Article

Transcriptomic Analysis of the Developing Testis and Spermatogenesis in Qianbei Ma Goats

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Abstract: The achievement of reproductive competence in male mammals is dependent on the testis. Goat testis' development and spermatogenesis involve physiological events with high complexity. In the current work, 6 testes were respectively collected from immature, sexually mature and physically mature Qianbei Ma goats (1, 6 and 12 months old, respectively). RNA-Seq was carried out to reveal changes in testis mRNA expression levels in Qianbei Ma goats at various developmental stages, and gene expression profiling at different ages was established. Totally 18 libraries were established for screening genes and pathways associated with testis development and spermatogenic processes. Totally 9,724 upregulated and 4,153 downregulated genes were identified between immature (I) and sexually mature (S) testes; 7 upregulated and 3 downregulated genes were detected between sexually mature (S) and physically mature (P) testes, and approximately 4% of genes were alternately spliced between the I and S groups. Selected genes were verified by qRT-PCR, in agreement with sequencing data indicating their reliability. Those genes have critical functions in various developmental stages of goat testicular development and spermatogenesis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were carried out to evaluate differentially expressed genes (DEGs). GO analysis suggested DEGs were involved in "reproduction process", "channel activity" and "cell periphery part" between I and S, and in "ion transport process", "channel activity" and "transporter complex part" between S and P. KEGG analysis indicated that pathways such as "glycerolipid metabolism", "steroid hormone biosynthesis" and "MAPK signaling pathway" may be involved in testis development and spermatogenesis. Genes including *IGF1*, *TGFB1*, *TGFBR1* and *EGFR* may regulate the development of the testis from immature to sexually mature, which may be key candidate genes for the development of goat testis. These findings provide novel insights into goat testicular development and spermatogenesis.

Keywords: Qianbei Ma goat; Testis development; RNA-Seq; mRNA expression

1. Introduction

The testis represents a critical organ of the male reproductive system in mammals, producing spermatozoids and androgens. Spermatogenesis constitutes a developmental event, which produces haploid spermatozoa from diploid spermatogonial stem cells during meiosis in the testis [1]. Spermatogenesis comprises three steps, including (1) spermatogonial proliferation and differentiation, (2) meiotic division of spermatocytes and (3) spermatozoid maturation. Spermatogonial stem cell differentiation produces spermatogonia, which after DNA replication produce primary spermatocytes. Next, secondary spermatocytes/spermatids are generated after DNA replication and meiosis from primary spermatocytes. Haploid sperm cells after four or more morphological alterations give rise to sperm cells, including chromatin condensation, acrosome generation, flagella formation and cytoplasmic decrease [2]. Besides spermatogenic cells,

 doi:10.20944/preprints202304.1269.v1

spermatogenic mechanisms involve many somatic cells in the testicle, including Sertoli and Leydig cells. In the testis of mammals, spermatogenesis is carried out in seminiferous tubules, where germ cells are associated with Sertoli cells. Associated genes in the latter cells have critical functions in precise steps of spermatogenesis [3].

RNA sequencing (RNA-Seq) allows an expression profiling of genes and may help map and quantify the transcriptome [4,5]. This approach offers many advantages compared to other transcriptomic tools, including high resolution/sensitivity, a broad dynamic range of gene expression, and the identification of new transcript sequences and splice isoforms of previously reported genes [6]. Ramsköld [7] evaluated multiple tissues from mammals by RNA-Seq and reported most genes are specifically expressed in testicular samples. Additionally, considering RNA-Seq-based expression patterns, Djureinovic [8] categorized 20,050 putative human genes, which showed specific expression in the human testicle, whereas 26 additional tissue types were present in 7 people. Their evaluation revealed the testicular tissue had by far the largest quantity of tissue-specific genes. Using microarray analysis, Anand detected differentially expressed genes (DEGs) in testicular samples compared to other tissues, identifying 2868 upregulated transcripts and 2011 downregulated mRNAs [9]. The testicle appears to have a higher degree of metabolic activity relative to other normal tissues. Most current reports assessing the association of testicular development with spermatogenesis have been performed in the human or mouse species, and the goat is scarcely examined.

The Qianbei Ma goat is a fine goat breed in Guizhou Province, which adapts to harsh climatic conditions and prolonged breeding. Normal testicular development and sperm formation is very critical for ensuring high-level semen production and perpetuating species continuity, and genes highly contributing to various steps of testicular development and spermatogenesis likely have functions in fertility. However, gene expression in testicular development and sperm formation in goats is mostly undefined, which deserves further investigation. The present work aimed to perform transcriptome profiling of immature and mature Qianbei Ma goat testis specimens by RNA-Seq and bioinformatic assessment. The findings provide novel insights into the mechanisms regulating goat testicular development and sperm formation.

2. Materials and Methods

2.1. Ethics statement.

This study had approval from the Animal Ethics Committee of Guizhou University (Guiyang, China) (No. EAE-GZU-2021-P024, Guiyang, China; March 30, 2021).

