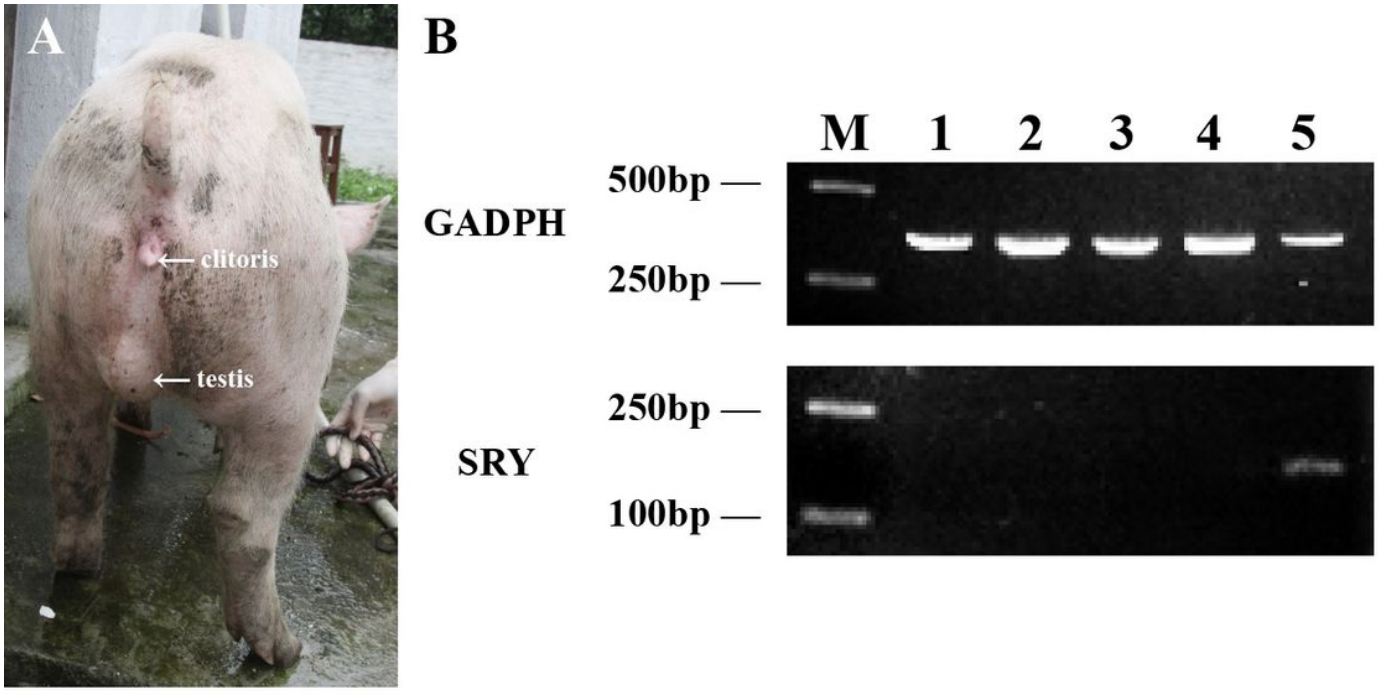

Figure 1

The phenotypic and genetic identification of XX-DSD pigs. A: The external genitalia of XX-DSD pigs. B: The SRY and GADPH detection. M represented the DNA marker; 1-3 were XX-DSD pigs; 4 was normal female pig, while 5 was male.

