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Integrated miRNAome and mRNAome analysis identifies candidate genes that mediate testis development promoted by vitamin E in sheep*

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Vitamin E is known to improve testis development, yet the underlying mechanism responsible for this process remains poorly understood. To elucidate the mechanism, through which vitamin E facilitates testiscular development, we hypothesised that vitamin E improves testis development by promoting testis cell proliferation, while miRNAs also play a crucial role in the process. In order to test this hypothesis, we isolated primary testis cells from prepubertal sheep. Using the CCK-8 (Cell Counting Kit-8) assay we found that 800 μM vitamin E supplementation enhanced cell proliferation. This was verified through western blot analysis of Ki67 and PCNA (proliferating cell nuclear antigen). Subsequently, cell cycle distribution induced by vitamin E was determined in the cells. Vitamin E markedly decreased the proportion of cells in the G1 phase and increased those in the S and G2/M phases in response to vitamin E supplementation. Meanwhile, vitamin E significantly increased expression of Cyclin B1 and Cyclin B3. Through the miRNAome and transcriptome technology, we identified a number of correlation pairs including miR-107-BLM (BLM RecQ like helicase), miR-107-REL (REL proto-oncogene) and miR-493-3p-KLHL25 (kelch like family member 25), that are known to be involved in the regulation of correlation pairs participating in the cell cycle. Together these data demonstrate that 800 μM vitamin E promotes testis cell proliferation and its action may be mediated by miR-107 and miR-493-3p that target the *BLM*, *REL* and *KLHL25*.

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Vitamin E serves as a very important lipid-soluble vitamin for animal reproduction, its supplementation increases the diameter of seminiferous tubules [Momeni et al. 2012] and testis weight [Rao *et al*. 2001]. At the same time, we also found that dietary vitamin E could improve testis size, density of testis cells, histological feature of the seminiferous tubules and epididymis development in sheep and goats [Luo *et al*. 2011, Zhu *et al*. 2009]. However, the specific mechanism, by which vitamin E affected testis development has been poorly elucidated.

Some studies refered to the relationship between vitamin E and miRNA expression [Fang *et al*. 2019, Gaedicke *et al*. 2008, Park *et al*. 2011, Khanna *et al*. 2013]. Vitamin E can inhibit the activity of nuclear exoribonucleases by 3'-phosphoadenosine 5'-phosphate (PAP) so that primary miRNAs may be kept from being degraded, which facilitates production of mature miRNAs [Fang *et al*. 2019]. In addition, α-tocopherol imparted an impact on the expression of miR-199a-5p and miR-29b [Park *et al.* 2011, Khanna *et al.* 2013]. A diet lacking vitamin E resulted in the lower expression of miRNA-122a, which was related to lipid metabolism [Gaedicke *et al.* 2008]. If miRNA mediate testicular development induced by vitamin E, how will they work? Some studies demonstrated that a great number of additional miRNAs regulated the proliferation of Leydig, Sertoli and germ cells in testes [Rakoczy *et al.* 2013, Yao *et al*. 2016, Wu *et al*. 2011].

Based on the above-mentioned literature sources it has been stated that vitamin E promotes testis development and organ development is tightly determined by cell proliferation and apoptosis [Bryant *et al*. 1984, Raff *et al.* 1992], in addition, miRNAs play a vital role in male reproduction and testis cell proliferation. So we put forward the hypothesis that vitamin E enhances testis development by affecting cell proliferation in the prepubertal testis through the regulation of miRNAs. Our research aims at exploring the mechanism that vitamin E promotes testis development so that it provides significant references for animal husbandry.

Material and methods

Cell isolation and culture

The experimental animal protocols were approved by the China Agricultural University Animal Care and Use Committee, Beijing, China. Testes were removed from 2-month-old Dorper×thin tailed Han crossbred sheep. After rinsing with 75% ethanol (ANNJET, Dezhou, China) and then with PBS (phosphate-buffered saline) containing 1% penicillin/streptomycin (Hyclone, Logan, Utah, USA), the tunica albuginea was removed. The testicular parenchyma was cut into a paste and testicular cells were obtained through digestion with 5 mg/ml collagenaseI(Sigma, NY, USA) for 15 min at 37ºC. The suspension was filtered through two cell strainers (Corning, NY, USA, mesh size 40 μM and 70 μM, respectively). Individual cells were then

cultured in 100 mm culture dishes (Corning, NY, USA) in DMEM (Dulbecco's Modified Eagle Medium) /F12 (1:1) medium (Hyclone, Logan, Utah, USA) containing 15% fetal bovine serum (Gibco, Waltham, Massachusetts, USA), and 1% penicillin/ streptomycin (Hyclone, Logan, Utah, USA).

