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Transcriptional Regulation of Spermatogenesis-Related Gene *CDK16* in Banna Mini-Pig Inbred Line

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HD and SX conceived the study and participated in design and coordination. ZL conducted a literature search, HH and FY performed laboratory operations. XZ and JH performed the bioinformatics analysis, review and editing.

Key words
Banna mini-pig inbred line (BMI), Whole-transcriptome sequencing, *CDK16*, Functional annotation, Transcriptional regulatory

ABSTRACT

CDK16 is known to have significant involvement in various biological processes of mammalian spermatogenesis. Here, the whole-transcriptome sequencing was performed on the testes of 12-month-old adult boars of Banna mini-pig inbred line (BMI), which identified the *CDK16* gene as being highly expressed in the BMI testes. The initial average expression of the *CDK16* gene in BMI testes was found to be 4,385, with a corrective average expression value (TPM) of the corresponding transcript ENSSSCT00000035953.3 being 54.3489. The full-length coding region of *CDK16* obtained from BMI testes was 1,509 bp (GenBank accession number: OP094608). Gene structure analysis revealed that the *CDK16* gene was located on chromosome X of the pig genome and comprised 16 exons. Additionally, Protein structure analysis showed that CDK16 consisted of 502 amino acids with a conserved S_TKc domain. Phylogenetic analysis indicated that the amino acid sequence of CDK16 was highly conserved across mammalian species in evolution. PPI network, KEGG and GO analyses suggested that CDK16 interacted with 50 proteins, with mainly involving in the cell cycle, cellular senescence, p53 signaling pathway, protein kinase activity, phosphorylation, and G1/S transition of mitotic cell cycle. Furthermore, correlation analysis between these proteins and RNA-seq data from BMI testes revealed that CDK16 was significantly associated with CDC6, CDKN1A, BARD1, CCNF, ACTR1B, CDKN1B, CKS2, CCND1, and CCNY. Functional annotations indicated that CDK16 was mainly involved in 9 GO terms, including four molecular functions (MF), three cellular components (CC), and two biological processes (BP). Furthermore, the ceRNA network analysis showed that BMI *CDK16* was primarily regulated by six miRNAs including ssc-miR-21-3p, ssc-miR-296-3p, ssc-miR-296-5p, ssc-miR-370, ssc-miR-490 and ssc-miR-532-3p. Overall, our findings revealed the crucial role of *CDK16* in BMI spermatogenesis, representing a promising candidate for further investigation.

INTRODUCTION

Banna mini-pig inbred (BMI) line, which has been bred through extreme inbreeding method such as full-sibling or parent-offspring mating since 1980 in an isolated environment at Xishuangbanna, Southwest China, is a valuable laboratory animal resource and a potential candidate for porcine to human xenotransplantation (Huo *et al*., 2022; Liu *et al*., 2022). However, in recent years, the reduced reproductivity in male pigs has become a significant constraint on the expansion of the BMI population (Wang *et al*., 2022). Therefore, the study of molecular characteristics and transcriptional regulations of genes expressed in BMI testis is necessary to better understand the regulatory mechanism of BMI spermatogenesis.

Cyclin-dependent kinase 16 (CDK16, also known as PCTAIRE-1 or PCTK1) is a member of the PCTAIRE family, which is a highly conserved subfamily of cyclin-

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dependent kinases (CDKs) (Mikolcevic *et al*., 2012a). CDKs are serine/threonine kinases that regulate cell cycle turnover in eukaryotic cells and their activity depends on regulatory subunit cyclin (Hartwell, 1974). The three kinases CDK16, CDK17 and CDK18 in vertebrates are highly similar, with a typically central kinase domain of CDKs and unique N-terminal and C-terminal extensions involved in cell cycle (Cole, 2009; Malumbres, 2014). *CDK16* is widely expressed in mammalian tissues, and highly expressed in the testis and brain (Le Bouffant *et al*., 2000). During mammalian spermatogenesis, AMPK phosphorylated CCNY binding with CDK16, which activates the kinase activity of CDK16 and maintains its stability, making CDK16 play an indispensable role in regulating spermatogenesis (Campbell *et al*., 2020; Dohmen *et al*., 2020; Mikolcevic *et al*., 2012b; Zi *et al*., 2015). In mice lacking CDK16, spermatozoas in the epididymis were thin, bent, impaired motility, malformed head, eventually leading to infertility (Mikolcevic *et al*., 2012b). CDK16 is an important kinase that affects the development of neuronal dendrites and regulates the release of neurotransmitters through the phosphorylation of NSF (N-ethylmaleimide-sensitive fusion protein) to affect the function of dendrites (Fu *et al*., 2011). CDK16 affects secretion of growth hormone by binding to phosphorylation of the NSF (Liu *et al*., 2006). CDK16 mediates integrin-dependent spindle orientation and secretory cargo transport by interacting with the COPII complex (Iwano *et al*., 2015; Palmer *et al*., 2005). In addition, CDK16 phosphorylates p27 and p53 via ubiquitination to regulate spindle formation during mitosis process, which is associated with various cancers, such as breast cancer (Li *et al*., 2022; Xie *et al*., 2018; Yanagi *et al*., 2014).

Although aforementioned study has investigated the role of *CDK16* in other species, further analysis focusing on transcriptional regulatory in BMI pig is necessary to accelerate our comprehensive perception of *CDK16*. in this study, we conducted transcriptome sequencing to quantify the expression levels of *CDK16* mRNA, miRNAs, and lncRNAs in BMI testes. Additionally, we utilized RT-PCR technology to amplify the full-length coding sequence of *CDK16*. We performed molecular and functional analyses of the *CDK16* gene and corresponding protein, including protein-protein interaction and correlation analyses. Through annotating the *CDK16* gene, we constructed a ceRNA transcriptional regulatory network and identified the GO terms, miRNAs, and lncRNAs that interact with *CDK16*. This study highlights the significance of *CDK16*'s high expression in BMI testis, which can serve as a valuable resource for exploring the potential mechanisms and functions of *CDK16* gene during the process of BMI-related spermatogenesis.