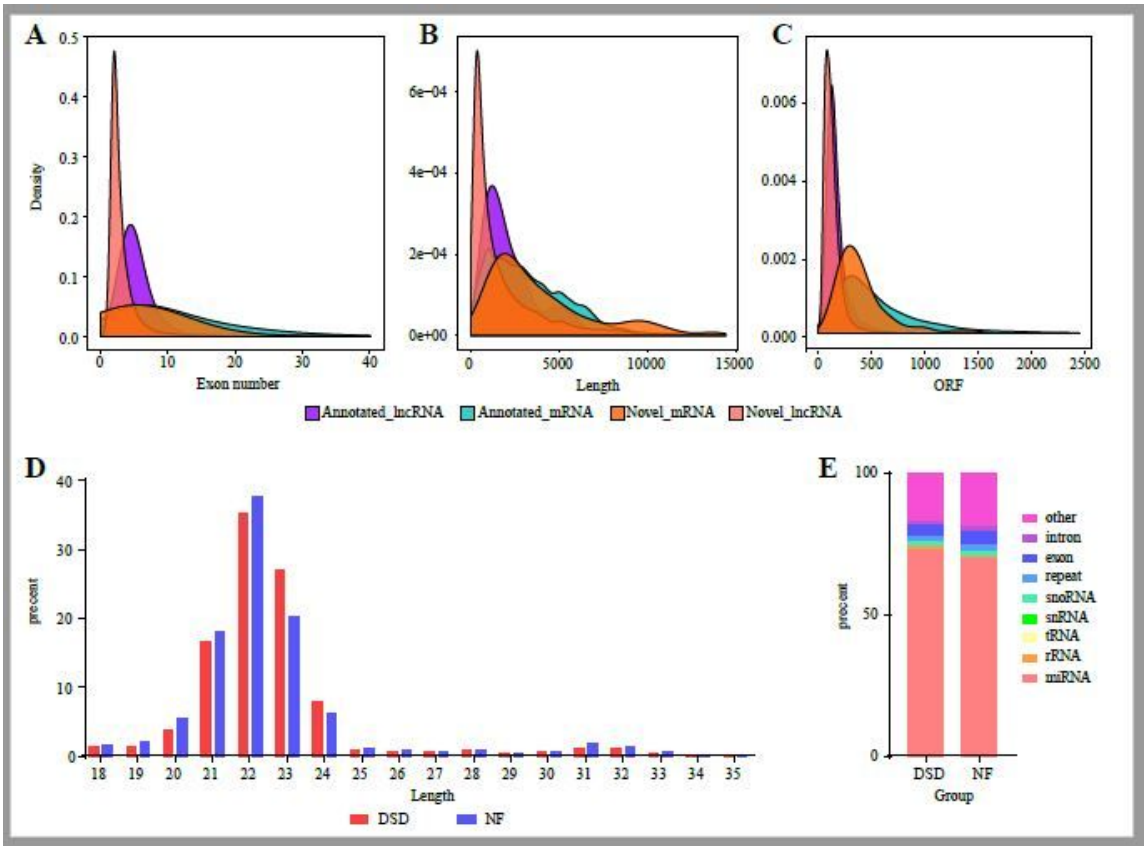

Figure 2

Identification of lncRNA and miRNA. A-C: Comparison of lncRNA and mRNA with respect to the exon number, transcript length and ORF length. D-F: Length and type distribution of all the small RNA in the DSD and NF group.

