Reproductive Sciences
2019, Vol. 26(8) 1094-1104
© The Author(s) 2018
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1933719118805869
journals.sagepub.com/home/rsx

Differentially Expressed lncRNAs After the Activation of Primordial Follicles in Mouse

Liping Zheng, PhD[1,2], Ruichen Luo, MSc[1,2], Tie Su, MSc[1,2], Liaoliao Hu, PhD[1,2], Fengxin Gao, MSc[3], and Xiaoning Zhang, PhD[2,3]

Abstract

The activation of primordial follicles is critical to ovarian follicle development, which directly influences female fertility and reproductive life span. Several studies have suggested a role for long noncoding RNAs (lncRNAs) in ovarian function. However, the precise involvement of lncRNAs in the initiation of primordial follicles is still unknown. Here, an in vitro culture model was used to investigate the roles of lncRNAs in primordial follicle activation. We found that primordial follicles in day 3 mouse ovaries were activated after culturing for 8 days in vitro, as indicated by ovarian morphology changes, increases in primary follicle number, and downregulation of mammalian Sterile 20-like kinase messenger RNA (mRNA) and upregulation of growth differentiation factor 9 mRNA. We next examined lncRNA expression profiles by RNA sequencing at the transcriptome level and found that among 60 078 lncRNAs, 6541 lncRNA were upregulated and 2135 lncRNA were downregulated in 3-day ovaries cultured for 8 days in vitro compared with ovaries from day 3 mice. We also found that 4171 mRNAs were upregulated and 1795 were downregulated in the cultured ovaries. Gene ontology and pathway analyses showed that the functions of differentially expressed lncRNA targets and mRNAs were closely linked with many processes and pathways related to ovary development, including cell proliferation and differentiation, developmental processes, and other signaling transduction pathways. Additionally, many novel identified lncRNAs showed inducible expression, suggesting that these lncRNAs may be good candidates for investigating mouse primordial follicle activation. This study provides a foundation for further exploring lncRNA-related mechanisms in the initiation of mouse primordial follicles.

Keywords

lncRNA, RNA sequencing, primordial follicles, activation in vitro

Introduction

Nowadays, increasing numbers of women are attempting to preserve ovarian function to delay reproductive aging or treat ovarian dysfunction. In the primordial follicles, germ cells arrest at prophase I and become surrounded by a thin ring of granulosa cells, forming the primordial follicle pool, the size of which reflects the ovarian reserve during a female's reproductive life. Under the coordinated and synergistic action of key molecules, primordial follicles can be activated and undergo a growth process that leads to either maturation or death.[1,2] Follicle activation is an irreversible process, and excessive death or overactivation of primordial follicles in the primordial follicle pool might lead to primary ovarian insufficiency (POI) and even to infertility.[3] However, little is still known of the key regulatory factors and the regulatory mechanisms controlling selective or primitive activation, and better understanding of these processes will contribute to the improved diagnosis and treatment of ovarian disorders such as POI.

Long noncoding RNAs (lncRNAs), defined as ncRNAs longer than 200 bp, regulate a wide variety of biological functions and various physiological processes, including cell growth, proliferation, apoptosis, fat metabolism, neuronal patterning, and tumorigenesis.[4-7] The lncRNAs can act as a signal, decoy, guide, or scaffold to regulate gene expression on transcriptional, posttranscriptional, translational, posttranslational, and epigenetic levels.[8-11] Notably, several lncRNAs, such as HOTAIR,[12] HOST2,[13] and ANRIL,[14] serve as pivotal regulators in the biological processes of epithelial ovarian cancer. Furthermore, some studies have indicated an involvement of lncRNAs in the ovary during development. For example, several reports have demonstrated abnormal lncRNA expression profiles in mouse ovary caused by β-crystallin B2 knockout and different lncRNA expression patterns in cumulus cells isolated from patients with polycystic ovary syndrome and in duck ovary in different phases.[15-17] However, whether lncRNAs are involved in the activation of primordial follicles is still unknown.

[1] Jiangxi Medical College, Nanchang University, Nanchang, China
[2] Jiangxi Provincial Key Laboratory of Reproductive Physiology and Pathology, Nanchang University, Nanchang, China
[3] Institute of Life Science and School of Life Science, Nanchang University, Nanchang, China

Corresponding Author:
Xiaoning Zhang, PhD, 999 Xuefu Rd, Nanchang 330031, People's Republic of China.
Email: zhangxiaoning@ncu.edu.cn

To gain new insight into the currently limited understanding of the biological process of primordial follicle activation, here we examined lncRNA expression profiles after activation of primordial follicles in in vitro cultured ovaries from 3-day-old mice. We identified and validated differentially expressed lncRNAs and performed a global evaluation of lncRNA-regulated gene ontology (GO) terms, signaling pathways, and related genes based on their expression profiles and bioinformatics analysis.

Materials and Methods

Ovary Culture

Three-day-old Kun-Ming female mice were obtained from the Centre of Experimental Animals, Nan Chang University (Nan Chang, China). Ovary cultivation in vitro was performed using the Waymouth system (Waymouth MB 752/1 culture; Sigma St. Louis, USA) supplemented with 0.23 mM sodium pyruvate, 3 mg/mL bull serum albumin, 100 IU/mL penicillin G, 100 IU/mL streptomycin, and 10% (vol/vol) fetal bovine serum.[18] Briefly, 3-day-old female mice were sterilized by alcohol and killed according to ethical guidelines.[19] Ovaries were isolated and washed with culture medium containing no serum. Five to 6 ovaries were placed on a gelatin sponge with 300 μL of culture medium in each well of a 24-well plate. Three wells containing ovaries served as the "control group" and were stored at −80°C for subsequent analysis. Three wells containing ovaries were cultured in a 5% CO_2 incubator (Heal Force, Hong Kong, China) at 37°C for 8 days and served as the "cultured group." For a separate analysis using a primordial follicle activator, 3 wells containing ovaries were treated with 40 μM bpV(pic) (Sigma) and cultured in a 5% CO_2 incubator at 37°C for 8 days; this group served as the "bpV(pic) group." The medium was changed every 48 hours by replacing half of the medium with fresh medium. All experiments were carried out according to the guidelines of the Institutional Animal Ethics Committee of Nanchang University (Nanchang, People's Republic of China).

Hematoxylin and Eosin Staining

Control and cultured ovaries (n = 10, each group) were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. Morphologic changes in ovaries were observed under a light microscope (OLYMPUS IX73P2F, Tokyo, Japan) at ×200 magnification.

RNA Isolation and Quality Control

Total RNA was extracted from the ovaries (control group, n = 50; cultured group, n = 50; bpV(pic) group, = 10) using TRIzol (Invitrogen, Düsseldorf, Germany), according to the manufacturer's instructions. The concentration and purity of the total RNA samples were evaluated using a NanoPhotometer (IMPLEN, Munich, Germany) with optical density measurements at $1.8 < A_{260}/A_{280} < 2.2$. The integrity of RNA was checked by electrophoresis in 1.5% agarose gels.