CCK-8 Assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to measure the effect of vitamin E on testis cell proliferation [Wang *et al*. 2012]. Testis cells were seeded in 96-well plates (Corning), with 3×10^4 cells per well, in 5 replications. Using the kit, cell counting was performed 24 h after vitamin E (α-tocoperol acetate, Zhejiang Jin Dakang Animal Health Products Co., Ltd.) at different concentrations, was added to the cells. Vitamin E was dissolved in ethanol (Beijing Chemical Works, Beijing, China) and when added to the media it accounted for 0.5% (v/v) of the total volume. The final concentration of vitamin E in the culture medium was 0 μM (control), 100 μM, 200 μM, 400 μM, 800 μM, 1000 μM, 1400 μM and 1600 μM, respectively.

Determination of intracellular vitamin E levels

Intracellular vitamin E content was determined according to a method described previously [Noguchi *et al*. 2003]. One million primary testis cells were collected, which was followed by adding 2 volumes of chloroform/methanol mixture (2/1, v/v) to the cells. After drying under a N_2 atmosphere the chloroform layer was replaced with ethanol and the intracellular vitamin E content was determined with the HPLC (High Performance Liquid Chromatography, Agilent, Palo Alto, CA, USA).

Western blot analysis

Western blot was performed as described previously [Tao *et al*. 2018]. Briefly, cells from the vitamin E group (800 μM) along with the control were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Extracted proteins were resolved in a 12% (PCNA), 5% (Ki67), 12% (Cyclin B1) and 10% (Cyclin B1) SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel as a seperation gel and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, pore size 0.22 μm). After blocking with 5% BSA (Amresco, Emeryville, CA, USA) at 37 ºC for 60 min, the membranes were incubated with primary antibodies against Ki67 (Abcam/ab15580), PCNA (Abcam/ab18197), Cyclin B1 (Bioss/bs-23016R), Cyclin B3 (Bioss/bs-7884R) and GAPDH (Abcam/ab22555). Subsequently, the membranes were washed with TBS-T (Tris-Buffered Saline-Tween) 4 times, 5 min each time and incubated with a secondary antibody (goat anti-mouse IgG; HRP, santa/Sc-2005, and goat anti-rabbit IgG; HRP, santa/Sc-2004). The bands obtained were analysed using the Quantity One v.4.6.2 software (https://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software?ID=1de9eb3a-1eb5-4edb-82d2-68b91bf360fb, Bio-Rad, Hercules, CA, USA).

Cell cycle distribution assay

The DNA Content Quantitation Assay (Solarbio Life Science, Beijing, China) was used to assess cell cycle distribution. As above, the experiment included 10 replications in each group, the control and the treatment group (800 μM α-tocopherol). In each replication we harvested 1×10^6 cells to fix with 70% ethanol at 4ºC overnight, then RNase A and propidium iodide were added. Cell cycle distribution was determined by means of a Flow Cytometer (BD FACSCalibur, BD Biosciences, New Jersey, USA) and the Flowjo 10 software (https://www.flowjo.com/solutions/flowjo/downloads, BD Biosciences, New Jersey, USA) was used to analyse the results.

miRNAome analysis

For the experiment two groups, the control and the vitamin E group (800 μM α-tocopherol) were created; each group comprised 3 replications. After a 24-hour treatment with vitamin E the control and treated cells were collected and immediately placed into liquid nitrogen until use in the miRNAomes and transcriptomes analyses.

Total RNA (Ribonucleic acid) was isolated from the samples using Trizol (Qiagen, Hilden, Germany) according to the manufacturer's protocol; the concentration of total RNA was measured by Qubit3.0 (Invitrogen, Waltham, Massachusetts, USA). The quality of the isolated RNA was assessed using an Agilent 2100 Bioanalyzer (Aligen, Santa Clara, CA, USA) and RNA with a RIN (RNA integrity number) value of at least 7 was used for further analysis.

Small RNA sequencing libraries were constructed according to the Illumina TruSeq Small RNA Sample Preparation protocol. In brief, the 3' and 5' RNA adapter, specifically modified to target the ends of small RNA molecules, was ligated to 1 mg of high-quality total RNA. Reverse transcription was performed to generate cDNA (complementary DNA) libraries. Then, PCR (Polymerase chain reaction) was used to amplify and add unique index sequences to each library. The library preparations were sequenced on an Illumina Hiseq 2500 platform (Illumina, San Diego, CA, USA) and 50bp single-end reads were generated.