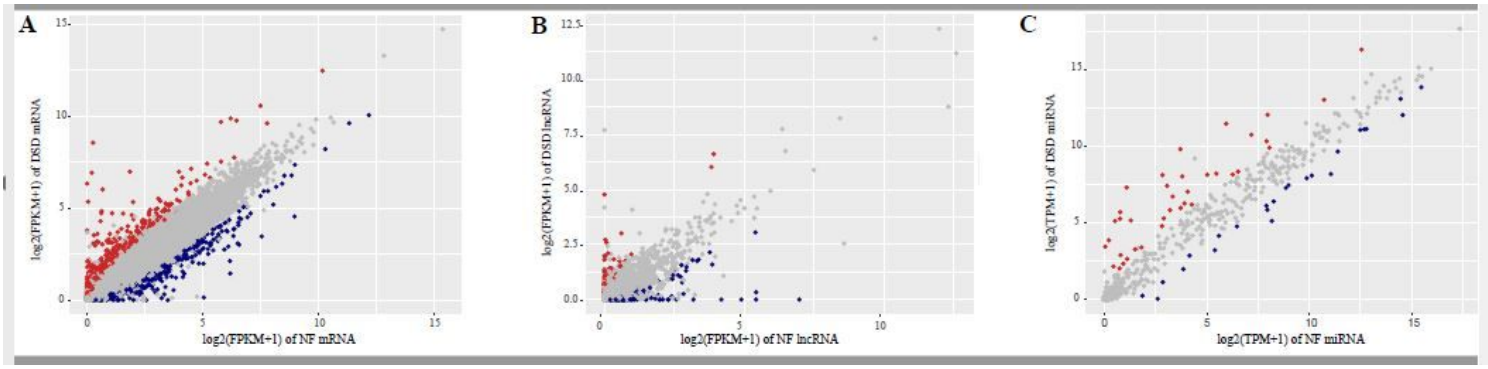

Figure 3

Expression of mRNAs (A), lncRNA (B) and miRNA (C) in NF vs. DSD. Red spots indicate up-regulated genes, and blue spots represent down-regulated genes.