Strand-Specific Transcriptome Sequencing

Qualified total RNA was used for strand-specific transcriptome sequencing, which was conducted using an Illumina HiSeq 4000 system with a PE100 strategy (Illumina, San Diego, California), according to the manufacturer's instructions, as developed by BGI Wuhan Pharmaceutical Co Ltd (Wuhan, People's Republic of China).

Bioinformatic Analysis

The raw data produced by the Illumina HiSeq 4000 platform were subjected to quality control tests, including removing the adaptors as well as empty reads and then filtering the low-quality reads. The clean reads were aligned to reference sequences using Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT)[20] and then assembled by StringTie.[21] The coding capacity of transcripts was predicted by Coding Potential Calculator,[22] txCdsPredict (http://hgdownload.soe.ucsc.edu/admin/jksrc.zip), Coding-Non-Coding Index,[23] and Pfam database.[24] The gene expression level was calculated using the fragments per kilobase of exon per million fragments mapped (FPKM) method.[25] Differentially expressed lncRNAs and messenger RNAs (mRNAs) were identified via fold-change filtering based on DEGseq software.[26]

The lncRNA-targeted genes were predicted based on *cis* and *trans* regulatory principles. For each lncRNA, we calculated the Pearson and Spearman correlation coefficients of its expression value with that of each mRNA. The mRNAs that were coexpressed with the lncRNA of interest were defined as having Pearson and Spearman correlations >0.8 and $P < .05$. The mRNA loci within 10 kb upstream or 100 kb downstream of the lncRNA were defined as *cis*-regulated target genes. RNAplex analysis,[27] a tool for rapid searches of RNA–RNA interactions, was conducted on lncRNA trans-targeted genes to quickly find possible hybridization sites for a query lncRNA in the mRNA database with free energies less than $-30\ \text{kJ}\cdot\text{mol}^{-1}$.

Gene Ontology (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathway analyses were applied to determine the functional roles of the differentially expressed lncRNA targets and mRNAs. The GO terms and KEGG pathways with corrected *P* values <.05 were considered as being significantly enriched by the differentially expressed genes.

Quantitative Polymerase Chain Reaction

Total RNA (1 μg) was reverse-transcribed to complementary DNA using PrimeScript RT reagent kits with gDNA Eraser (TaKaRa Bio Inc, Otsu, Japan), according to the manufacturer's instructions. The quantitative polymerase chain reaction (qPCR) reaction was performed as follows: denaturation at 98°C for 30 seconds, followed by 45 cycles of denaturation at 98°C for 5 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. The qPCR was performed with the StepOnePlus Real-Time PCR Systems (Applied

Table 1. qPCR Primers of Examined lncRNA Genes.

Noncode ID	Tm (°C)	Length (bp)	Primers (5′→3′)
Primers for lncRNAs			
NONMMUT067382.2	60	116	F: CGATGGGCTCCCTAACACAA R: AATCTCCCATGCACCCTGGA
NONMMUT054732.2	60	287	F: GATCCCAAGCAACACACACG R: AGCACCCCAGAGACTACCAA
LTCONS_00063190	60	159	F: TCAGAGCTGACCAACCGAAG R: ATGAGCCTTGGTGAGCACAT
NONMMUT133105.1	60	145	F: ACAAGGACGAACCTGCACTT R: GGTGGTGACAGAGTAGAGGC
NONMMUT010291.2	60	129	F: GCACATCCGTGTTTGGAGAC R: TGTGCTCCACCCTGCTTAAC
LTCONS_00097867	60	129	F: CAGGCTCCGGTTTTTACCAG R: CCAAGCACCTTCCTCCACTTG
LTCONS_00043857	60	285	F: ATACGGAATGTGGCAGGTGG R: TTATTGCCCTGTGAAGGCGT
NONMMUT091171.1	60	158	F: ATAAAGTGGCTTGGGAGGGTC R: AGCCCTTTTCGGGATTCTCTG
NONMMUT108708.1	60	262	F: AGCGCTCTAAGTGGGTTCTG R: GCGGTCCCATTTCTCCTTGT
NONMMUT117230.1	60	274	F: TCACCGGGTCCGTGTAGAAA R: AGTGAGACCCACGACCATCC
Primers for internal control			
β-actin (mouse)	60	127	F: TGGAGAAGAGCTATGAGCTGCCTG R: GTAGTTTCATGGATGCCACAGGAT

Abbreviations: lncRNA, long noncoding RNA; qPCR, quantitative polymerase chain reaction.

Biosystems, Foster City, California) using the SYBR Premix DimerEraser Kit (TaKaRa), according to the manufacturer's instructions. The β-*actin* gene was used as an internal control to normalize the related gene expression levels. Both mRNA and lncRNA expression levels were expressed as $\log_2 2^{-\Delta\Delta CT}$. Samples were run 3 times with good reproducibility. Melting curves were constructed to check the specificity of PCR products. The primer sequences used for qPCR are shown in Table 1. All primers were obtained from GENEWIZ, Inc (Suzhou, People's Republic of China).

Statistical Analysis

Results are expressed as the mean (standard deviation of the mean). All statistical analyses were performed using SPSS version 19.0 (IBM Corp, Armonk, New York). Comparisons between control and activated groups were performed using 1-way analysis of variance, followed by Fisher least significant difference post hoc test. $P < .05$ was regarded as statistically significant, and $P < .01$ was considered extremely statistically significant.

Results

Activation of Mouse Primordial Follicles

We first isolated and treated ovaries from 3-day-old mice as described in the Methods to establish the control group (ovaries from 3-day-old mice) and cultured group (ovaries from 3-day-old mice cultured for 8 days). Microscopic examination of mouse ovaries after 8-day incubation (the cultured group) showed marked initiation of follicle growth compared with mouse ovaries from 3-day-old mice in the control group. The single layer of granulosa cells in the cultured group changed from flattened to cuboidal in shape, and oocyte growth initiated from primordial follicles to the primary follicle stage (Figure 1A-D). The number of primary follicles also increased in the cultured group compared with the control (Figure 1E).

To confirm activation of primordial follicles, we examined the mRNA expression of 2 markers of mouse primordial follicle activation (mammalian Sterile 20-like kinase and growth differentiation factor 9 [*MST* and *GDF9*])[18,28] using qPCR. As shown in Figure 1F, MST mRNA levels were downregulated in primary follicles in ovaries from the cultured group, while GDF9 mRNA levels were upregulated compared with levels in control ovaries, confirming the activation of primordial follicles in the cultured group.

RNA-seq Data

A total of 107 543 054 to 221 135 178 raw reads were yielded in the control and cultured groups using Illumina HiSeq 4000. Following quality trimming and adapter clipping, clean reads (168 000 960 and 215 667 076 for the control group, and 105 820 630 and 186 154 192 for the cultured group) were aligned to the reference transcriptome database of *Mus musculus* with HISAT. The uniquely mapping ratio of clean reads ranged from 76.97% to 79.32% and the Q30 of reads was ≥94.20% (Table 2).