Raw data were processed using a bioinformatic pipeline. First, low quality reads were removed. The reads were then trimmed by removing the 3' prime adaptors. Finally, the sequence lengths, ranging from 18 to 30 nt, were chosen in the following processes. Conserved miRNAs (microRNA) were identified by comparing the sRNA (small RNAs) reads with the known miRNAs collected in miRbase (http://www.mirbase.org/, University of Manchester, England) [Griffiths *et al.* 2006] using Bowtie 1.1.1 (http://bowtie bio.sourceforge.net/manual.shtml, Johns Hopkins University, Baltimore, USA), allowing one mismatch. Novel miRNAs were identified from the unmatched reads. Putative miRNA precursors were identified by miRDeep2 (https://www.mdc-berlin.de/n-rajewsky#t-data,software&resources, Max Delbrück Center for Molecular Medicine, Berlin, Germany). Only those with precursors found in the genome were identified as conserved or novel miRNAs. Potential miRNA targets were predicted using miRanda (http://cbio.mskcc.org/microrna_data/manual.html, Computational

Biology Center of Memorial Sloan-Kettering Cancer Center, New York, USA) [Dai et al. 2011] with the default parameters. Different expression analyses were performed by the edgeR (http://www.bioconductor.org/packages/3.0/bioc/html/edgeR.html) and the limma package (http://bioconductor.org/packages/release/bioc/html/limma.html). Function annotation of the target genes and enrichment analyses for the differentially expressed miRNAs were performed with KOBAS 3.0 (https://bioinformaticshome.com/tools/rna-seq/descriptions/KOBAS.html, Beijing University, China). Parameters for classifying significantly differentially expressed miRNAs were ≥ 2-fold differences ($|\log_2 FC(\text{Fold Change})| \geq 1$) and $P < 0.05$ in the transcript abundance.

mRNAome analysis

Cell treatment and RNA isolation were taken as the above-mentioned methods. Libraries for sequencing were constructed with the NEB Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA). Poly A-tailed mRNA molecules were enriched from 1 μg total RNA using the NEB Next Poly (A) mRNA Magnetic Isolation Module kit (New England Biolabs). The mRNA were fragmented into approximately 200 base pairs. First-strand cDNA was synthesised from the mRNA fragments using reverse transcriptase and random hexamer primers, then the second-strand cDNA was synthesised using DNA polymerase I and Rnase H. The end of the cDNA fragment was subjected to an end repair process that included the addition of a single 'A' base, followed by ligation of the adapters. Products were purified and enriched by PCR to amplify the library DNA. The final libraries were quantified using the KAPA Library Quantification kit (KAPA Biosystems, Cape Town, South Africa) and an Agilent 2100 Bioanalyzer (Agilent). After the quantitative reverse transcription-polymerase chain reaction, the libraries were subjected to paired-end sequencing with the pair end 150-base pair reading length on an Illumina HiSeq sequencer (Illumina).

The sheep genome (Ensembl Oar_v3.1, http://uswest.ensembl.org/Ovis_aries/Info/Annotation) was used as the reference. Sequencing quality was assessed with FastQC (Version 0.11.5, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, The Babraham Institute, Cambridge, UK) and low quality data were filtered using NGSQC (v0.4, http://www.nipgr.res.in/ngsqctoolkit.html, National Institute of Plant Genome Research (NIPGR), New Delhi, India). The clean reads were aligned to the reference genome using HISAT2 (http://daehwankimlab.github.io/hisat2/, Johns Hopkins University, Baltimore, USA) with default parameters. The processed reads from each sample were aligned using HISAT2 (Johns Hopkins University, USA) against the reference genome. Gene expression analysis was performed using Cuffquant and Cuffnorm (Cufflinks 2.2.1, http://cole-trapnell-lab.github.io/cufflinks/releases/v2.2.1/, University of California, Berkeley, USA and Johns Hopkins University, Baltimore, USA).

Cuffdiff (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/, Harvard University, Cambridge, USA) was used to analyse the DEGs (differentially expressed genes)

between samples. The standardisation method of Cuffdiff was geometric, with the per-condition and pooled as the discrete model. Thousands of independent statistical hypothesis testing were conducted on DEGs separately. Parameters for classifying significant DEGs were ≥2-fold differences ($|\log_2 FC| \geq 1$, FC: the fold change of expressions) and $P < 0.05$ in the transcript abundance.

The annotation of the DEGs were performed based on the information obtained from the ENSEMBL database (https://uswest.ensembl.org/index.html, Wellcome Sanger Institute, Cambridgeshire, UK; EMBL - European Bioinformatics Institute, Cambridge, UK; European Molecular Biology Laboratory, Heidelberg, Germany), NCBI (https://www.ncbi.nlm.nih.gov/, National Center for Biotechnology Information, Bethesda, Maryland), Uniprot (https://www.uniprot.org/, EMBL - European Bioinformatics Institute, Cambridge, UK; SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland; Protein Information Resource, Washington, D.C. USA), GO (Gene Ontology) (http://geneontology.org/, the Gene Ontology Consortium), and KEGG (https://www.genome.jp/kegg/, Kyoto Encyclopedia of Genes and Genomes) databases (Kyoto University, Kyoto, Japan). The enrichment analysis was performed using Omicsbean (http://www.omicsbean.cn/, geneforhealth, Shanghai, China).