MATERIALS AND METHODS

Sample collection

Testis samples of four 12 months old adult BMI boars were obtained by surgical castration and then washed with a PBS buffer. Subsequently, small sections were crosscut from the middle area of the parenchyma, including the seminiferous tubules, and any fat and fascia tissues were removed before immediately immersed the samples into liquid nitrogen and storing at -80°C. The animal procedures were approved by the Research Ethics Committee of Yunnan Agricultural University (No. YNAUREC2021096), and were conducted according to guideline for care and use of laboratory animals established by the National Research Council (2017).

Transcriptome sequencing and expression analysis of CDK16 *gene*

Total RNAs were extracted from testicular samples using oligo(dT) beads (BeyoMag™ Oligo $(dT)_{25}$ Magnetic Beads). RNA-seq and miRNAs libraries were prepared by Novogene (Tianjin, China) and sequenced on Illumina Hiseq 4000 and Novaseq 6000 platforms, respectively. The raw data were analyzed using the fastp software, filtering low-quality data, and removing adaptors, sequences with N ratio greater than 10% and all A bases. The filtered data were aligned with the rRNA reference sequence (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/003/025/GCF_000003025.6_Sscrofa11.1/GCF_000003025.6_Sscrofa11.1_rna_from_genomic.fna.gz), and sequences that matched with reference sequences were removed using Bowtie2 (V.2.1.0). The pig reference genome (Sus_scrofa.Sscrofa11.1.dna.toplevel.fa) and the annotations file (Sus_scrofa.Sscrofa11.1.102.gtf) were downloaded from Ensembl, and the genomic index was constructed using STAR (V.2.5.2a). The data were aligned with the pig reference genome and the original expression and corrected TPM values were calculated with Feature Counts (V.2.0.1) and Salmon (V.1.5.1), respectively. The expression of *CDK16* transcript ENSSSCT00000035953.3 in BMI was obtained and visualized using the Gviz (V.1.40.1) package of R. The quality control analysis of small RNA-seq was similar to the RNA-Seq analysis. The reads matching with porcine rRNAs, tRNAs, snRNAs, and snoRNAs of the RFAM14.8 database (http://rfam.xfam.org/) were removed, and the remaining sequences were aligned to miRBase22.1, and miRNAs were quantified using miRdeep2 (V.2.0.1.3).

Gene amplification of CDK16

The *CDK16* gene were amplified using cDNA from BMI testes and a pair of primes (F: GCCTACTTCGGCGCTAACA, R: TGAGTCAGGCGGGTAGGTAG.) designed based on the transcript ENSSSCT00000035953.3. The 25 μL reaction system contained Premix Taq™ (12.5 μL), 10 μM primer F and primer R (1 μL each), 50 ng/μL cDNA (1 μL), and H_2O (9.5 μL). The amplification program consisted of 95°C 5 min; 94 °C 30 sec, 58 °C 45 sec, 72 °C 2 min, 35 cycles; 72 °C 5 min. The products are sequenced by Kunming Tsingke Biotechnology Co., Ltd.

Functional analysis of CDK16 protein

To further investigate the structure of CDK16 protein, we conducted a series of functional analysis. We obtained the open reading frame (ORF) through NCBI's ORF finder. Subsequently, we utilized ProtParam to analyze the molecular weight (Mw), molecular formula, isoelectric point (PI), as well as number of positively and negatively charged residues of the protein. We further investigated the conserved domain, secondary structure, hydrophobic structure, transmembrane helix, signal peptide and tertiary structure of the CDK16 protein using SMART, SOPMA, Prot Scale, TMHMM 2.0, Signal P 5.0 and I-TASSER, respectively. To compare the homology of CDK16 amino acids, we employed MegAlign and visualized the results using the ggplot2 package of R. Finally, we generated a phylogenetic tree of CDK16 protein using MEGA-X and visualized using ITOL (V6).

The protein-protein interaction

To further delve the proteins that interacts with CDK16, we employed String11.5 to construct a network of protein-protein interactions. Specifically, Sus Scrofa was selected as the species with a confidence level of 0.4. Next, we performed functional enrichment analysis of GO and KEGG for the proteins predicted with $P<0.05$ using the cluster Profiler 4.0 package of R. To assess the correlation between these enriched proteins and the expression levels of *CDK16* obtained from RNA-seq, we utilized the cor. test function in R. Finally, we used R to generate visualization of the results.

Functional annotation and transcriptional regulation of CDK16

To obtain a comprehensive understanding of the biological process associated with *CDK16*, we annotated it based on Uniprot and EggNOG-mapper (V2) databases. To identify miRNAs and lncRNAs that regulated *CDK16,* we analyzed RNA-seq data using miRanda 3.3 and RNAhybrid 2.1.2. Finally, we visualized the network of transcriptional regulation using Cytoscape 3.9.1.

RESULTS

Expression and structural characteristics of CDK16 *gene*

To provide a global insight on the express profile of *CDK16* gene, we conducted transcriptome sequencing on four BMI testis samples. Our findings revealed that the average raw count expression of the *CDK16* gene was 4,385, and the normalized TPM expression of corresponding transcription ENSSSCT00000035953.3 was 54.3489. Furthermore, our analysis indicated that *CDK16* was localized on chromosome X of pig genome Sscrofa11.1, with a total length of 12,694 bp. The RNA sequencing data provided information on the expression abundance, exons and introns of *CDK16*, as well as the location of the gene on the chromosome. This information can be used to better understand the gene structure and potential regulatory mechanisms that control *CDK16* expression.