2.2. Animal handling and sample collection.

Permission was granted to geld 18 healthy Qianbei Ma goats in Fuxing Husbandry Co., Ltd., Zunyi, Guizhou, China. Goat ages were obtained from goat farming records. There were 6 immature goats (1 month old, before sexual maturation, i.e., samples I1, I2, I3, I4, I5 and I6), 6 sexually mature goats (6 months old, after sexual maturation but before physical maturation, i.e., samples S1, S2, S3, S4, S5 and S6) and 6 physically mature goats (12 months old, after physical maturation, i.e., samples P1, P2, P3, P4, P5 and P6). We surgically collected the right testes from the 18 goats by castration after anesthesia, followed by storage in RNA/DNA sample protector (Servicebio, Wuhan, China). The testis from each goat was cut longitudinally, and a small amount (3-5 g) of the parenchyma, including seminiferous tubules and Leydig cells, underwent snap freezing in liquid nitrogen and was taken back to the lab for further studies. All castrated goats remained in Fuxing Husbandry Co., Ltd. (Guizhou, China) after our study, for fatten feeding.

2.3. RNA quantitation and quality

Total RNA extraction was carried out from the testicular tissue in groups I, S and P using TRIzol reagent (Servicebio, Wuhan, China) and RNeasy RNA purification kit (Servicebio, Wuhan, China) with DNase as directed by the manufacturer. A NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, USA) was utilized to assess RNA purity and amounts. RNA quality assessment utilized

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1% agarose gel electrophoresis. High-quality RNA samples (OD 260/280 of 1.8-2.0, integrity>7.0 and 28S:18S above 1.0) were sequenced on an Illumina NovaSeq 6000 system, generating 150-bp paired end reads.

2.4. Transcriptome sequencing.

Totally 18 libraries were generated. The 6 obtained from immature testis samples were termed I1, I2, I3, I4, I5 and I6; the 6 from sexually mature specimens were S1, S2, S3, S4, S5 and S6, and the 6 from physically mature samples were P1, P2, P3, P4, P5 and P6. Approximately 5 μg RNA/sample constituted the input material for RNA sample preparation. Index-coded samples were clustered with NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® according to a protocol provided by the manufacturer. Upon clustering, the prepared libraries underwent sequencing on an Illumina NovaSeq 6000（Illumina, USA）. The image data of the sequences yielded by the high-throughput sequencor underwent conversion into sequence data (reads) by CASA V A base recognition to obtain fastq files. Raw RNA-Seq fastq data next underwent filtration with Fastp v to exclude adapter-containing, N-containing and low-quality (quality score below 20) reads, resulting in clean reads, which were mapped to the goat (Capra hircus) (ARS1.2) reference genome [10] using HISAT2.

2.5. Quantification of gene expression.

Reads mapped to a given gene were counted with featurerts for estimating the expression of various gene transcripts. Gene expression was determined from million mapped reads per kilobase (FPKM) values [11], the current commonest approach to estimate gene expression [12].

2.6. Differential expression analysis.

The DESeq2 software was used to analyze differential expression between treatment and control groups. The Benjamini-Hochberg algorithm was utilized for adjusting p values (p-adj) to control for false discovery rate. $|\log2\ (\text{FoldChange})| \geq 1$ & padj<0.05 was set as the significance threshold for differential expression [13].

2.7. GO and KEGG enrichment analyses of DEGs.

GO and KEGG analyses of DEGs were implemented with ClusterProfiler, correcting for gene length bias. KEGG is an information database based on molecular findings, particularly via genome sequencing and additional high-throughput techniques to produce large-scale molecular data sets, allowing a deep understanding of biological systems (http://www.genome.jp/kegg/) [14]. GO and KEGG terms with $|\log2\ (\text{FoldChange})| \geq 1$ and padj<0.05 were deemed to be DEGs with significant enrichment [15].

2.8. Prediction of new transcripts and alternative splicing analysis.

StringTie was utilized to build and identify previously reported and new transcripts from HISAT2 alignment data. StringTie utilizes a network-flow algorithm with optional *de novo* assembly to splice transcripts. Compared with cufflinks, StringTie has the following advantages: it (1) yields more complete transcripts; (2) assembles more accurate transcripts, (3) better estimates the transcript's expression level and (4) has greater splicing speed [16,17]. rMATS (http://rnaseqmats.sourceforge.net/index.html) was utilized to classify AS events, which were assessed in various samples separately.

2.9. qRT-PCR for RNA-Seq data validation.

TGFBR1, *TGFB1*, *EGFR*, *IGF1*, *MAPK3* and *SMAD4* were examined by qRT-PCR to validate RNA-Seq findings. Total RNA (1000 ng) was utilized to produce complementary DNA (cDNA) with 2×SYBR Green qPCR Master Mix None ROX (Servicebio, Wuhan, China) at 25°C (5 min), 42°C (30

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min) and 85°C (5 s). The primers used for qRT-PCR are shown in Table 4. A CFX96 Real-Time PCR system (Bio-Rad, USA) was utilized for amplification in 15-μL reactions containing 2×qPCR Mix (7.5 μL), forward and reverse primers (10 pmol/μL, 0.75 μL each), cDNA (1000 ng/μL, 2 μL), and Nuclease-Free water (4 μL). The reaction conditions were: 1 cycle at 95°C (30 s), followed by 40 cycles at 95°C (15 s), 60°C (30 s), with fluorescence signals collected every 0.5°C increase from 65°C to 95°C. Melting curves were utilized to assess primer specificity. Assays were carried out in triplicate, and *GAPDH* was utilized for normalization in data analysis by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers utilized in qRT-PCR.