miRNAomes-transcriptomes integrated analysis

To identify significant negative miRNA-mRNA (Messenger RNA) interacting pairs, we standarised the expression value and estimated the Pearson correlation coefficient and the p-value of differentially-expressed miRNAs and mRNAs using the R statistics software (https://www.r-project.org/, the University of Auckland, Auckland City, New Zealand). Based on the correlation coefficient < -0.80 and $P < 0.05$, significantly negative related pairs were screened. Further, we assessed the target genes of candidate miRNAs by miRanda (http://www.microrna.org/, Computational Biology Center of Memorial Sloan-Kettering Cancer Center). Finally, candidate miRNA-mRNA correlation pairs were identified and a network graph was made by igraph (http://igraph.org/, Harvard University, Cambridge, USA and Eötvös University, Budapest, Hungary).

Statistical analysis

CCK-8, cell cycle and Western blot were analysed by IBM SPSS 25.0 (https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-25, Armonk, NY, USA), using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. The graphs of CCK-8, cell cycle and Western blot were made by the GraphPad Prism 5 software (https://www.graphpad.com/support/prism-5-updates/, San Diego, CA, USA). Differences with a $P<0.05$ were considered statistically significant, while $P <0.01$ indicated highly significant differences.

Results and discussion

Cell number assay

Results of the CCK-8 assay indicated that vitamin E effectively enhanced cell proliferation, especially at the 800 μM concentration (Fig. 1). There were no significant differences between the non-treated control and the vehicle-treated group. Compared with the control, any other concentrations of vitamin E enhanced testicular cell proferation ($P<0.01$). Based on these results we concluded that vitamin E could enhance cell proliferation and its most effective in vitro concentration was 800 μM.

Results of the assay indicated that in response to vitamin E the percentage of dividing cells was higher than that in the control group. Similarily, our previous in vivo study revealed that dietary Vitamin E increased the density of somatic and germ cells in the testes [Luo *et al*. 2011, Zhu *et al*. 2009]. Besides, supplementation of vitamin E increased the number of germ cells in testes and the epididymis [Qu *et al.* 2019]. In addition, we determined intracellular vitamin E content by HPLC and confirmed that vitamin E was indeed absorbed by the cells (Fig. 2).

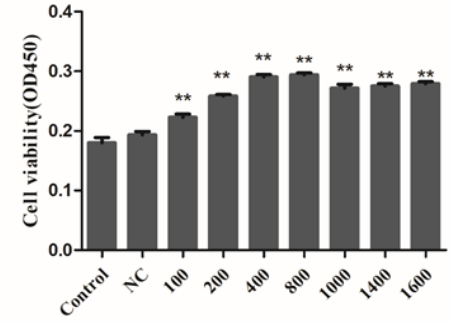

Fig. 1. Effect of vitamin E on testis cells proliferation in a CCK-8 assay. (**represents $P<0.01$. NC represents negative control. The control, 100, 200, 400, 800, 1000, 1400 and 1600 mean groups of exposure to different dosages of vitamin E).

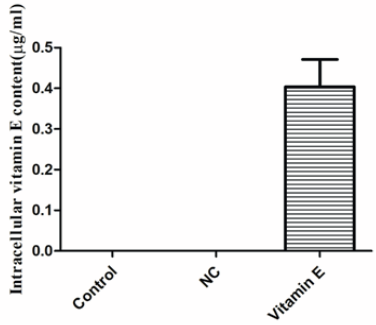

Fig. 2. Effect of supplemental vitamin E on intracellular vitamin E content (NC represents negative control. Vitamin E group means addition of 800 μM.)

As above, the two proteins are widely used to evaluate cell proliferation [Muskhelishvili *et al*. 2003]. The expression level of the two candidate proteins indicates the extent of cell proliferation. The exact function of Ki67 is unclear, but PCNA is known to participate in DNA replication [Juríková *et al*. 2016]. Both proteins were reported to be highly expressed during the S phase (DNA synthesis phase) of the cell cycle [Celis *et al*. 1984, Celis *et al.* 1985]. This was also confirmed by the result of the western blot analysis, unequivocally demonstrating that vitamin E promotes cell proliferation. Western blot indicated that 800 μM vitamin E considerably supported testis cell proliferation. Expression levels of Ki67 and PCNA in the vitamin E group were markedly higher than those in the control ($P<0.01$) (Fig. 3A and 3B). Although we confirmed that vitamin E indeed affected cell proliferation, whether cell cycle