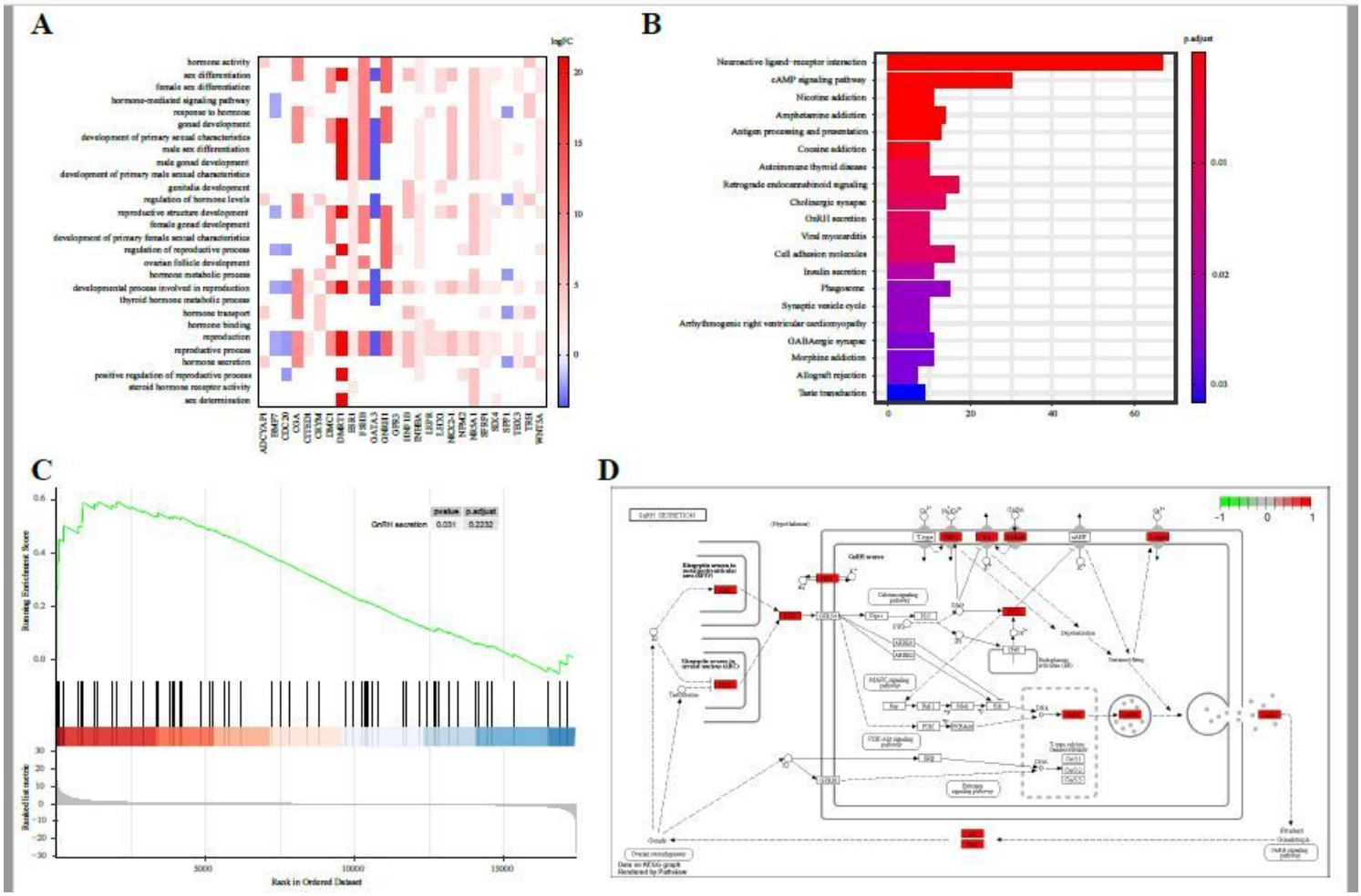

Figure 4

The function of analysis of DEMs. A: Heatmap of DEMs enriched in the sex development and hormone signaling related GO terms. B: KEGG pathway analyses. C: KEGG-GSEA analysis of GnRH secretion. D: Up-regulated DEMs enriched in the GnRH secretionpathway.