Primer name	Gene ID	Primer sequence	Fragment length	annealing temperature
GAPDH-F	XM_005680968.3	ATGTTTGTGATGGGCGTGAA	153 bp	60 ℃
GAPDH-R		GGCGTGGACAGTGGTCATAAGT		
TGFBR1-F	XM_018052233.1	TTCAAACGTGCTGACATCTATGC	128 bp	60 ℃
TGFBR1-R		ACTGATGGATCGGAAGGTACAAG		
SMAD4-F	XM_018039535.1	CATAACAGCACTACCACCTGGACT	173 bp	60 ℃
SMAD4-R		GGATGATTAGAAATAGGAGGCTGG		
TGFB1-F	NM_001314142.1	CAACAATTCCTGGCGCTACCT	183 bp	60 ℃
TGFB1-R		ATGTCCACTTGAAGCGTGTTATCC		
EGFR-F	XM_018067044.1	CCGTGCGATTCAGTAACAACC	194 bp	60 ℃
EGFR-R		GGTCAATTTCTGGCAGTTCTCCTC		
IGF1-F	NM_001285697.1	AATCAGCAGTCTTCCAACCCAA	114 bp	60 ℃
IGF1-R		AGCAAGCACAGGGCCAGATA		
MAPK3-F	XM_018040780.1	CTGGACCGGATGTTGACCTTTA	138 bp	60 ℃
MAPK3-R		CTCCTTCAGTCGTTCCTTGGG		

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3. Results

3.1. Gene expression profiling during testicular development in Qianbei Ma goats.

To determine genes associated with testicular development and sperm formation, 18 libraries, including 6 each from immature (I1, I2, I3, I4, I5 and I6), sexually mature (S1, S2, S3, S4, S5 and S6) and physically mature (P1, P2, P3, P4, P5, P6) testes, underwent sequencing. Table 1 shows an overview of the information pertaining to raw and clean reads for all libraries. Error rates and GC contents for various libraries were determined for their quality control (Table 2). All 18 libraries had a high quality.

Table 2. Statistics of RNA-Seq data quality.

Sample name	Library number	Raw reads (n)	Clean reads (n)	Error rate	Q20	Q30	GC pct
I1	1	40405582	39128916	0.03	97.36	93.09	50.99
I2	2	43841646	42366502	0.03	97.41	93.2	51.16
I3	3	39433208	38237518	0.03	97.42	93.25	51.42
I4	4	42396474	40926952	0.03	97.43	93.23	51.26
I5	5	45463988	43854898	0.03	97.41	93.19	51.1
I6	6	39351212	38000364	0.03	97.48	93.36	50.91
S1	7	44934642	43739660	0.03	97.51	93.41	52.09
S2	8	43025142	41894994	0.03	97.39	93.17	52.36
S3	9	45845898	44647422	0.03	97.59	93.55	52.15
S4	10	41237336	40020062	0.03	97.37	93.11	52.29
S5	11	41130946	39901252	0.03	97.36	93.09	51.09
S6	12	41242240	40019490	0.03	97.51	93.4	51.9
P1	13	45377178	44131516	0.03	97.48	93.34	52.27
P2	14	44664918	43577078	0.03	97.49	93.36	52.06
P3	15	43792502	42601198	0.03	97.47	93.32	51.98
P4	16	43921138	42702250	0.03	97.5	93.39	52.17
P5	17	43223452	42134186	0.03	97.44	93.27	52.14
P6	18	45571906	44622618	0.03	97.3	92.91	50.37

Error rate: overall sequencing error rate for the data; Q20 and Q30: percentages of total bases with Phred values above 20 and 30, respectively; GC pct: percentage of C and G among the 4 bases in clean reads.

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Clean reads underwent alignment to the goat reference genome ARS1.2 that is commonly utilized for goats [10], with HISAT2. Table 3 shows an overview of the information about uniquely mapped clean reads.

Table 3. Statistics of reads aligned with the reference genome.