We annotated the transcript ENSSSCT00000035953.3 of *CDK16* gene using Gviz 1.40.1 and identified that it contained 16 exons and 15 introns. Moreover, all four BMI testis samples exhibited highly consistent positive expression (Fig. 1A). We performed RT-PCR to amplify 1,786 bp of *CDK16* gene including 1,509 bp CDS (Accession No. OP094608 in GenBank) (Fig. 1B). Open reading frame (ORF) analysis indicated that the 1,509 bp fragment that encoded 502 amino acids was the genuine ORF encoding CDK16 protein among the 11 ORFs that were identified (Figs. 1C, D).

Structural and functional analysis of CDK16

To provide further evidence for evaluating the function of CDK16 protein, we assess the various characteristics of CDK16 protein. Our results reflected that the molecular weight (Mw), molecular formula, isoelectric point (PI), negative charge residues (Asp+Glu), and positive charge residues (Arg+Lys) of BMI CDK16 were 56.38 kDa, $C_{2497}H_{4005}N_{699}O_{755}S_{15}$, 6.92, 70, 69, respectively. The analysis of protein's hydrophobicity demonstrated that the 273th amino acid exhibited the maximum hydrophobic value of 1.956, while the 110th amino acid displayed the minimum hydrophobic value of -2.822, with the hydrophobic N-terminal and hydrophilic C-terminal. CDK16 contained phosphorylation sites but lacked signaling peptides and transmembrane structures. The secondary structure of BMI CDK16 protein was composed mainly of random coils (228 amino acids, 45.42%), followed by alpha helices (190 amino acids 37.85%), extended strands (59 amino acids, 11.75%), and the least amount of beta turns (25 amino acids, 4.98%) (Fig. 2A). The tertiary structure of CDK16 was consistent with its secondary structure composition, containing

four substructures: Random coil, alpha helices, extended strands, and beta turns (Fig. 2B). Additionally, structurally, CDK16 possessed a conserved domain S_TKc (171-452), which is a protein kinase domain (Fig. 2C).

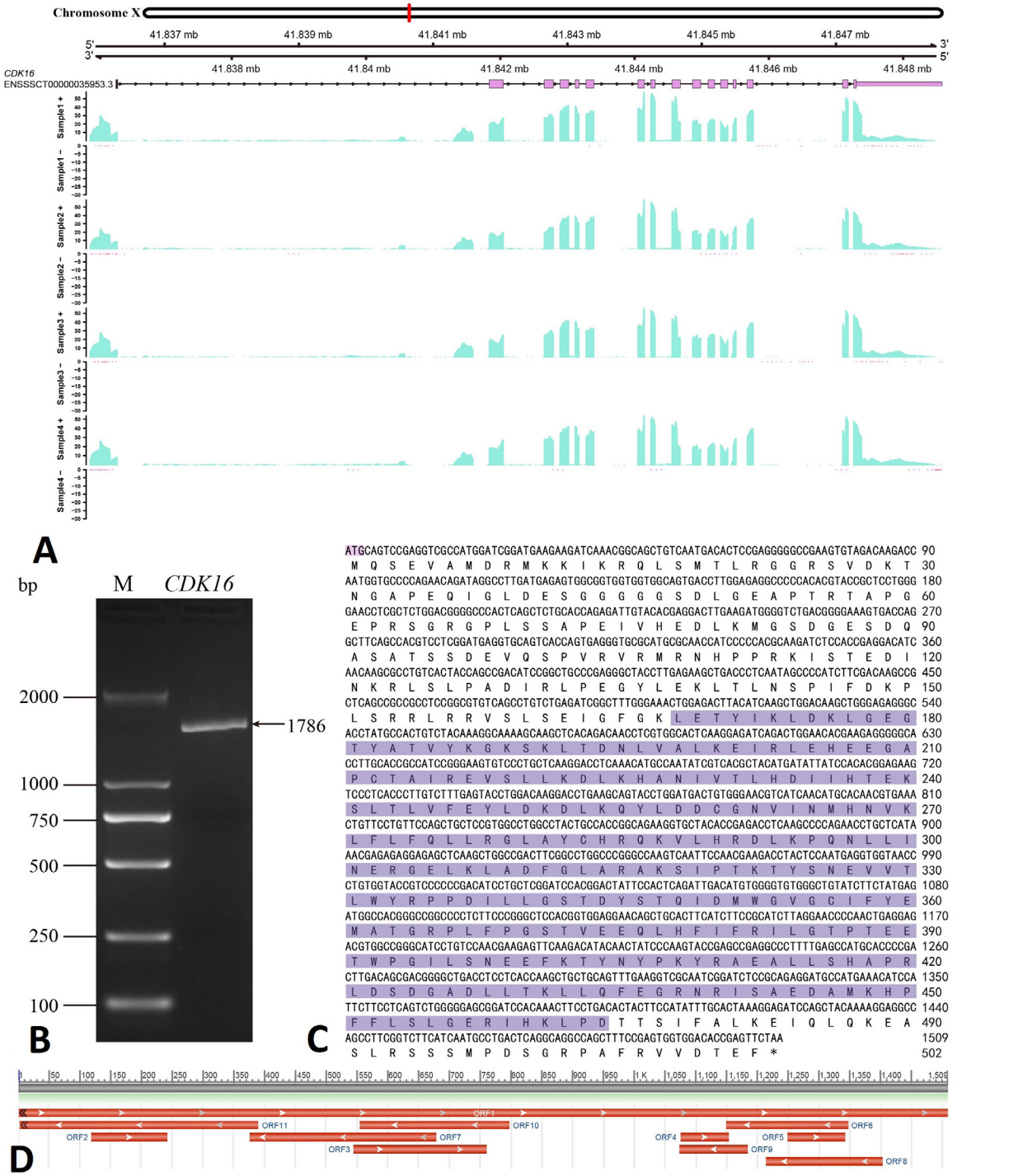

Fig. 1. Analysis of expression and encoding protein of *CDK16*. A, chromosome location and exon, intron abundance of *CDK16* based on RNA-seq. B, RT-PCR product of CDK16. M. DL2000 DNA Marker. C, *CDK16* CDS and encoding amino acids. The colored shade represents the conserved domain S_TKc (amino acid positions 171-452). D, ORF analysis of *CDK16* cDNA.