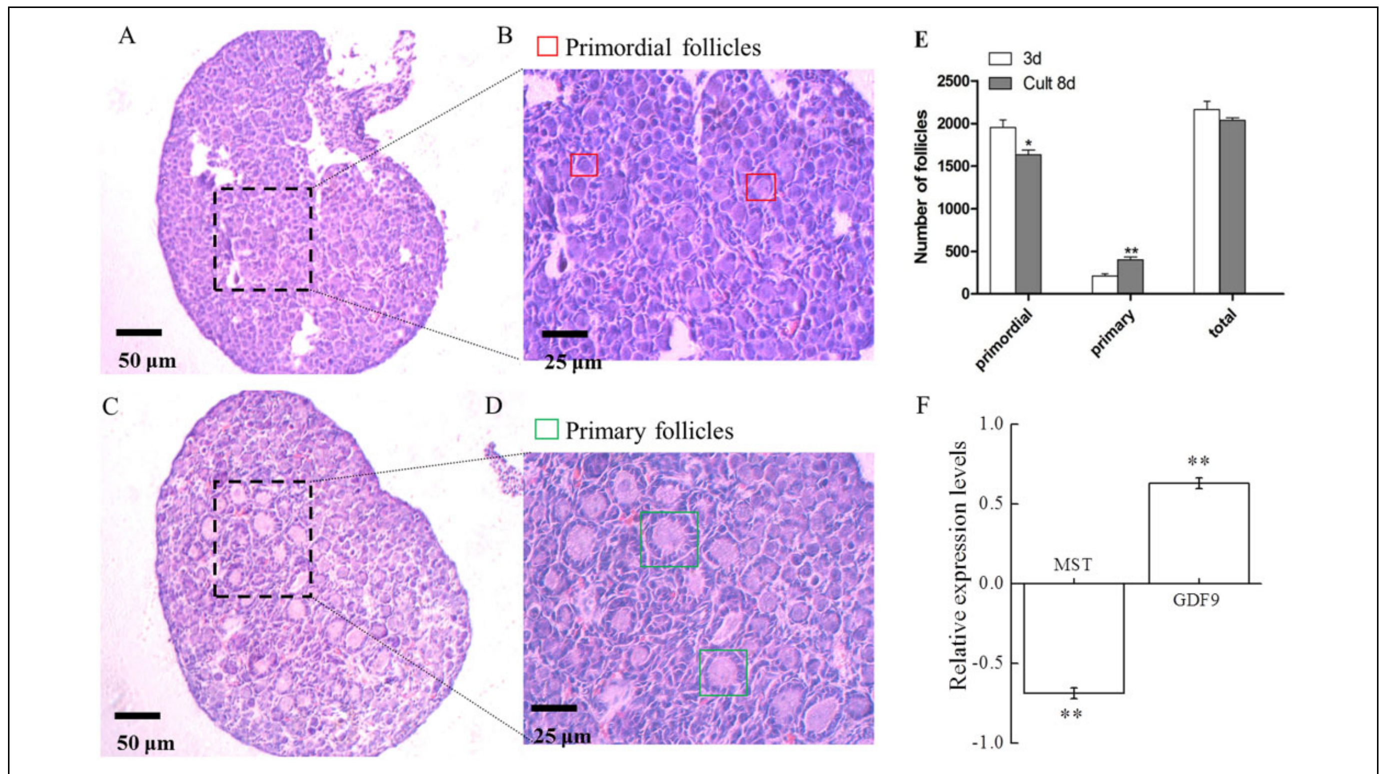

Figure 1. Activation of mouse primordial follicles. A and B, Primordial follicles of mouse ovaries at 3 days (control group). C and D, Primary follicles of mouse ovaries after culturing 8 days in vitro (cultured group). E, The numbers of primordial follicles and primary follicles in the control group and cultured group, respectively. F, The mRNA expression levels of markers of mouse primordial follicle activation (*MST* and *GDF9*). Data are presented as mean (SD; n = 3). **P* < .05 versus control group; ***P* < .01 versus control group. mRNA indicates messenger RNA; SD, standard deviation.

Table 2. Data of RNA-seq.

Sample	RawReads	CleanReads	CleanReadsRatio	UniquelyMappingRatio	Q30
Con1	172 154 636	168 000 960	97.59%	76.97%	94.20%
Con2	221 135 178	215 667 076	97.53%	77.48%	96.54%
Treat1	191 638 124	186 154 192	97.14%	79.32%	96.52%
Treat2	107 543 054	105 820 630	98.40%	77.45%	94.68%

After assembly and analysis, we identified 60 078 known lncRNA transcripts from 47 459 known annotated lncRNA genes and predicted 4088 novel lncRNA transcripts from 3717 lncRNAs with the Coding Potential Calculator (Figure 2A and C). The average expression levels of lncRNAs were lower than those of mRNAs in both control and cultured groups (Figure 2D).

Long Noncoding RNA and mRNA Expression Profiles

As shown in Figure 3, there were 60 078 detectable lncRNAs in mouse primordial follicles. After the activation of mouse primordial follicles, there were 6541 lncRNAs with upregulated expression and 2135 lncRNA with downregulated expression ($\geq$2.0 fold-change, $P < .05$). In comparison, 4171 mRNAs were upregulated and 1795 were downregulated ($\geq$2.0 fold-change, $P < .05$). The detailed information of the differentially expressed lncRNAs and mRNAs is provided in Supplemental Tables S1 and S2.

Gene Ontology Analysis and KEGG Pathway Analysis

Gene ontology analysis showed that genes with aberrant mRNA expression after activation of mouse primordial follicles were mainly involved in the following biological processes: immune system process, cell communication, cell differentiation, regulation of mitogen-activated protein kinase (MAPK) cascade, positive regulation of ERK1 and ERK2 cascade, regulation of I-κB kinase/Nuclear Factor Kappa B (NF-κB) signaling, and other processes (Table 3). For the differentially expressed lncRNA-targeted genes, highly enriched GO terms mainly included immune system process, developmental