SAMPLE	TOTAL READS	TOTAL MAP	UNIQUE MAP	MULTI MAP	POSITIVE MAP	NEGATIVE MAP
I1	39128916	37563635(96.00%)	35626251(91.05%)	1937384(4.95%)	17793049(45.47%)	17833202(45.58%)
I2	42366502	40625554(95.89%)	38612044(91.14%)	2013510(4.75%)	19294015(45.54%)	19318029(45.60%)
I3	38237518	36735876(96.07%)	34685761(90.71%)	2050115(5.36%)	17323488(45.30%)	17362273(45.41%)
I4	40926952	39282398(95.98%)	37389786(91.36%)	1892612(4.62%)	18672350(45.62%)	18717436(45.73%)
I5	43854898	42092675(95.98%)	40255938(91.79%)	1836737(4.19%)	20105549(45.85%)	20150389(45.95%)
I6	38000364	36500392(96.05%)	34501227(90.79%)	1999165(5.26%)	17230278(45.34%)	17270949(45.45%)
S1	43739660	42076928(96.20%)	40551689(92.71%)	1525239(3.49%)	20259699(46.32%)	20291990(46.39%)
S2	41894994	40318684(96.24%)	39006285(93.10%)	1312399(3.13%)	19484346(46.51%)	19521939(46.60%)
S3	44647422	43061457(96.45%)	41514183(92.98%)	1547274(3.47%)	20739259(46.45%)	20774924(46.53%)
S4	40020062	38465949(96.12%)	36829538(92.03%)	1636411(4.09%)	18396451(45.97%)	18433087(46.06%)
S5	39901252	38334062(96.07%)	36942595(92.59%)	1391467(3.49%)	18452256(46.24%)	18490339(46.34%)
S6	40019490	38522907(96.26%)	36912252(92.24%)	1610655(4.02%)	18439977(46.08%)	18472275(46.16%)
P1	44131516	42514601(96.34%)	41073585(93.07%)	1441016(3.27%)	20518750(46.49%)	20554835(46.58%)
P2	43577078	41944011(96.25%)	40303516(92.49%)	1640495(3.76%)	20133389(46.20%)	20170127(46.29%)
P3	42601198	41016519(96.28%)	39440892(92.58%)	1575627(3.70%)	19701050(46.25%)	19739842(46.34%)
P4	42702250	41098071(96.24%)	39528322(92.57%)	1569749(3.68%)	19745068(46.24%)	19783254(46.33%)
P5	42134186	40510371(96.15%)	38849502(92.20%)	1660869(3.94%)	19405731(46.06%)	19443771(46.15%)

 doi:10.20944/preprints202304.1269.v1

P6	44622618	38550615(86.39%)	37036723(83.00%)	1513892(3.39%)	18501103(41.46%)	18535620(41.54%)

Sample: sample name; total reads: number of clean reads upon quality control; total map: number (percentage) of reads aligned to the reference genome; unique map: number (percentage) of reads aligned to a unique region of ARS1.2 (subsequently analyzed for quantitation), multi map: number (percentage) of reads with alignment to many locations of ARS1.2; positive and negative maps: numbers (percentages) of reads with alignment to the positive and negative strands of the reference genome, respectively.

Additionally, an overview of the percentages of clean reads mapped to exon borders (junction reads) is shown in Table 4. The RNA-Seq data have been submitted to NCBI (accession number BioProject: PRJNA879963).

Table 4. Statistics of reads alignment to genomic regions.

Sample name	Exonic region	Intronic region	Intergenic region
I1	4466151533 (79.48%)	611822545 (10.89%)	541085383 (9.63%)
I2	4779622398 (78.65%)	761383464 (12.53%)	535946498 (8.82%)
I3	4432093997 (80.66%)	570346947 (10.38%)	492580965 (8.96%)
I4	4614052978 (78.53%)	738532433 (12.57%)	523236881 (8.90%)
I5	5160159063 (81.96%)	605739527 (9.62%)	530252297 (8.42%)
I6	4298336210 (78.73%)	599791632 (10.99%)	561800986 (10.29%)
S1	5004805293 (79.49%)	606545683 (9.63%)	684973145 (10.88%)
S2	4891821345 (81.09%)	533206687 (8.84%)	607543338 (10.07%)
S3	5173907638 (80.30%)	600212860 (9.32%)	668983341 (10.38%)
S4	4579030919 (79.56%)	552189052 (9.59%)	623968138 (10.84%)
S5	4488498885 (78.27%)	613561446 (10.70%)	632649316 (11.03%)
S6	4595667229 (79.73%)	543550434 (9.43%)	624459696 (10.83%)
P1	5058268703 (79.52%)	636322977 (10.00%)	666220762 (10.47%)
P2	4921081484 (78.42%)	644761157 (10.27%)	709616381 (11.31%)
P3	4871901450 (79.39%)	593069117 (9.66%)	671905236 (10.95%)
P4	4918914052 (80.00%)	601562419 (9.78%)	628332353 (10.22%)
P5	4886718972 (80.63%)	554456620 (9.15%)	619529425 (10.22%)
P6	4658554613 (80.75%)	537138992 (9.31%)	573327628 (9.94%)

Exonic, intronic and intergenic regions: numbers (percentages) of reads with alignment to the exonic, intronic and intergenic regions, respectively.

3.2. Alternative splicing data.

Alternative splicing (AS) represents a commonly encountered phenomenon in eukaryotes, which could lead to the production of various protein forms at different times under different circumstances, increasing species/body fitness. Although AS research is a known subfield of molecular biology, only in recent years has this subfield attracted sufficient attention. AS is critical for the complex proteomes and functions found in higher organisms. In this work, AS events were categorized into 5 types with rMATS (http://rnaseq-mats.sourceforge.net/index.html). By

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determining the types and amounts of AS events, and analyzing each AS type, a large number of AS events were found in testicular development and sperm formation. In the present work, we detected 13,877 differential genes between immature (I) and sexually mature (S) testes, of which 544 underwent AS events, and 10 differential genes were detected between sexually mature (S) and physically mature (P) testes, with no detected AS events (padj≤0.05). Therefore, approximately 4% of genes showed AS between I and S, and no gene had AS between S and P in this study. The above findings indicated AS was very important in the complexity of gene expression during testis development, especially in the period from immaturity to sexual maturity.