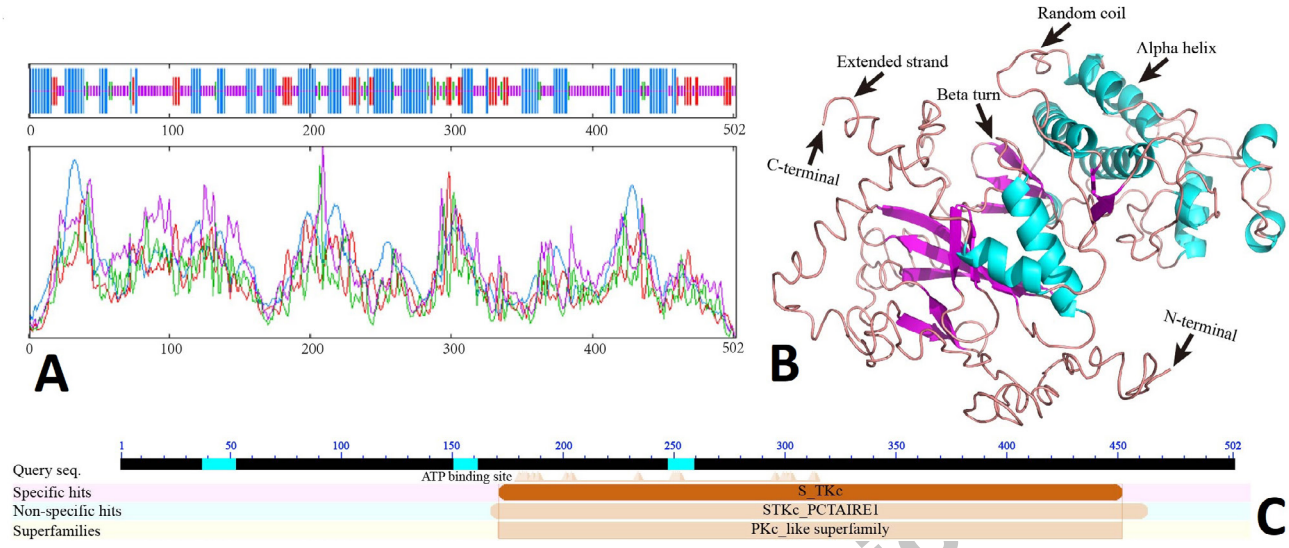

Fig. 2. The spatial structure and conserved domains of CDK16 protein. A, Secondary structure of CDK16 protein. The red, pink, green and blue vertical lines represent extended strand, random coil, beta turn and alpha helix, respectively. B, Tertiary structure of CDK16 protein. The N-terminal, C-terminal, α-helix, β-sheet, β-turn and random coil are highlighted with thick arrows. C, The conserved domains S_TKc (171-452).

Amino acid homology and phylogenetic tree of multi-species

To investigate whether amino acid sequences was conserved among mammals, we conducted homology analysis. Our findings revealed that over 80% of CDK16 sequences were identical across 14 mammalian species, including BMI-pig, *Equus caballus* (XP_003365828.2), *Leopardus geoffroyi* (XP_045328451.1), *Felis catus* (XP_011289962.3), *Enhydra lutris kenyoni* (XP_022363796.1), *Ursus arctos* (XP_026369273.1), *Papio anubis* (XP_003917680.1), *Homo sapiens* (NP_148978.2), *Hylobates moloch* (XP_032612681.1), *Rattus norvegicus* (XP_006256679.2), *Mus musculus* (NP_035179.1), *Capra hircus* (XP_017899153.1), *Bos taurus* (XP_010820178.1) and *Bos mutus* (ELR53820.1) (Fig. 3A). Our results indicated that, from an evolutionary standpoint, these 14 species were clearly divided into five clades. The first clade contained *Ursus arctos, Enhydra lutris kenyoni, Leopardus geoffroyi* and *Felis catus*, which were classified as Carnivora. The second clade included BMI-pig and *Equus caballus*, both of which belonged to livestock. The third clade consisted of *Homo sapiens, Hylobates moloch* and *Papio anubis*, which were classified into Primates. The fourth clade included *Bos taurus, Bos mutus and Capra hircus*, which were classified into Artiodactyla. The last clade consisted of *Rattus norvegicus* and *Mus musculus*, which were classified Rodentia (Fig. 3B), suggesting our reasonable classification standard. Furthermore, functional domain analysis found that CDK16 protein had the same conserved domain S_TKc in all 14 species, indicating that it was highly conserved across mammals (Fig. 3C).

Protein interaction network and gene correlation analysis

To fully excavate the landscape of proteins interacting with BMI CDK16, we constructed the protein-protein interaction networks, identifying 50 interacting proteins (Fig. 4A). To gain a deeper understanding of the functions of these proteins in conjunction with CDK16, we conducted enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). Among the top ten KEGG pathways with $P < 0.05$, the proteins were significantly enriched in the processes such as cell cycle, cellular senescence, progesterone-mediated oocyte maturation, viral carcinogenesis, and p53 signaling pathway (Fig. 4B), while in the top ten GO pathways, these proteins were mainly involved in protein kinase activity, phosphorylation, G1/S transition of mitotic cell cycle, centrosome, serine/threonine protein kinase complex (Fig. 4C). Furthermore, our analysis of the correlation between these proteins and RNA-seq data implied that CDK16 was significantly correlated with CDC6, CDKN1A, BARD1, CCNF, ACTR1B, CDKN1B, CKS2, CCND1, and CCNY ($P < 0.05$) (Fig. 4D). Intriguingly, we found CCNY, a protein regulating cell cycle progression exhibited the most significant correlation with CDK16 ($P < 0.99999$) (Table I).