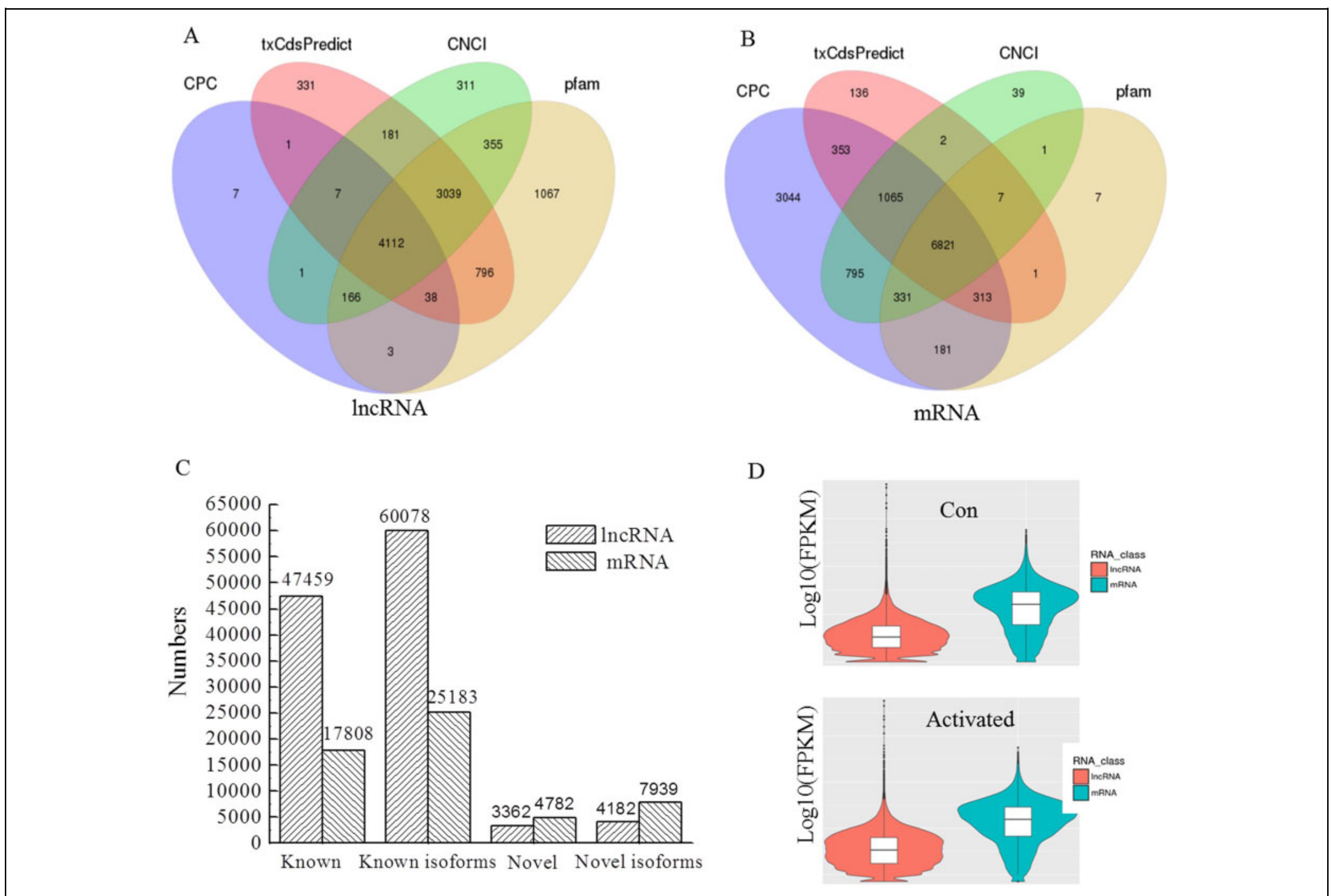

Figure 2. Assembly and analysis of RNA-seq. A, Novel lncRNAs and (B) novel mRNAs predicted by Coding Potential Calculator (CPC), txCdsPredict, Coding-Non-Coding Index (CNCI), and Pfam database. C, The numbers of lncRNAs and mRNAs in mouse ovaries. D, The average expression levels of lncRNAs and mRNAs in the control and cultured groups. lncRNA indicates long noncoding RNA; mRNA, messenger RNA. The color version of this figure is available online.

process, cell differentiation, positive regulation of cell proliferation, regulation of cyclic guanosine monophosphate (cGMP)-mediated signaling, and regulation of cytokine production (Table 4).

For the differentially expressed lncRNA targets, the most significant pathways were those involved in MAPK, NF-κB, Ras, Notch, and tumor necrosis factor (TNF) signaling pathways and other pathways (Table 5). The KEGG pathway analysis of differentially expressed mRNAs showed they were involved in TNF, MAPK, Ras-related protein 1 (Rap1), hypoxia inducible factor-1 (HIF-1), vascular endothelial growth factor (VEGF), cyclic Adenosine monophosphate (cAMP), and Notch signaling pathways (Table 6).

Quantitative Polymerase Chain Reaction Validation

To confirm the reliability of our sequencing data, we randomly selected 10 lncRNAs from the pool of lncRNAs with a fold change >2.0 and analyzed their expression levels by qPCR after initiation of mouse primordial follicles. As shown in Figure 4, the qPCR results were consistent with the sequencing data and showed the same trend of dysregulation for each lncRNA. To confirm our findings, we used bpV(pic), an inhibitor of phosphatase and tensin homolog deleted on chromosome ten (PTEN) that functions as a primordial follicle activator,[29] to further evaluate the lncRNA expression pattern after initiation of mouse primordial follicles. As described in Methods, we treated ovaries obtained from 3-day-old mice with bpV(pic), cultured them in vitro for 8 days, and performed qPCR. The results showed that 5 lncRNAs among the abovementioned 10 lncRNAs displayed a similar expression trend (Figure 4).

Discussion

The precise mechanisms that govern the regulation of follicle activation are still largely unknown, and the specific molecular changes that mediate the initiation of growth and development of both the oocyte and surrounding granulosa cells are not understood. An increasing number of studies have linked dysregulation of lncRNAs to a wide range of biological processes, including in the ovarian development and ovarian cancer,[15,30,31] which have suggested that lncRNAs play important