To determine AS types associated with testicular genes, AS events were compared between the S and I groups. The five known types of AS events include retained intron (RI), mutually exclusive exon (MXE), alternative 3′ splice site (A3SS), alternative 5′ splice site (A5SS) and skipped exon (SE). All five types of AS were found in the S vs I group comparison. RI, MXE, A3SS and SE were found in the P vs S group comparison. These findings showed SE as the commonest AS event amounting to 368 in S vs I. Other identified AS events were RI (48), MXE (110), A3SS (66), and A5SS (54) (Fig. 1).

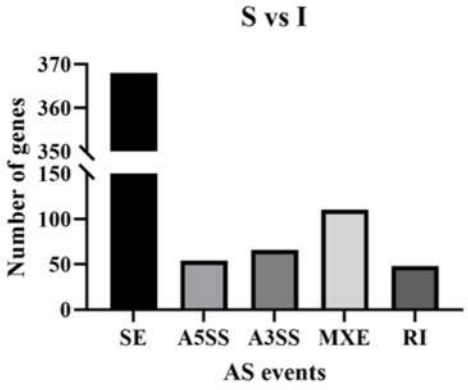

Figure 1. AS events among genes for immature and sexually mature group comparisons.

3.3. Analysis of DEGs.

DEGs were determined with |log2 (FoldChange)| ≥1 and padj<0.05. As a result, 9,724 upregulated and 4,153 downregulated genes were detected between the I and S groups, and 7 upregulated and 3 downregulated genes were found between the S and P groups (Fig. 2A1, Fig. 2A2). Venn diagrams showed the I and S libraries had 13451 genes in common, the S and P libraries had 17,448 genes in common (Fig. 2B). Figure 2C depicts hierarchical clustering, with DEGs for the 18 libraries grouped into three clusters. The above data suggested the I, S and P libraries had differential expression patterns overall, but similar repetitive expression commonalities across developmental stages.

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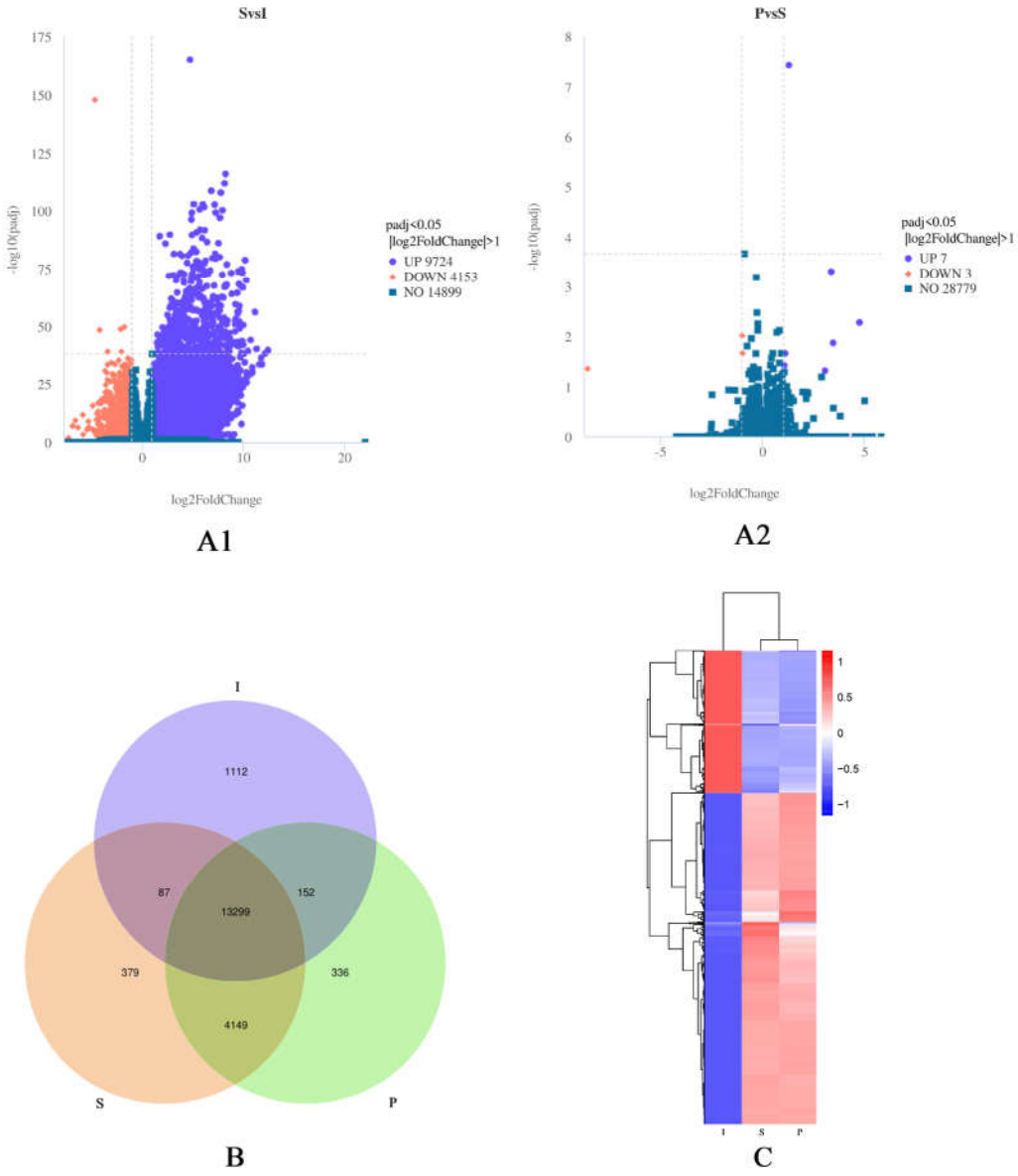