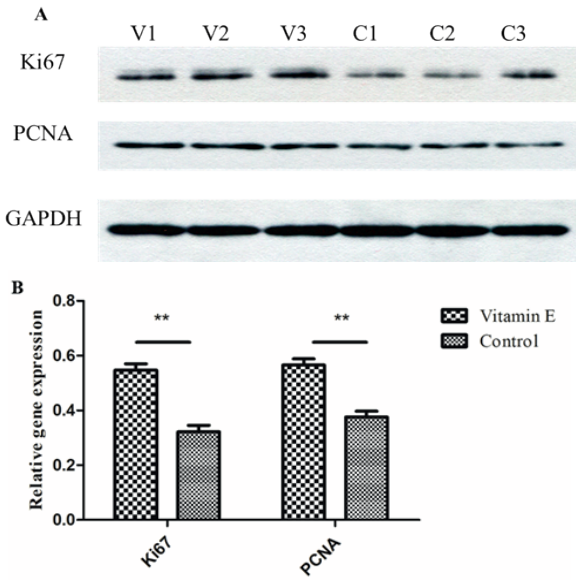

Fig. 3. Western blot analysis for Ki67 and PCNA (V, vitamin E group; C, control). A - results of Western blot; B - results of density analysis. **represents P<0.01; V1,V2, V3 represent three replications in vitamin E group; C1,C2, C3 represent three replications in control.

distribution was regulated by vitamin E was unknown. Thus we made the further cell cycle analysis.

Cell cycle distribution

The cell cycle consists of 4 phases, G1, S, G2 and M. The G1 phase is a gap phase, during which the cell prepares to replicate its DNA. During the S phase of the cell cycle DNA synthesis takes place [Manders *et al*. 1992]. The G2 phase is another gap phase that follows DNA synthesis, while the M phase is also known as the mitotic phase, during which two daughter cells are produced [Nurse 1990]. In this study we determined and analysed the distribution of cells in the various phases of the cell cycle in response to vitamin E supplementation of the culture medium (Fig. 4). Compared with the control, vitamin E decreased the proportion of cells in the G1 phase ($P<0.01$). At the same time, the frequency of cells that were in the S phase increased as a result of vitamin E supplementation of the culture media ($P<0.01$). In addition, the treated cells had a propensity to be in the G2/M phase of their cell cycle ($P<0.05$). The results showed that vitamin E decreased the proportion of cells in the G1 phase, while at the same time it could increase the percentage of cells in the S phase and G2/M compared with the control. In other words, vitamin E decreased cell arrest in the G1 phase and promoted cell transition from G1 to DNA sythesis and the mitosis phase. These results suggest that vitamin E affects the cell cycle, increases the percentage of cells undergoing DNA synthesis and thus promotes cell proliferation.

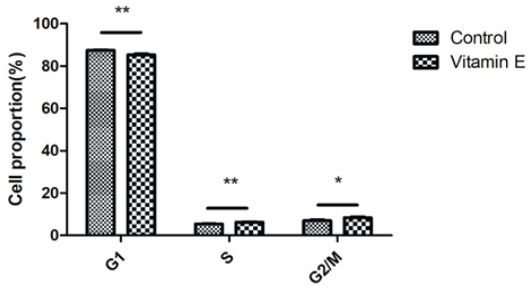

Fig. 4. Effect of vitamin E on primary testis cell cycle distribution. *represents P<0.05; **represents P<0.01. G1, S, G2/M represents three phases of cell cycle.

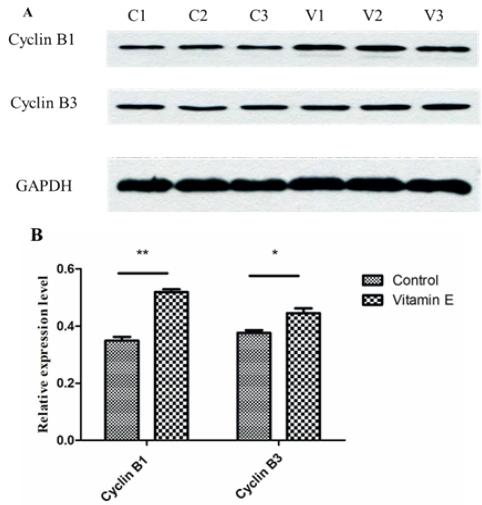

Fig. 5. Western blot analysis for Cyclin B1 and Cyclin B1. A - results of Western blot; B - results of density analysis. V1,V2, V3 represent three replications in vitamin E group; C1,C2, C3 represent three replications in control. *represents P<0.05; **represents P<0.01.