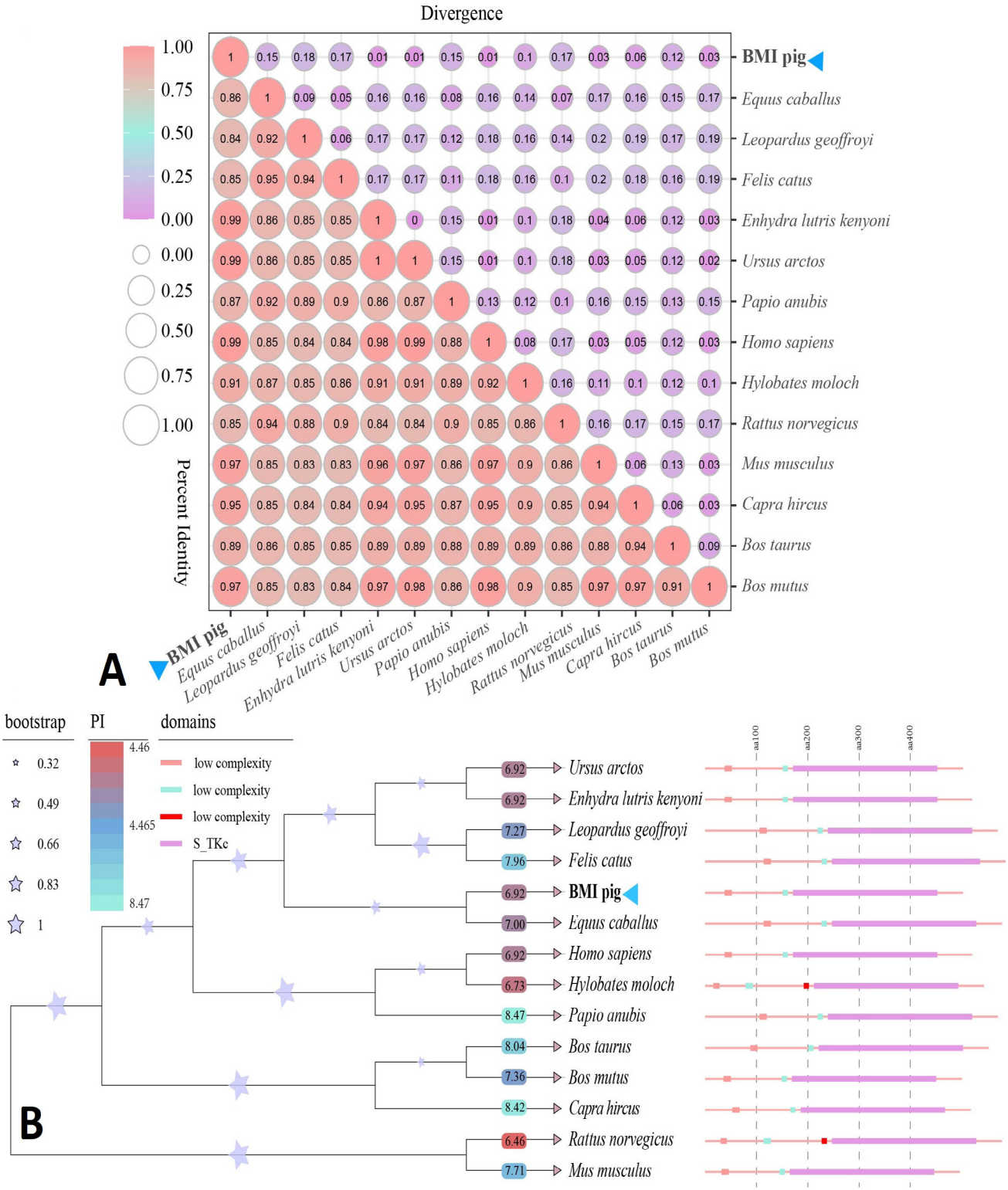

Fig. 3. Amino acid sequences analysis of CDK16 across 14 mammalian species. A, The homology analysis of CDK16 among 14 species. B, Phylogenetic tree of CDK16 from 14 species.