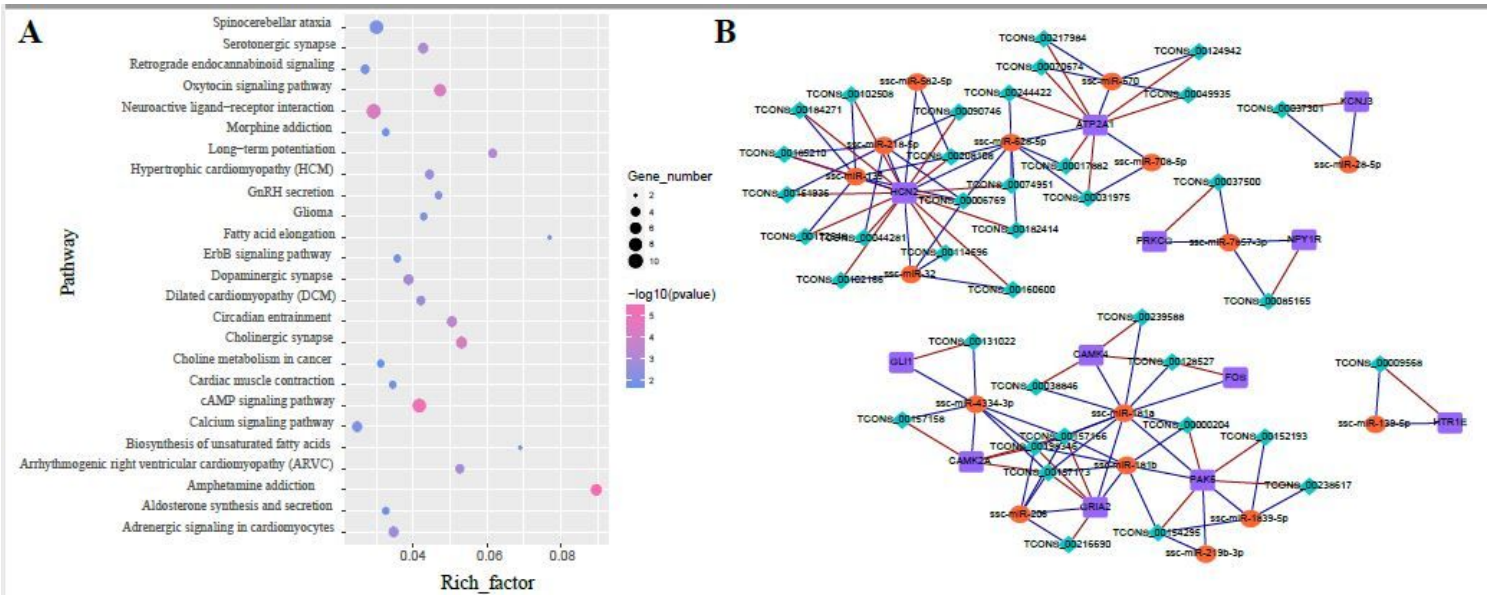

Figure 5

Function analysis and sub-CeNET construction of candidate RNA. A: KEGG pathway of DEMs in the CeRNA net word. B: Sub-CeNET constructed with 12 related DEMs and all DELns and DEMis in the hypothalamus.

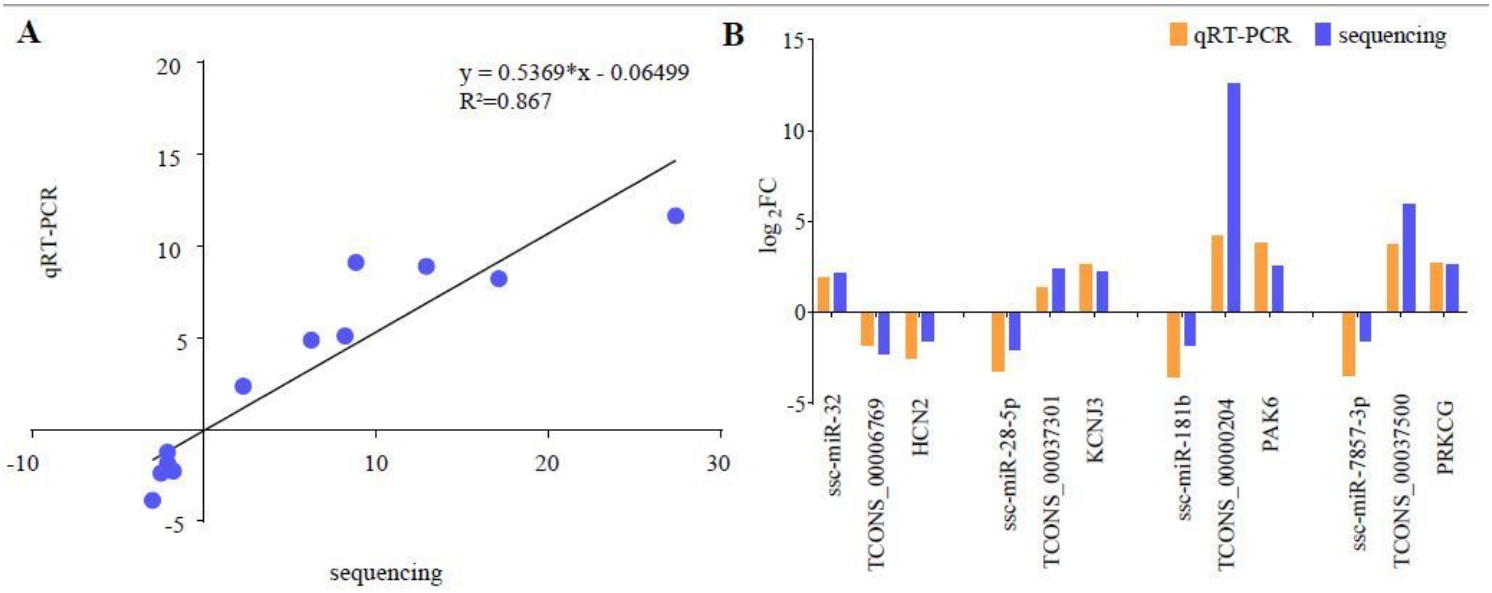

Figure 6

qRT-PCR analysis to confirm the sequencing data. A: The correlation analysis between the result ofsequencing and RT-PCR. B: The expression profiles of four co-expression competing triplets.

Supplementary Files

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