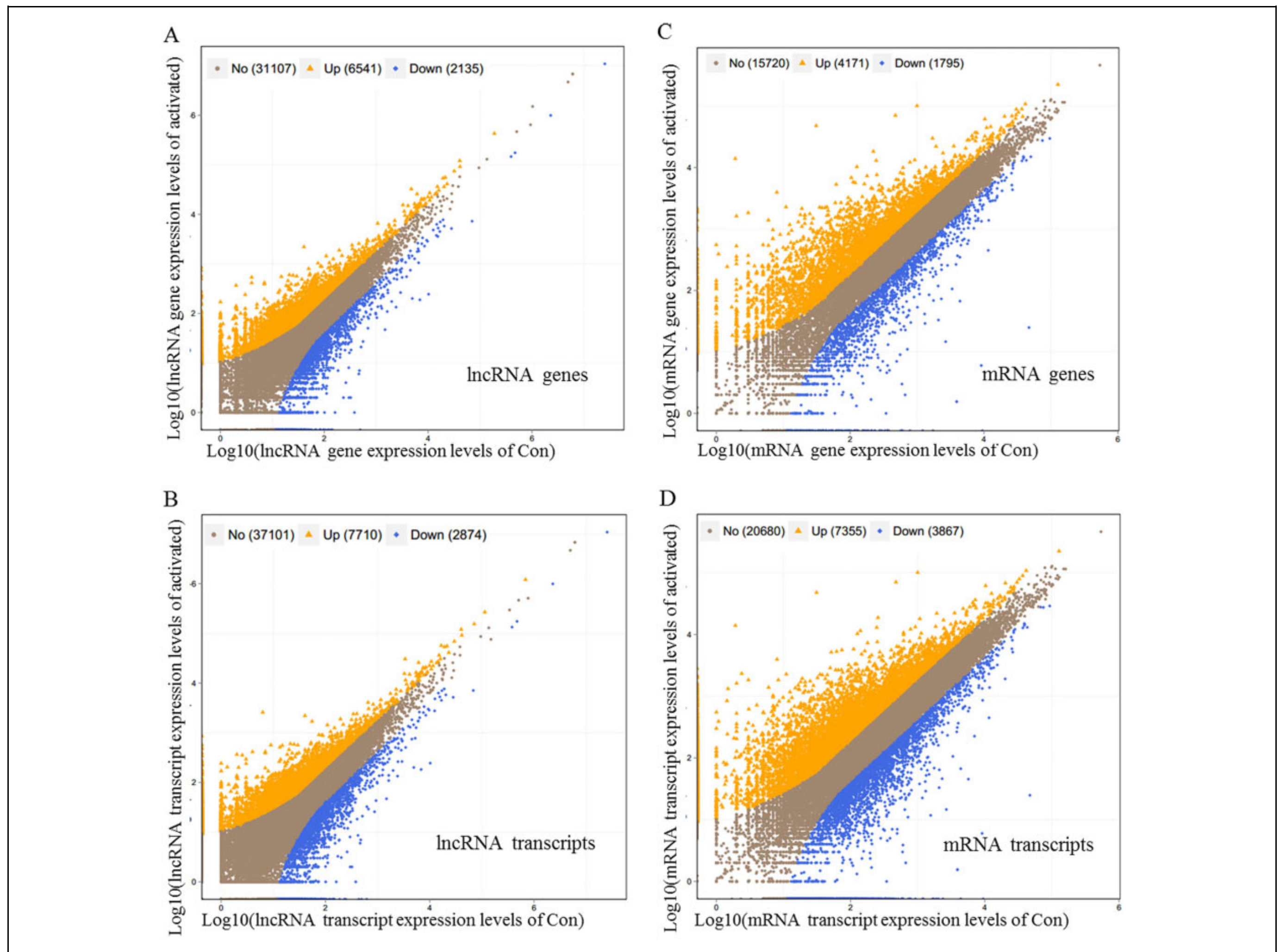

Figure 3. The lncRNA and mRNA expression profiles after activation of mouse primordial follicles. The differentially expressed genes were analyzed using DEGseq software based on the FPKM method ($\geq$2.0 fold-change with $P < .05$). Differentially expressed lncRNA genes (A), mRNA genes (B), lncRNA transcripts (C), and mRNA transcripts (D). lncRNA indicates long noncoding RNA; mRNA, messenger RNA. The color version of this figure is available online.

roles in ovarian function. The mechanisms that regulate primordial follicle activation, one of the most important processes in the female reproductive life, have not been fully elucidated, especially at the global transcriptional level. Our study, for the first time, demonstrates the potential role of lncRNAs in the initial phase of follicle development.

The activation of primordial follicles in vitro has served as a model to investigate ovarian development in various species, including rodents,[32] cattle,[33] baboons,[34] and humans.[35] Our results showed that under the in vitro cultured conditions, mouse primordial follicles developed to primary follicles and the single layer of granulosa cells changed from flattened to cuboidal, suggesting that the primordial follicles might be activated. Previous studies reported that GDF9 mRNA expression was significantly elevated,[28] while MST mRNA levels were reduced[18] after activation of primordial follicles. GDF9 can stimulate the transition from primary to secondary follicles, maintaining ultrastructural integrity of the follicles, suggesting that GDF9 plays an important role in the development of primordial follicles.[36,37] In contrast, MST serves as negative regulator of activation of primordial follicles.[18] The present study confirmed this expression pattern in ovaries cultured for 8 days in vitro. Together these observations indicate that the primordial follicles cultured under our conditions were activated, indicating that our model may be used to explore the mechanism of primordial follicle activation.[32]

A recent study revealed that the lncRNA Meg3 mediated premature ovarian failure via the p53-p66Shc pathway, hinting that lncRNAs may be involved in activation of primordial follicles.[38] In our study, we identified 6541 upregulated and 2135 downregulated lncRNAs during the activation of primordial follicles, suggesting potential roles for these lncRNAs in controlling primordial follicle activation and maintenance of quiescence. To gain insight into the function of the differentially expressed lncRNAs, GO term and KEGG pathway annotation analysis were applied to the lncRNA target gene pools

Table 3. Significant GO Terms for the Differentially Expressed lncRNAs Target Genes.

GO-Target-Biological_Process-Term	*P* Value
Immune system process	3.57e-12
Regulation of signal transduction	1.47e-11
Developmental process	6.04e-11
Regulation of macromolecule metabolic process	2.38e-09
Regulation of gene expression	2.66e-08
Regulation of RNA metabolic process	5.48e-07
Regulation of intracellular signal transduction	5.96e-07
Regulation of transcription DNA-templated	9.09e-07
Regulation of nucleic acid-templated transcription	1.12e-06
Regulation of RNA biosynthetic process	1.42e-06
Cell differentiation	1.52e-06
Regulation of metabolic process	1.88e-06
Regulation of cell adhesion	4.23e-05
Positive regulation of transcription from RNA polymerase II promoter	5.44e-05
Regulation of phosphorylation	.00012
Positive regulation of RNA metabolic process	.00143
Negative regulation of nucleic acid-templated transcription	.00681
Positive regulation of cell proliferation	.01119
Regulation of cGMP-mediated signaling	.01336
Regulation of cytokine production	.03141

Abbreviations: cGMP, cyclic guanosine monophosphate; GO, gene ontology; lncRNA, long noncoding RNA.

Table 4. Significant GO Terms for the Differentially Expressed mRNAs.

GO-Biological_Process-Term	*P* Value
Immune system process	9.94e-36
Regulation of cell communication	1.54e-22
Cell differentiation	2.00e-20
Positive regulation of signal transduction	3.41e-14
Regulation of MAPK cascade	1.44e-10
Regulation of phosphorylation	2.41e-10
Positive regulation of molecular function	2.23e-08
Negative regulation of signaling	2.91e-08
Regulation of macromolecule metabolic process	9.06e-08
Positive regulation of transcription from RNA polymerase II promoter	7.63e-07
Positive regulation of ERK1 and ERK2 cascade	1.38e-06
Positive regulation of sequence-specific DNA binding transcription factor activity	6.05e-05
Regulation of protein serine/threonine kinase activity	.00014
Vascular endothelial growth factor receptor signaling pathway	.00016
Reproductive process	.00040
Regulation of I-κB kinase/NF-κB signaling	.00339
Negative regulation of phosphorylation	.00360
Positive regulation of protein serine/threonine kinase activity	.00396
Negative regulation of cytokine-mediated signaling pathway	.00668
Positive regulation of nucleic acid-templated transcription	.02330

Abbreviations: cAMP, cyclic Adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GO, gene ontology; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NF-κB, Nuclear Factor Kappa B; PKG, protein kinase G; PPAR, peroxisome proliferators-activated receptor.