To further explore the cell cycle affected by vitamin E, we determined the expression of Cyclin B1 and Cyclin B3 (Fig. 5). Cyclin B1 (CCNB1) plays a crucial role in the transition from G2 to M [Strauss *et al*. 2017]. Downregulation of Cyclin B1 could inhibit cell proliferation [Müssnich *et al*. 2015]. Our western blot analysis demonstrated that vitamin E could increase the expression of Cyclin B1 (P<0.01) and Cyclin B3 (P<0.05). The results are consistent with our results that vitamin E promoted cell proliferation and up-regulated Cyclin B1 expression.

miRNAome analysis

As above, the total number of high-quality raw reads was around 121.44 million, with an average of 20.24 million reads per library (control and Vitamin E). When only small RNA with an appropriate length (18-30 nt) was considered, the clean reads rate

was 90.84%. Mapping to the miRBase and the reference genome resulted in a number of known miRNAs and novel RNAs.

As above, no known miRNAs were identified. As for novel miRNAs, we found 13 up-regulated miRNAs and 10 down-regulated miRNAs (Fig. 6).

MiRanda, with the threshold value set as score ≥140 and Energy ≤ -20kcal/mol, was able to predict target genes. A total of 13 up-regulated miRNAs were predicted to target 104 genes, while 10 down-regulated miRNAs targeted 65 genes. Functions and pathways of the target genes were analysed by the KOBAS 3.0 software. The top 20 GO terms, including the biological process, cellular component and molecular

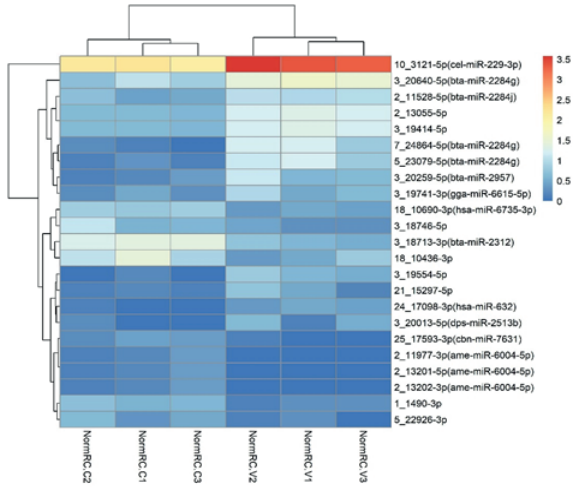

Fig. 6. Numbers of differentially expressed novel miRNAs in the miRNAome. V1,V2, V3 represent three replications in vitamin E group; C1,C2, C3 represent three replications in control. The novel miRNAs was displayed in the right side. The expression level of each miRNA in vitamin E group and control was shown by the color of heat map.

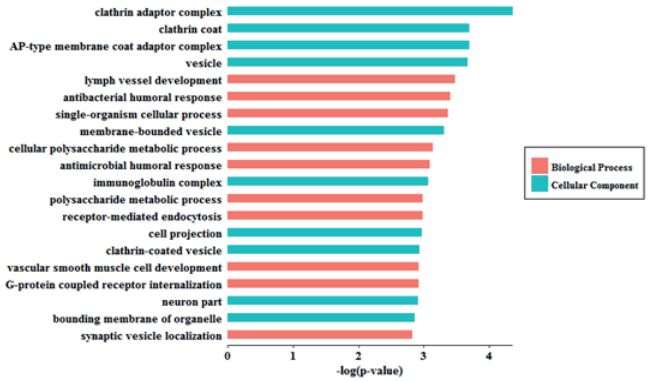

Fig. 7. GO enrichment analysis of novel miRNA target genes in the miRNAome. The vertical axis displayed top 20 enriched GO terms. The horizontal axis showed the significance of GO terms.

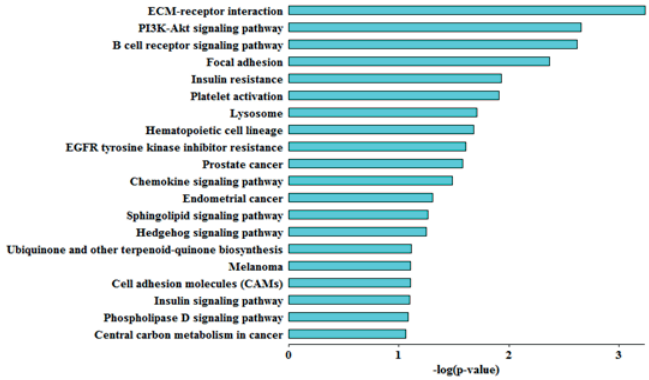

Fig. 8. KEGG pathway analysis of novel miRNA target genes in the miRNAome. The vertical axis displayed top 20 enriched KEGG pathway. The horizontal axis showed the significance of KEGG).

function, and the top 20 KEGG pathways identified are shown in Figure 7 and Figure 8, respectively. However, no key functions and pathways related to male reproduction were observed.