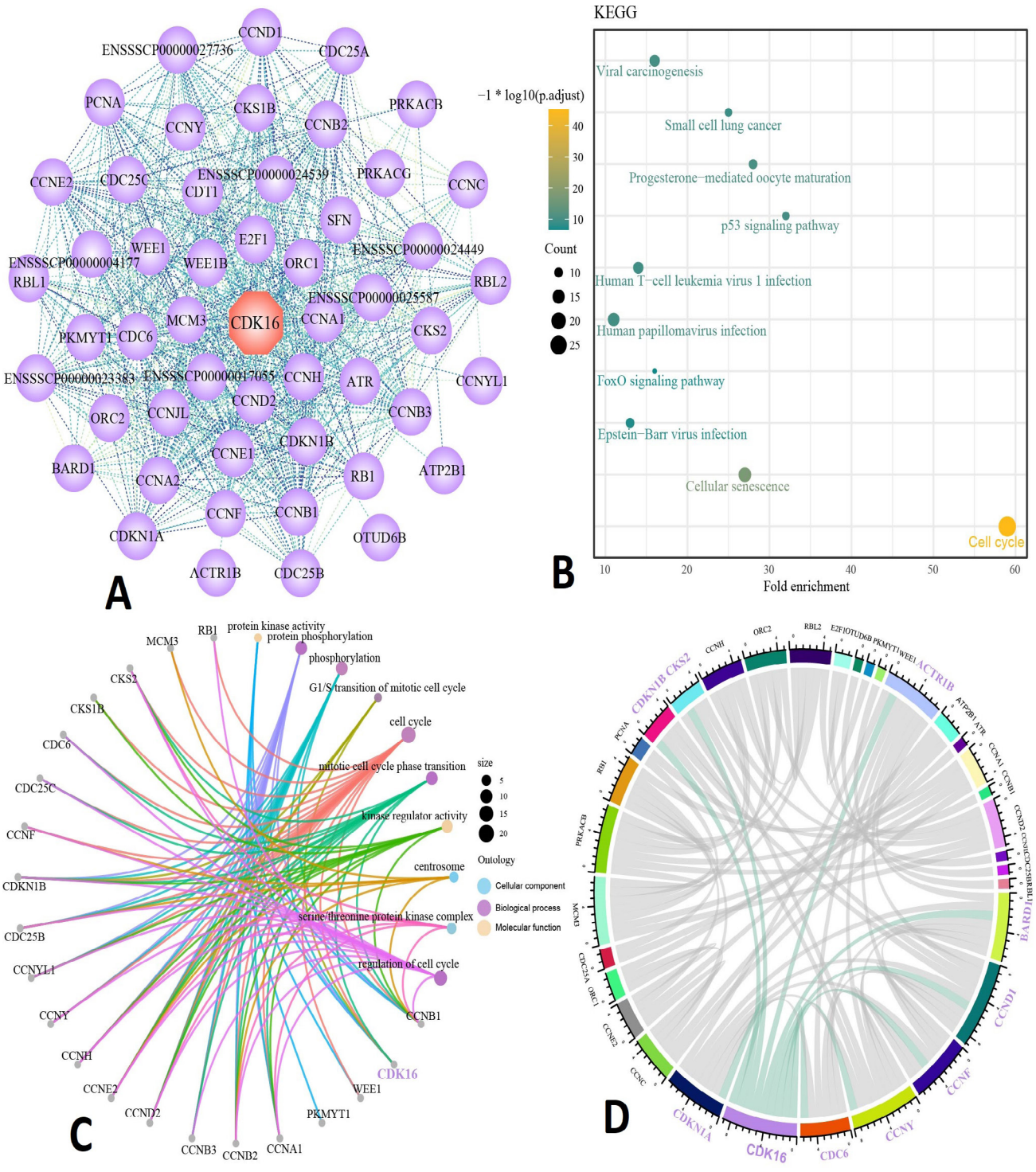

Fig. 4. Interaction network of CDK16. A, Protein-protein Interaction networks. The lines connected proteins indicated the interactions with each other, and more lines indicated higher confidence level. B, Interaction network of KEGG enrichment of CDK16. C, Interaction network of GO enrichment of CDK16, including proteins and the pathways related to biological process, cellular component and molecular function. D, Chord plot of genes that were correlated with CDK16. The wathet lines within the circle indicated significant correlation.

Table I. Genes interacting significantly with the *CDK16*.

Node1	Node2	Correlation	P value
CDK16	CDC6	0.99696	0.04960
CDK16	*CDKN1*A	0.99745	0.04546
CDK16	BARD1	0.99847	0.03514
CDK16	CCNF	0.99866	0.03287
CDK16	ACTR1B	0.99881	0.03104
CDK16	CDKN1B	0.99923	0.02492
CDK16	CKS2	0.99944	0.02119
CDK16	CCND1	0.99991	0.00822
CDK16	CCNY	0.99999	0.00104

Regulatory network of CDK16-miRNA-lncRNA

To better parse the biological functions of *CDK16*, we performed functional annotation. Regarding cellular component (CC), *CDK16* was mainly involved in three GO terms, including cytosol, plasma membrane, and microtubule cytoskeleton; In terms of molecular function (MF), CDK16 was primarily associated with four GO terms, including ATP binding, protein kinase activity, nucleotide binding, and kinase activity; With regard to biological process (BP), CDK16 mainly linked to two GO terms, including protein phosphorylation and phosphorylation (Fig. 5). Through our investigation of the interaction between *CDK16* and miRNA, we identified that *CDK16* was dominantly regulated by six miRNAs, including ssc-miR-296-5p, ssc-miR-296-3p, ssc-miR-21-3p, ssc-miR-490, ssc-miR-532-3p and ssc-miR-370. Moreover, we found that eight and seven lncRNAs competitively bound ssc-miR-370 and ssc-miR-296-5p with *CDK16*, respectively, and two lncRNAs competitively bound ssc-miR-296-3p and ssc-miR-532-3p with *CDK16*, respectively, and one lncRNAs competitively bound ssc-miR-21-3p and ssc-miR-490 with *CDK16*. Our study also indicated that a single gene could be regulated by multiple miRNAs, whereas a miRNA could be competitively bound by both mRNA and lncRNA, which was consistent with the molecular mechanism of functional genes, suggesting these miRNAs might have vital roles in posttranscriptional regulation of *CDK16*.