Table 5. Significant KEGG Pathways for the Differentially Expressed lncRNA Target Genes.

KEGG-Target-Pathway	*P* Value
MAPK signaling pathway	9.67e-06
NF-κB signaling pathway	1.15e-05
Cell adhesion molecules (CAMs)	4.72e-05
Ras signaling pathway	8.49e-05
Focal adhesion	.00032
Notch signaling pathway	.00034
TNF signaling pathway	.00146
GnRH signaling pathway	.00330
TGF-β signaling pathway	.00391
PPAR signaling pathway	.00416
cGMP-PKG signaling pathway	.00485
Thyroid hormone signaling pathway	.00538
Oxytocin signaling pathway	.00545
Apoptosis	.00592
Adherens junction	.01688
Calcium signaling pathway	.01693
cAMP signaling pathway	.02205
T-cell receptor signaling pathway	.02984
Rap1 signaling pathway	.03310
Wnt signaling pathway	.04973

Abbreviations: cAMP, cyclic Adenosine monophosphate; cGMP, cyclic guanosine monophosphate; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long noncoding RNA; MAPK, mitogen-activated protein kinase; NF-κB, Nuclear Factor Kappa B; PKG, protein kinase G; PPAR, peroxisome proliferators-activated receptor; TGF-β, transforming growth factor β; TNF, tumor necrosis factor.

Table 6. Significant KEGG Pathways for the Differentially Expressed mRNAs.

KEGG-Pathway	*P* Value
B-cell receptor signaling pathway	5.36e-12
TNF signaling pathway	1.40e-10
MAPK signaling pathway	3.51e-08
Rap1 signaling pathway	3.97e-07
HIF-1 signaling pathway	7.41e-07
VEGF signaling pathway	3.36e-06
Apoptosis	4.00e-06
NF-κB signaling pathway	9.81e-06
Toll-like receptor signaling pathway	1.30e-05
PI3K-Akt signaling pathway	6.54e-05
Ras signaling pathway	.00015
Calcium signaling pathway	.00017
cAMP signaling pathway	.00142
ErbB signaling pathway	.00218
Notch signaling pathway	.00485
Phosphatidylinositol signaling system	.00561
cGMP-PKG signaling pathway	.01020
Insulin signaling pathway	.02828
PPAR signaling pathway	.03158
Estrogen signaling pathway	.04579

Abbreviations: cAMP, cyclic Adenosine monophosphate; ErbB, Erythroblastic Leukemia Viral Oncogene Homolog; HIF-1, hypoxia inducible factor-1; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NF-kB, Nuclear Factor Kappa B; PPAR, peroxisome proliferators-activated receptor; Rap-1, Ras-related protein 1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

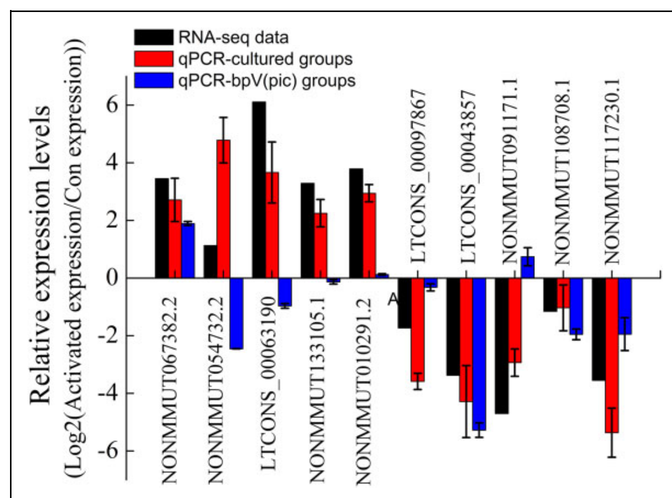

Figure 4. Validation of RNA-seq data by qPCR. Total RNA was isolated from mouse ovaries in control and cultured groups as well as bpV(pic) groups. qPCR was performed to detect RNA expression levels. β-actin was used as a loading control to normalize RNA expression levels. Data are expressed as the mean (SD; n = 3). qPCR indicates quantitative polymerase chain reaction. The color version of this figure is available online.

predicted by *cis* and *trans* mechanisms.[39] Our results showed that macromolecule metabolic processes (regulation of macromolecule metabolic process, regulation of RNA metabolic process, regulation of RNA biosynthetic process, regulation of metabolic process, and positive regulation of RNA metabolic process) were dramatically activated, suggesting the follicles are generally more metabolically active, as anticipated, during the early development stages.[40] We found that many cell proliferation and differentiation-related GO terms were significantly activated, such as regulation of gene expression, regulation of intracellular signal transduction, regulation of transcription DNA-template, and regulation of signal transduction, which will facilitate the activation of primordial follicles.

Previous studies showed that interleukins exert activities in the ovary,[41] neonatal immune activation depletes the ovarian follicle reserve, and accelerated follicle maturation alters ovarian acute inflammatory mediators.[42] Interestingly, our results revealed that the immune system process and regulation of cytokine production-related genes were induced in activated follicles, suggesting these factors might also play a role in the coordination of the primordial follicle to primary follicle transition. A similar finding was also reported by Kezele et al. The authors identified 80 upregulated genes and 44 downregulated genes between the primordial and primary follicle stages using microarray gene chips in rats.[43] In our study, we identified 4171 upregulated mRNAs and 1795 downregulated mRNAs in ovaries cultured for 8 days in vitro compared with controls. These differentially expressed mRNAs were also enriched in the abovementioned GO terms, such as immune system, cell communication, cell differentiation, signal transduction, and reproductive process. However, the precise functions of these genes in primordial follicle development remain to be elucidated. In addition, changes in cell adhesion are very important for follicular assembly,[44] and modifying the culture environment (medium components) can substantially enhance the survival and growth of early-stage follicles.[45] Here, we found that KEGG pathways of cell adhesion molecules, focal adhesion, and adherens junction changed after activation of primordial follicles in in vitro cultured conditions. We propose that a precisely controlled culture condition could ensure activation of primordial follicles and mimic the in vivo process of primordial follicle activation.