Figure 2. (A)1: Volcanic plot of differentially expressed genes (DEGs) between the immature and sexually mature groups. **(A)2**: Volcanic plot of DEGs between the sexually mature and physically mature groups. Highly significant differences in the expression of up- (purple) and down- (red) regulated genes were observed between immature (I) and sexually mature (S) testes, and between sexually mature (S) and physically mature (P) testes. Blue indicates no differential expression. **(B)**: Venn diagram depicting gene expression patterns. The numbers of uniquely and commonly (FPKM>1) expressed genes are shown. **(C)**: Clustering of differentially expressed genes. The overall FPKM hierarchical clustering map was obtained with log10 (FPKM + 1) for normalization. Red and blue represent high and low expression levels, respectively.

3.4. GO analysis of DEGs.

GO analysis was carried out to examine the functions of DEGs in testicular development. Totally 106 GO terms associated with "biological processes", "molecular functions" and "cellular components" were markedly enriched between immature (I) and sexually mature (S) testes. Approximately 43 GO terms belonged to "biological process", and mostly involved "protein phosphorylation process", "transport process" and "reproduction process". Approximately 56 GO terms belonged to "molecular functions", and mostly involved "channel activity", "transporter activity" and "receptor activity". Approximately 7 GO terms belonged to "cellular components", and mostly involved "cell periphery part", "plasma membrane part" and "myosin complex part". Totally

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36 GO terms associated with "biological processes", "molecular functions" and "cellular components" had significant enrichment in the sexually mature (S) vs physically mature (P) DEG groups. About 5 GO terms belonged to "biological processes", and mostly involved "ion transport process" and "immune system process". Approximately 21 GO terms belonged to "molecular functions", and mostly involved "channel activity", "chemokine activity" and "cytokine activity". About 10 GO terms belonged to "cellular components", and mainly involved "transporter complex part", "channel complex part" and "component of plasma membrane part".

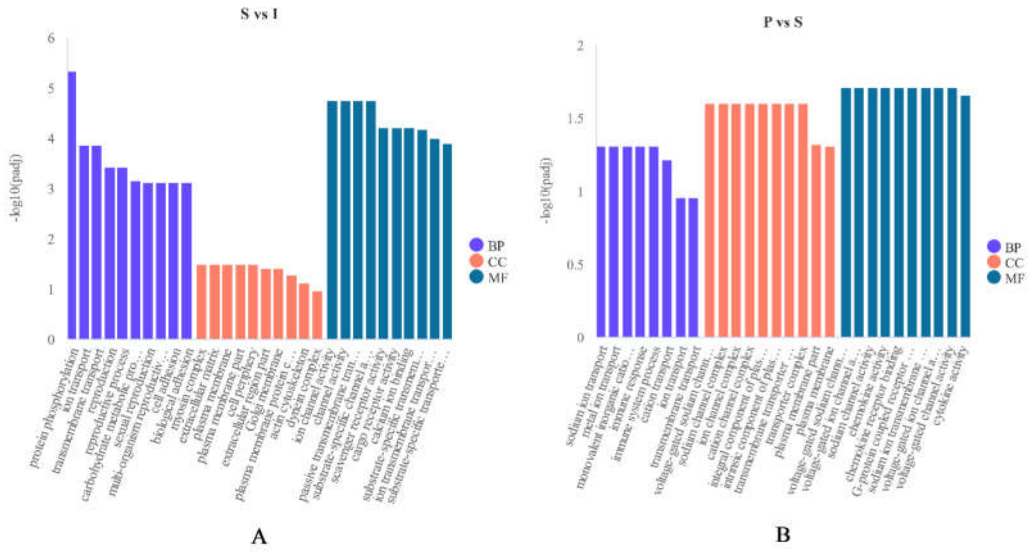

Figure 3. (A): Most enriched GO terms between immature (I) and sexually mature (S) testes. **(B)**: Most enriched GO terms between sexually mature (S) and physically mature (P) testes. Abscissas and ordinates represent enriched GO terms and significance levels of GO enrichment, respectively. Purple, "biological processes"; red, "cellular components"; blue, "molecular functions".

3.5. KEGG pathway analysis of DEGs.

KEGG pathway analysis of DEGs (|log2 (FoldChange)| ≥1 and padj<0.05) was carried out. For I vs S, 8 KEGG pathways were upregulated, including "glycerolipid metabolism", "protein digestion and absorption" and "steroid hormone biosynthesis". Totally 90 pathways were downregulated, including "lysosome", "MAPK signaling pathway" and "chemokine signaling pathway" (Fig. 4A). For S vs P, 7 KEGG pathways were upregulated, including "oxidative phosphorylation", "thermogenesis" and "steroid biosynthesis". However, no pathways were significantly downregulated (Fig. 4B).