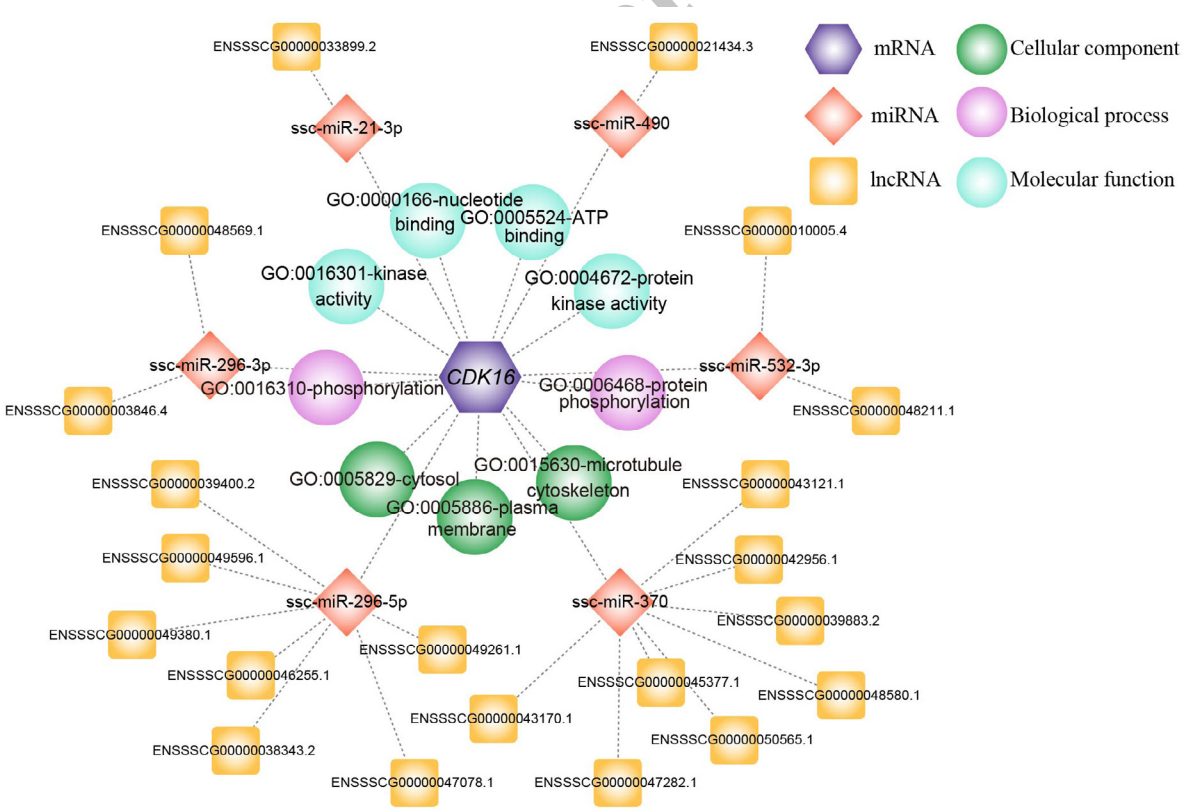

Fig. 5. The functional annotation of *CDK16* and regulatory network of *CDK16*-miRNA-lncRNA. The different elements of this network were represented by hexagon, rhombus, square, and sphere, which correspond to *CDK16* mRNA, miRNA, lncRNA and GO pathways including green cellular component, pink biological process and blue molecular function.

DISCUSSION

In this study, using Illumina Noveseq sequencing technology, we analyzed the expression of *CDK16* gene in BMI testis, and matched the corresponding transcript ENSSSCT00000035953.3 in Ensembl database. By conducting RT-PCR on BMI testicular cDNA, we obtained a 1,786 bp sequence of the *CDK16* gene, with a coding region of 1,509 bp that encoded 502 amino acids. Eleven open reading frames (ORFs) proved that this fragment is the true ORF encoding CDK16 protein. This gene is significantly involved in various biological processes of spermatogenesis in mammals, and its expression level is higher in BMI, indicating that this gene plays an important role in spermatogenesis. In mice, by isolating CDK16, it was found to interact with CCNY and exhibit kinase activity. Mice lacking CDK16 developed normally, but the male mice were sterile. CDK16-deficient spermatozoa had malformed heads and excess residual cytoplasm, suggesting an important role for CDK16 in spermatogenesis (Mikolcevic *et al*., 2012b).

Our analysis of the protein structure indicated that CDK16 had a central kinase domain S_TKC, which is highly similar to those found in the CDK family and PCTAIRE subfamily. In addition, CDK16 also contained a unique N-terminal and C-terminal, with the former being longer and the latter shorter. These regions contained cyclins regulatory phosphorylation sites and were involved in cyclin binding (Dixon-Clarke *et al*., 2017; Malumbres, 2014; Shehata *et al*., 2015). Comparing the amino acid sequences of CDK16 across 14 mammals, we found that BMI shared over 80% similarity with other species, and was most closely related to horse in evolution. Moreover, our analysis of the structural domains of CDK16 in these mammals implied a high homology in the S_TKC domain, suggesting that CDK16 was highly conserved across multiple mammalian species. This shows that in the process of species evolution, the function of this gene has a significant adaptive advantage for survival and reproduction, and the encoded protein plays a key role in the structure and function of organisms. Therefore, it is of great significance to clarify the structure of the gene and the structure of the encoded protein.

We comprehensively annotated CDK16 to screen for the important KEGG and GO terms pathway terms associated with CDK16. Our analysis revealed that the significantly enriched pathways were mainly involved in biological process, such as cell cycle, cellular senescence, viral carcinogenesis, and p53 signaling pathway. By matching proteins screened from GO terms with gene expression data obtained from our RNA-seq, we revealed a significant correlation between CDK16 and CDC6, CDKN1A, CDKN1B, BARD1, CCNF, ACTR1B, CKS2, CCND1, and CCNY. CDC6 is an indispensable element in the pre-replication complex that initiates DNA replication in eukaryotic cells, facilitating the replicative helicase MCM2-7, Orc1-6 and Cdt1 to load into DNA (Bleichert *et al*., 2017). In addition, the significance of CDC6 in male germ cells of *Drosophila* has been reported (Ranjan *et al*., 2022). CDKN1A and CDKN1B, both members of the cell cycle-dependent kinase inhibitor (CDKI) family, are crucial to regulate the cell cycle progress and maintain the stability of the genome (Kreis *et al*., 2019). CDKN1A is involved in various important biological processes including DNA repair, apoptosis, autophagy (Kreis *et al*., 2019). CDK16 regulates the cell cycle of CDKN1B, particularly in cutaneous squamous cell carcinoma and non-small cell lung cancer (NSCLC), where overexpression of CDK16 inhibits CDKN1B expression, resulting in proliferation of tumor cell (Yanagi *et al*., 2017). Moreover, CDK16 negatively regulates p27 expression via processes of ubiquitination and protein degradation (Wang *et al*., 2018). BARD1 regulates apoptosis by binding to p53 and catalyzing its phosphorylation (Feki *et al*., 2005). Moreover, BRCA1/BARD1 complex mediate the deposition of ubiquitin molecules to different substrate proteins to regulate processes such as DNA repair, transcription and cell-cycle (Witus *et al*., 2021). CCNF (Cyclin F) identifies an E3 ubiquitin ligase complex and mediates substrate degradation, thereby regulating the cell cycle, DNA replication and repair, and cell cycle checkpoint (Galper *et al*., 2017; Tetzlaff *et al*., 2004). ACTR1B, a member of actin-related proteins (ARPs) family, plays a key role in regulating actin polymerization, primary cilia morphogenesis, and intracellular signaling pathway, affecting the frequency of ciliation and cilia-related signaling (Drummond *et al*., 2018). Additionally, ACTR1B also plays a crucial role in dynactin-associated biological processes, such as spindle formation and chromosome movement (Tey *et al*., 2016). Cyclin-dependent kinase subunit 2 (CKS2) is an essential regulator of maturation-promoting factor (MPF) activity during cell cycle and embryogenesis, and CKS2 is involved in cell cycle via interacting with CDK1 and cyclin B1, and mice lacking CKS2 resulted in infertility (Spruck *et al*., 2003). CCND1 (Cyclin D1) is a nuclear protein necessary for accumulating in G1 phase and degraded in S phase (Baldin *et al*., 1993). CCND1 binding CDK4/6 transmits mitogenic signals during mitosis, and misexpressed CCND1 can result in solid tumors and hemopathies (Tchakarska and Sola. 2020). Cyclin Y (CCNY) regulates the cell cycle and stimulate the kinase activity of CDK16, enhancing the Wnt/β-catenin signaling during mitosis (Zeng *et al*., 2016; Dohmen *et al*., 2020). Overall, these protein interacting