The KEGG annotation revealed that the identified genes were involved in important signaling pathways related to proliferation (MAPK, Ras, peroxisome proliferators-activated receptor [PPAR], and Wnt pathways), survival (transforming growth factor β [TGF-β] and NF-κB pathways), metabolism (GnRH pathways), apoptosis (TNF, cell receptor, and calcium pathways), and cell differentiation and development (notch, cyclic guanosine monophosphate-protein kinase G [cGMP-PKG], cAMP, Rap1, thyroid hormone, and oxytocin pathways). Most of these pathways have already been reported to participate in primordial follicle activation.[46] The proto-oncogene c-src and c-erbB2 are involved in primordial follicle activation and growth through the PKC and MAPK signaling pathways.[47,48] Notch signaling-related genes play a pivotal role in the initiation of primordial follicle growth, both in vivo and in vitro.[49,50] The apoptosis of random oocytes in oocyte nests is required for primordial follicle assembly,[51] and TNFα appears to be involved in this process.[52] In addition, an important role for the TGF-β signaling pathway in controlling primordial follicle activation, especially in granulosa cell growth, by modulating the cell–cell communication and microenvironment has recently been discovered.[53,54] Many studies have demonstrated that several key family members of the TGF-β pathway, such as the TGF-β family member activin, play a pivotal role in CYP19A expression through the ActRIB-Smad2 pathway and thus in follicular development.[55] BMP-4, BMP-7, and AMH promote primary follicle transition.[56,57] These findings showing that the TGF-β signaling pathway is critical for primordial follicle development. Remarkably, most KEGG pathways for the differentially expressed mRNAs were also enriched in similar pathways. Based on previous reports and our findings, we propose that the abovementioned pathways may contribute to primordial follicle activation.

Previous study has demonstrated that PTEN can inhibit the activation of dormant follicles.[29] Therefore, to further evaluate the lncRNA expression pattern in activated follicles, bpV(pic), an inhibitor of PTEN, was used to activate primordial follicles. We found that 5 lncRNAs among the selected 10 lncRNAs displayed a similar expression trend after treated with bpV(pic) compared with control groups; however, the other lncRNA expression patterns were inconsistent with RNA-seq and qPCR data. This might be attributed to the PTEN inhibitor exerting other functions, such as regulating the function of theca-interstitial cells and granulosa cells in ovary,[58,59] in addition to activating primordial follicles; these

effects may be responsible for the abnormality of some lncRNA expression patterns. Hence, we propose that the lncRNAs that showed similar expression patterns in both bpV(pic)-treated and nontreated ovaries cultured for 8 days in vitro should be the subjects of future studies on the activation of primordial follicles.

In conclusion, our study for the first time has determined genome-wide lncRNA expression profiles using strand-specific RNA sequencing after activation of primordial follicles with an in vitro culture model. Many lncRNAs and their associated signaling pathways may contribute to the activation of primordial follicles. The lncRNAs identified in the current study could serve as candidates for further investigation of the growth and development of primordial follicles and help expand our comprehensive understanding of follicular development.

Authors' Note

The RNA-seq data sets generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession number GSE103433. The animal experiments were conducted according to the guidelines of the institutional animal ethics committee of Nanchang University (Nanchang, People's Republic of China).

Acknowledgments

The authors thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by the National Natural Science Foundation of China (No. 81771583, 81260098 and 81760283), Natural Science Foundation of Jiangxi province (No. 20161BAB205213), Cultivation Plan for Young Scientists of Jiangxi Province (No. 20153BCB23031), Special Fund of Innovation for Post-graduate Students in Nanchang University (No. CX2017238), and the China Scholarship Council.

Supplemental Material

Supplemental material for this article is available online.

References

1. Ernst EH, Grondahl ML, Grund S, et al. Dormancy and activation of human oocytes from primordial and primary follicles: molecular clues to oocyte regulation. *Hum Reprod*. 2017;32(8):1684-1700.
2. Sun X, Su Y, He Y, et al. New strategy for in vitro activation of primordial follicles with mTOR and PI3K stimulators. *Cell Cycle*. 2015;14(5):721-731.
3. Kezele P, Nilsson E, Skinner MK. Cell-cell interactions in primordial follicle assembly and development. *Front Biosci*. 2002;7:d1990-d1996.
4. Hu X, Sood AK, Dang CV, Zhang L. The role of long noncoding RNAs in cancer: the dark matter matters. *Curr Opin Genet Dev*. 2017;48:8-15.
5. Svoboda P. Long and small noncoding RNAs during oocyte-to-embryo transition in mammals. *Biochem Soc Trans*. 2017;45(5):1117-1124.
6. Su Y, Wu H, Pavlosky A, et al. Regulatory non-coding RNA: new instruments in the orchestration of cell death. *Cell Death Dis*. 2016;7(8):e2333.
7. Hart RP, Goff LA. Long noncoding RNAs: central to nervous system development. *Int J Dev Neurosci*. 2016;55:109-116.
8. Nachtergaele S, He C. The emerging biology of RNA post-transcriptional modifications. *RNA Biol*. 2017;14(2):156-163.
9. Dykes IM, Emanueli C. Transcriptional and post-transcriptional gene regulation by long non-coding RNA. *Genomics Proteomics Bioinformatics*. 2017;15(3):177-186.
10. Wei JW, Huang K, Yang C, Kang CS. Non-coding RNAs as regulators in epigenetics (Review). *Oncol Rep*. 2017;37(1):3-9.
11. Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. *Mol Cell*. 2011;43(6):904-914.
12. Qiu JJ, Wang Y, Ding JX, Jin HY, Yang G, Hua KQ. The long non-coding RNA HOTAIR promotes the proliferation of serous ovarian cancer cells through the regulation of cell cycle arrest and apoptosis. *Exp Cell Res*. 2015;333(2):238-248.
13. Gao Y, Meng H, Liu S, et al. LncRNA-HOST2 regulates cell biological behaviors in epithelial ovarian cancer through a mechanism involving microRNA let-7b. *Hum Mol Genet*. 2015;24(3):841-852.
14. Qiu JJ, Lin YY, Ding JX, Feng WW, Jin HY, Hua KQ. Long non-coding RNA ANRIL predicts poor prognosis and promotes invasion/metastasis in serous ovarian cancer. *Int J Oncol*. 2015;46(6):2497-2505.
15. Gao Q, Ren H, Chen M, et al. Long non-coding RNAs regulate effects of beta-crystallin B2 on mouse ovary development. *Mol Med Rep*. 2016;14(5):4223-4231.
16. Huang X, Hao C, Bao H, Wang M, Dai H. Aberrant expression of long noncoding RNAs in cumulus cells isolated from PCOS patients. *J Assist Reprod Genet*. 2016;33(1):111-121.
17. Ren J, Du X, Zeng T, et al. Divergently expressed gene identification and interaction prediction of long noncoding RNA and mRNA involved in duck reproduction. *Anim Reprod Sci*. 2017;185:8-17.
18. Xiang C, Li J, Hu L, et al. Hippo signaling pathway reveals a spatio-temporal correlation with the size of primordial follicle pool in mice. *Cell Physiol Biochem*. 2015;35(3):957-968.
19. Association APOEaVM. 2000 Report of the AVMA panel on Euthanasia. *J Am Vet Med Assoc*. 2001;218(5):669-696.
20. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12(4):357-360.
21. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol*. 2015;33(3):290-295.