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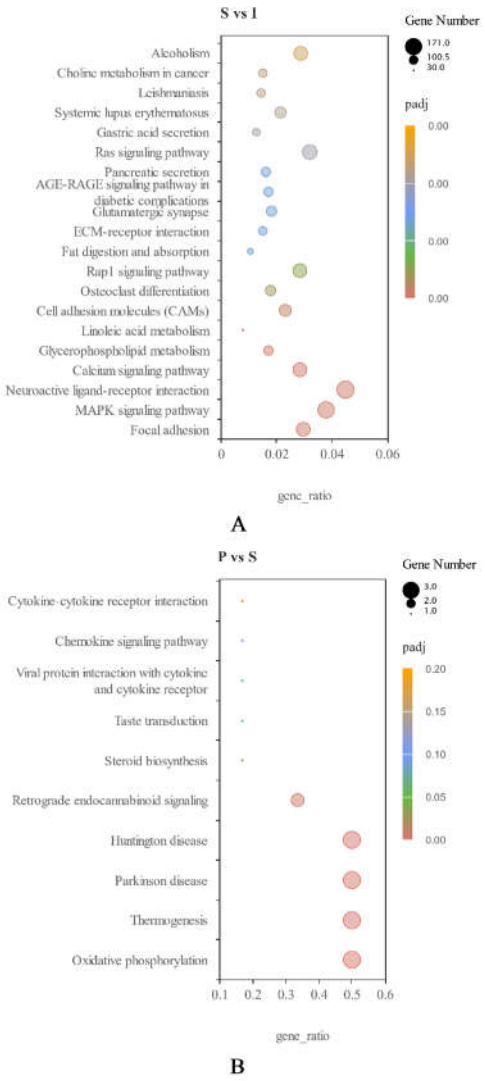

Figure 4. (A): Scatter plot of differentially expressed KEGG genes in immature (I) and sexually mature (S) testes. **(B)**: Scatter plot of differentially expressed KEGG genes in sexually mature (S) and physically mature (P) testes. Ordinates and abscissas represent the names of KEGG pathways and gene ratios, respectively. Point sizes and colors represent the numbers of DEGs and the ranges of Q values, respectively.

3.6. qRT-PCR validation of DEGs.

To verify the DEGs in immature (I), sexually mature (S) and physically mature (P) testes, we selected 6 DEGs, including *TGFBR1*, *TGFB1*, *EGFR*, *IGF1*, *MAPK3* and *SMAD4*, to validate RNA-Seq data by qRT-PCR. qRT-PCR data corroborated RNA-Seq findings, suggesting the reliability of RNA-Seq data (Fig. 5).

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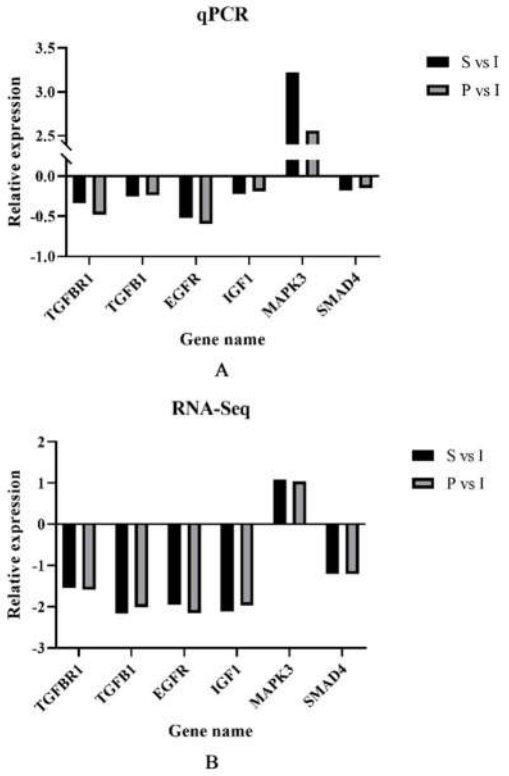

Figure 5. Relative expression of different genes in sexually mature (S) and physically mature (P) testes compared to immature (I) testes, respectively, using group I as a reference. **(A)**: qPCR relative expression of S vs I and P vs I. **(B)**: RNA-Seq data for S vs I and P vs I.

4. Discussion

With the current development of detection technology, more and more mRNAs specific to sperm have been reported. RNA-Seq has emerged as a tool for efficiently and inexpensively detecting new transcripts and genes. RNA-Seq methods have been broadly utilized to determine DEGs or gene expression patterns, new transcripts, AS events and SNPS, and have empowered studies examining porcine [18,19], cattle [20,21] and mouse [22,23] testicular development. In goats, the profiles of ovarian [24,25], uterine [26,27] and testicular [28,29] tissues under different conditions were recently compared by RNA-Seq. However, limited data on testicular development in goats are available. Breed and age represent major factors affecting testicular development. Here RNA-Seq was performed to build a complete dataset that details the spatiotemporal transcriptome of the testicular tissue in Qianbei Ma goats. Testicular growth and development constitute the key factors affecting goat reproduction. Therefore, identifying genes regulating testicular growth and development is critical. In this study, 13,887 genes were assessed by RNA-Seq in 6 immature, 6 sexually mature and 6 physically mature testes. Totally 9,724 genes were upregulated and 4,153 were downregulated between immature and sexually mature testes; 7 genes were upregulated and 3 were downregulated between sexually mature and physically mature testes. Using next-generation platforms, we determined most upregulated genes were associated with protein coding and may have functions in testicular development and sperm formation.