with CDK16 play unique and important roles in various biological processes, and their discovery provides further insight into the function of CDK16 in BMI.

MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that play a vital role in post-transcriptional regulation. MiRNA negatively regulated gene expression by binding to the 3'UTR of mRNA, which leads to the degradation of the target mRNA or the inhibition of its translation (Bartel, 2004). MiRNAs are highly conserved across species and involved in a variety of cell biological processes, such as cell cycle, cell proliferation, cell differentiation, and apoptosis (Bushati and Cohen, 2007). Our functional annotation of BMI *CDK16* gene and the construction of a ceRNA regulatory network identified 6 miRNAs that target *CDK16*, namely ssc-miR-296-5p, ssc-miR-296-3p, ssc-miR-21-3p, ssc-miR-370, ssc-miR-490 and ssc-miR-532-3p. The miR-296 precursor produces the highly conserved ssc-miR-296-5p and ssc-miR-296-3p, which have been associated with various diseases (Li *et al*., 2018). In glioblastoma, miR-296-5p activates glioblastoma cells via targeting caspase-8 (*CASP8*) and nerve growth factor receptor (*NGFR*), while miR-296-3p activates these cells via SOCS2/STAT3(Lee *et al*., 2017). In non-small cell lung cancer, miR-296-5p acts as a tumor suppressor by regulating the key mitotic kinase PLK1(Xu *et al*., 2016), while miR-296-3p regulates cell proliferation and apoptosis via down-regulating *CX3CR1* (Luo *et al*., 2016; Zhu *et al*., 2018). Additionally, miR-296-5p inhibits apoptosis and cartilage degradation in human via regulating TGF-β1/CTGF/p38MAPK pathway (Cao *et al*., 2020). The ssc-miR-21-3p, which is a member of the mir-21 family, has been associated with various cardiac dysfunction, such as heart failure, dilated cardiomyopathy, and myocardial infarction (Fontanella *et al*., 2021). Moreover, it has been found that miR-21-3p can induce pyroptosis in diabetes by suppressing the expression of androgen receptor (Shi *et al*., 2021). MiR-490-5p can facilitates cartilage degradation in humans by activating the PI3K/AKT pathway via its targeting of *PITPNM1*, whereas inhibiting miR-490-5p using the PITPNM1/PI3K/AKT can contributes to chondrogenesis in human adipose cells (Li *et al*., 2020). MiR-532-3p targeting *HMGA2* inhibits cells proliferation and extracellular matrix production, leading to apoptosis (Long *et al*., 2021). Moreover, miR-532-3p targeting *NOX2* results in reperfusion oxidative stress injury (Mao *et al*., 2020). MiR-370 facilitates the cell cycle and the proliferation of porcine preadipocytes, but inhibits adipogenic differentiation, indicating reduced triglyceride deposition (Chu *et al*., 2021). Our findings suggest that these miRNAs play crucial roles in regulating *CD16* function, making them intriguing candidates for follow-up studies.

CONCLUSION

This study performed RNA-seq technology to determine the expression level of the *CDK16* gene in the adult BMI testis and to obtain the full-length coding sequence of *CDK16* through RT-PCR. Bioinformatics analysis revealed the molecular characteristics of *CDK16* gene, its corresponding conserved protein domains, evolutionary relationships among multiple mammals, protein interaction network, and regulatory network of CDK16-miRNA-lncRNA. *CDK16* was found to be mainly involved in cell cycle, cellular senescence, p53 signaling pathway, protein kinase activity, and G1/S transition of mitotic cell cycle. Additionally, the correlation between *CDK16* and other genes such as *CDC6*, *CDKN1A*, *BARD1*, *CCNF*, *ACTR1B*, *CDKN1B*, *CKS2*, *CCND1* and *CCNY* were examined, with the strongest correlation observed between *CDK16* and *CCNY*. Furthermore, *CDK16* was found to be involved in 9 GO terms, including four molecular function, three cellular components, and two biological processes. This study not only provided insights into the transcriptional regulatory characteristics of the *CDK16* gene, but also enhanced our understanding of the gene landscapes. Our methodology represents a valuable reference for investigating the functions of genes in BMI testis, and demonstrated the potential of whole-transcriptome sequencing technology to unravel gene function related to spermatogenesis in pig.

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IRB approval

The animal procedures were approved by the Research Ethics Committee of Yunnan Agricultural University (No. YNAUREC2021096).Every effort was made to minimize pain and discomfort to the animals when conducting these experiments.

Ethical statement

This study was designed in accordance with the guiding principles of the 2013 revised Declaration of Helsinki.

Statement of conflict of interest

The authors have declared no conflict of interest.

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