RNA-Seq analysis

The total amount of high-quality raw reads was 240.59 million, 40.10 on average (control and vitamin E group). The clean reads rate was 90.49% on average. By mapping to the reference genome using the HISAT software, we acquired a total of 199.26 million reads. We identified 844 differetially expressed genes, including 321 up-regulated and 523 down-regulated genes between the vitamin E group and the control (FC>2 or FC<0.5, P<0.05) using the Cuffdiff software. After removing genes without gene symbols we ended up with 69 up-regulated and 176 down-regulated genes.

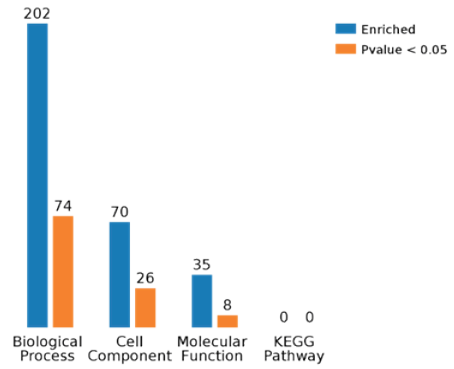

Fig. 9. Number of enriched significant GO terms and KEGG pathways in the transcriptome.

In addition, following the GO terms enrichment analysis we were able to identify 202 biological process terms, 70 cell component terms and 35 molecular function terms, which contained 74, 26 and 8 significant terms, respectively. None of the findings were associated with signaling pathways (Fig. 9).

The top 10 GO terms are shown in the Figure 10. We found that some of the GO terms were related to male reproduction, such as cellular response to luteinizing hormone stimulus and luteinizing hormone receptor activity. This finding made us further explore the situation using integrated analysis, and we speculated that a relatively high fold change might be a limiting factor that impedes the identification of key candidate genes.

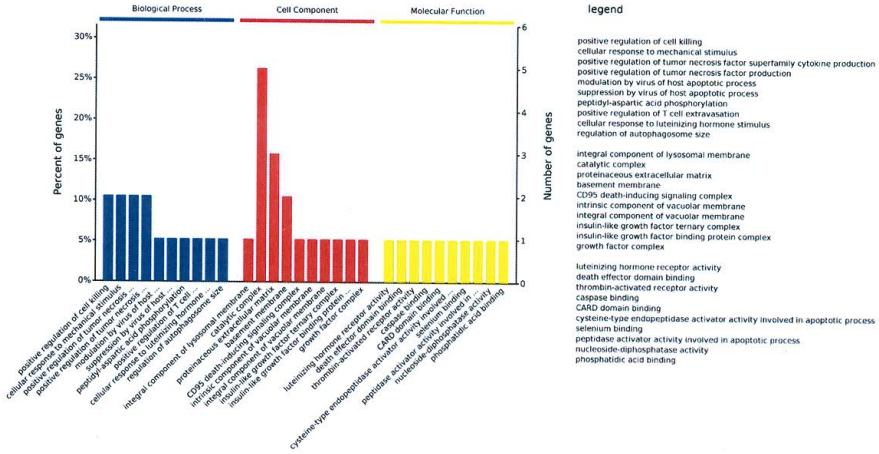

Fig. 10. Top 10 GO terms in biological process, cell component or molecular function in the transcriptome. The vertical axis displayed the numbers of genes of enriched GO terms. The horizontal axis showed the names of GO terms.

miRNAs-mRNAs targeting interacting pairs

In an effort to further investigate and identify the exact mechanisms involved in the mediation of vitamin E effects, we integrated the miRNAome and transcriptome technology to analyse key candidate miRNAs-genes. As expected, we were able to identify 15799 significantly negative related miRNA (FC>1.1 or FC<0.91, P<0.05) - mRNA (FC>1.5 or FC<0.67, P<0.05) pairs, using the R statistics software. Within the pairs, 87 targeting correlations existed between miRNAs and mRNAs (Fig. 11).

By means of the GO enrichment analysis, 114 terms were identified (P<0.05), consisting of 69 biological process terms, 18 cellular component terms and 27 molecular function terms. The top 20 significant enrichment terms are shown in Figure 12.