22. Kong L, Zhang Y, Ye ZQ, et al. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res*. 2007;35(Web Server issue): W345-W349.
23. Sun L, Luo H, Bu D, et al. Utilizing sequence intrinsic composition to classify protein-coding and long non-coding transcripts. *Nucleic Acids Res*. 2013;41(17):e166.
24. Punta M, Coggill PC, Eberhardt RY, et al. The Pfam protein families database. *Nucleic Acids Res*. 2012;40(Database issue): D290-D301.
25. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012;7(3):562-578.
26. Wang L, Feng Z, Wang X, Wang X, Zhang X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*. 2010;26(1):136-138.
27. Tafer H, Hofacker IL. RNAplex: a fast tool for RNA-RNA interaction search. *Bioinformatics*. 2008;24(22):2657-2663.
28. Asadi-Azarbaijani B, Santos RR, Jahnukainen K, et al. Developmental effects of imatinib mesylate on follicle assembly and early activation of primordial follicle pool in postnatal rat ovary. *Reprod Biol*. 2017;17(1):25-33.
29. Li J, Kawamura K, Cheng Y, et al. Activation of dormant ovarian follicles to generate mature eggs. *Proc Natl Acad Sci U S A*. 2010; 107(22):10280-10284.
30. Wang H, Fu Z, Dai C, et al. LncRNAs expression profiling in normal ovary, benign ovarian cyst and malignant epithelial ovarian cancer. *Sci Rep*. 2016;6:38983.
31. Liu YD, Li Y, Feng SX, et al. Long noncoding RNAs: potential regulators involved in the pathogenesis of polycystic ovary syndrome. *Endocrinology*. 2017;158(11):3890-3899.
32. O'brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod*. 2003;68(5):1682-1686.
33. Braw-Tal R, Yossefi S. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil*. 1997; 109(1):165-171.
34. Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE. Initiation of growth of baboon primordial follicles in vitro. *Hum Reprod*. 1997;12(9):1993-2001.
35. Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod*. 1999;14(10): 2519-2524.
36. Kedem A, Fisch B, Garor R, et al. Growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 both activate development of human primordial follicles in vitro, with seemingly more beneficial effects of GDF9. *J Clin Endocrinol Metab*. 2011;96(8):E1246-E1254.
37. Martins FS, Celestino JJ, Saraiva MV, et al. Growth and differentiation factor-9 stimulates activation of goat primordial follicles in vitro and their progression to secondary follicles. *Reprod Fertil Dev*. 2008;20(8):916-924.
38. Xiong Y, Liu T, Wang S, Chi H, Chen C, Zheng J. Cyclophosphamide promotes the proliferation inhibition of mouse ovarian granulosa cells and premature ovarian failure by activating the lncRNA-Meg3-p53-p66Shc pathway. *Gene*. 2017;596:1-8.
39. Bassett AR, Akhtar A, Barlow DP, et al. Considerations when investigating lncRNA function in vivo. 2014;3:e03058.
40. Mihm M, Bleach EC. Endocrine regulation of ovarian antral follicle development in cattle. *Anim Reprod Sci*. 2003;78(3-4): 217-237.
41. Wang LJ, Brannstrom M, Cui KH, et al. Localisation of mRNA for interleukin-1 receptor and interleukin-1 receptor antagonist in the rat ovary. *J Endocrinol*. 1997;152(1):11-17.
42. Fuller EA, Sominsky L, Sutherland JM, et al. Neonatal immune activation depletes the ovarian follicle reserve and alters ovarian acute inflammatory mediators in neonatal rats. *Biol Reprod*. 2017; 97(5):719-730.
43. Kezele PR, Ague JM, Nilsson E, Skinner MK. Alterations in the ovarian transcriptome during primordial follicle assembly and development. *Biol Reprod*. 2005;72(1):241-255.
44. Pepling ME. Follicular assembly: mechanisms of action. *Reproduction*. 2012;143(2):139-149.
45. Tagler D, Makanji Y, Tu T, et al. Promoting extracellular matrix remodeling via ascorbic acid enhances the survival of primary ovarian follicles encapsulated in alginate hydrogels. *Biotechnol Bioeng*. 2014;111(7):1417-1429.
46. Skinner MK. Regulation of primordial follicle assembly and development. *Hum Reprod Update*. 2005;11(5):461-471.
47. Du XY, Huang J, Xu LQ, et al. The proto-oncogene c-src is involved in primordial follicle activation through the PI3K, PKC and MAPK signaling pathways. *Reprod Biol Endocrinol*. 2012; 10:58.
48. Li-Ping Z, Da-Lei Z, Jian H, et al. Proto-oncogene c-erbB2 initiates rat primordial follicle growth via PKC and MAPK pathways. *Reprod Biol Endocrinol*. 2010;8:66.
49. Chen CL, Fu XF, Wang LQ, et al. Primordial follicle assembly was regulated by Notch signaling pathway in the mice. *Mol Biol Rep*. 2014;41(3):1891-1899.
50. Vanorny DA, Prasasya RD, Chalpe AJ, Kilen SM, Mayo KE. Notch signaling regulates ovarian follicle formation and coordinates follicular growth. *Mol Endocrinol*. 2014;28(4):499-511.
51. Hussein MR. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update*. 2005;11(2):162-177.
52. Marcinkiewicz JL, Balchak SK, Morrison LJ. The involvement of tumor necrosis factor-alpha (TNF) as an intraovarian regulator of oocyte apoptosis in the neonatal rat. *Front Biosci*. 2002;7: d1997-d2005.
53. Chen YC, Chang HM, Cheng JC, Tsai HD, Wu CH, Leung PC. Transforming growth factor-beta1 up-regulates connexin43 expression in human granulosa cells. *Hum Reprod*. 2015;30(9): 2190-2201.
54. Cheng JC, Chang HM, Fang L, Sun YP, Leung PC. TGF-beta1 up-regulates connective tissue growth factor expression in human granulosa cells through Smad and ERK1/2 signaling pathways. *PLoS One*. 2015;10(5):e0126532.
55. Nomura M, Sakamoto R, Morinaga H, Wang L, Mukasa C, Takayanagi R. Activin stimulates CYP19A gene expression in human ovarian granulosa cell-like KGN cells via the Smad2 signaling pathway. *Biochem Biophys Res Commun*. 2013;436(3):443-448.

56. Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod*. 2003;69(4):1265-1272.
57. Nilsson EE, Schindler R, Savenkova MI, Skinner MK. Inhibitory actions of anti-Mullerian hormone (AMH) on ovarian primordial follicle assembly. *PLoS One*. 2011;6(5):e20087.
58. Liu Z, Ren YA, Pangas SA, et al. FOXO1/3 and PTEN depletion in granulosa cells promotes ovarian granulosa cell tumor development. *Mol Endocrinol*. 2015;29(7):1006-1024.
59. Lan ZJ, Krause MS, Redding SD, et al. Selective deletion of Pten in theca-interstitial cells leads to androgen excess and ovarian dysfunction in mice. *Mol Cell Endocrinol*. 2017;444:26-37.