As shown in Figure 12, among the top 20 terms we could find DNA conformation change, condensed chromosome and DNA secondary structure binding, which were closely related to the cell cycle process. In addition, mitotic chromosome condensation,

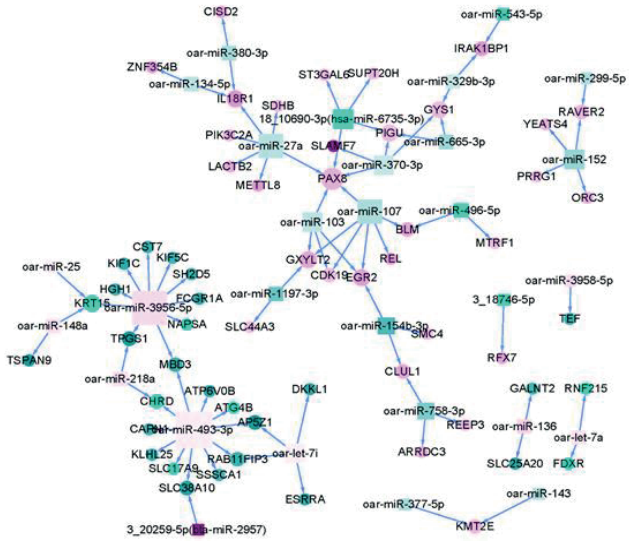

Fig. 11. Network of miRNAs-mRNAs Targeting Correlations.

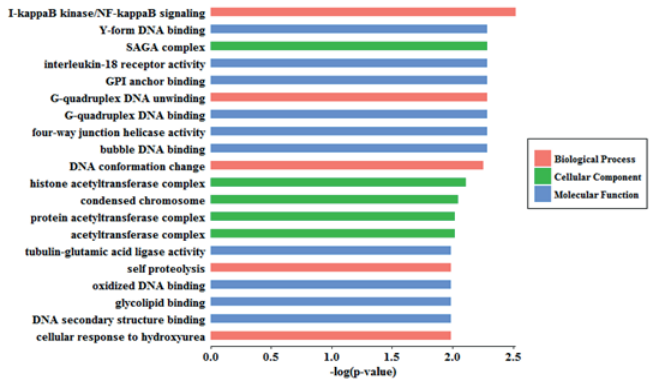

Fig. 12. The top 20 significant enrichment terms identified by intergration analysis. The vertical axis displayed top 20 enriched GO terms. The horizontal axis showed the significance of GO terms.

the mitotic G2 DNA damage checkpoint, mitotic G2/M transition checkpoint, negative regulation of G2/M transition of mitotic cell cycle, negative regulation of cell cycle G2/M phase transition, G2 DNA damage checkpoint and mitotic DNA damage checkpoint terms, among many others, were also significantly enriched, although these terms are not within the top 20 striking. By terms related to the cell cycle, we identified the key miRNAs-mRNAs pair, miR-107-BLM, which was closely related to cell proliferation. In addition, we found that two candidate genes, REL and KLHL25, that were reported previously to be associated with the process investigated here, were regulated by miR-107 and miR-493-3p, respectively.

Taking the relationship between vitamin E, testis cell proliferation and miRNAs into consideration, we applied the omic techniques to screen key candidate miRNAs and downstream target genes. Using the integrated analysis of the mRNAome and miRNAome, we identified 2 candidate miR-107 and miR-493-3p, which are associated with cell proliferation in sheep. In addition, miR-107 and miR-493-3p had a known function in cell cycle arrest and the mitotic process [Takahashi et al. 2009, Feng *et al*. 2012, Tambe *et al.* 2016]. The result is consistent with our results.

Further, by the integrated bioinformatic analysis of miRNAome and mRNAome we identified 3 candidate interacting correlation pairs, miR-107-BLM, miR-107-REL and miR-493-3p-KLHL25, which participate in the cell cycle process,. BLM is one of the RecQ family helicases, known to be associated with genetic disorders and genomic instability. It shows a high level of expression in the S phase and the G2/M transition, while its level sharply decreases during the G1 phase of the cell cycle [Dutertre *et al*. 2000]. In addition, up-regulated BLM ensures genomic stability in actively proliferating cells [Kawabe *et al*. 2000, Naim *et al.* 2009, Neff *et al*. 1999]. Depletion of BLM increases sister chromatid exchange and decreases genomic stability [Wang *et al.* 2013]. In our result, vitamin E could up-regulate BLM. Thus, vitamin E might play a role in maintaining genomic stability to guarantee that the process of cell proliferation goes on. Two additional candidate genes, REL and KLHL25, which are regulated by miR-107 and miR-493-3p, respectively, might also participate in the process. The knockdown of KLHL25 or knockout of REL significantly promoted cell proliferation [Zhang *et al.* 2016, Gilmore *et al.* 2004]. Our bioinformatic result also found that vitamin E could downregulate KLHL25 and REL. Based on the trial and bioinformatic analysis, we arrived at the following conclusion.

Our results first demonstrated that vitamin E is able to promote testis cell proliferation in vitro. In addition, we first studied cell cycle distribution and identified two miRNAs (miR-107 and miR-493-3p), as well as three targeting genes (*BLM, REL and KLHL25*), through which vitamin E may regulate the cell cycle phase and stimulate cell proliferation. These results provide a new insight into the mechanism, through which vitamin E promotes testicular development.

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