AS is an important mechanism in the regulation gene expression and promotes proteome diversity [30]. It is estimated about 95% of human multiple-exon gene expression is associated with AS events [31]. In metazoans, AS is critical for the production of various protein forms with functions in different cell events such as cell growth, differentiation and death [32]. Here, 5 AS events were observed, mostly involving ES. The effects of AS events on the functions of related genes can be predicted by a comprehensive analysis of AS events, and GO and KEGG analysis data [33,34]. In the current study, Sec insertion sequence binding protein 2 (*SECISBP2*) was the gene with the highest number of SE events, i.e., a total of 10 SE events. Mutation of *SECISBP2* alters thyroid hormone

 doi:10.20944/preprints202304.1269.v1

metabolism [35,36]. Thyroid hormones can modulate semen quality under physiological conditions by regulating testosterone and changing some semen indexes [37-39].

Combining previous relevant reports and KEGG and GO data in the current study, the genes involved in the regulation of testis development and sperm formation through protein phosphorylation were mainly *TGFB1*, *EGFR* and *IGF1*, which have critical functions in testis growth, hormone secretion, spermatogenesis and Leydig cell differentiation.

Transforming growth factor beta-1 (*TGFB1*) plays multiple biological roles, including the control of proliferative and differentiation potentials of cells [40]. *TGFB1* regulates tight junctions in Sertoli cells and controls spermatogenesis. It modifies the blood-testicular barrier (BTB) by downregulating tight junction proteins [41]. *TGFB1* may play an important role in testicular development because of its high expression in the immature testis and markedly reduced expression in sexual maturity, as spermatogenesis begins. A comparable expression pattern was found for TGFB receptor type 1 (*TGFBR1*) [42]. In addition, loss of *TGFB1* resulted in lower testosterone levels in the testis and serum, and decreased the ability to mate with females [43]. Based on previous studies, we speculated that *TGFB1* may not only directly regulate goat testicular development and sperm formation, but also ensure the normal development of male external genitalia and affect fertility.

Epidermal growth factor receptor (*EGFR*) represents a receptor gene for EGF and controls testicular function in the human, mouse, rat and livestock species as well as in alpacas [44]. EGF and *EGFR* are critical paracrine and/or autocrine modulators of testicular development and sperm formation, and regulate testosterone production by testicular interstitial cells [45,46]. We speculated that EGF and *EGFR* may also be expressed in various goat testicular cells, and can stimulate testosterone secretion, and regulate testis development and spermatogenesis.

Insulin-like growth factor I (*IGF1*) contributes to the regulation of testicular function [47]. Pitetti indicated growth factors of the insulin family play essential roles by controlling SC number, testis size and daily sperm production [48,49]. Both *IGF1* and its receptor *IGF1R* are expressed in testicles, and their hormones act directly on male gonads [50,51]. In immature testes, *IGF1* promotes the development of sustentacular cells, Leydig cells, and gonocytes. In mature testis, the *IGF1* gene induces spermatogenesis and regulates Leydig cell function [52,53]. *IGF1* may act as an autocrine/paracrine or endocrine signal to regulate testicular steroid production as well as germ cell and Sertoli cell functions [54]. *IGF1* plays different roles in testicular function at different stages of testicular development [47,55]. We speculated that high IGF1 and IGF1R protein amounts in the immature testis may suggest they highly promote the development and differentiation of sustentacular cells, Leydig cells and gonocytes in goat testis during sexual maturity.

Transcriptome data revealed the MAPK pathway is implicated in goat testicular development, while *TGFBR1*, *TGFB1*, *EGFR* and *IGF1* were enriched in this pathway and downregulated during sexual maturation, as key genes that regulate testis development and spermatogenesis [56,57]. Multiple reports suggest MAPK signaling is a critical regulator of testis growth and development, testis cell proliferation, differentiation and apoptosis, testosterone secretion, thus affecting male fertility [58-60]. One of the key downstream target genes of MAPK signaling is *IGF1*, which together with other genes in the pathway, controls testis cell proliferation, testis volume development, hormone secretion and spermatogenesis, and is often reported to be associated with male fertility [48,61,62]. We speculate that MAPK signaling is a critical regulatory pathway in goat testis development and spermatogenesis. As an essential male fecundity-related gene in the MAPK signaling pathway, *IGF1* modulates goat testis growth and development and can affect the functions of various cells of goat testis by regulating other downstream genes in the signaling pathway. During male goat sexual maturation, *IGF1* regulates the development of testis, spermatogenesis and hormone synthesis through the MAPK signaling pathway and other cooperative genes.

5. Conclusions

This study firstly used RNA-Seq to profile the expression of genes during testicular development in Qianbei Ma goats. We identified 544 genes with AS events between I and S, which suggests that AS of differential genes might be critical in the regulation of testicular development in goats. Totally

8 KEGG pathways were upregulated and 90 were downregulated between I and S. Totally 7 KEGG pathways were upregulated between S and P. Among the 6 screened DEGs (*TGFBR1*, *TGFB1*, *EGFR*, *IGF1*, *MAPK3* and *SMAD4*) *TGFBR1*, *TGFB1*, *EGFR*, *IGF1* and *MAPK3* belonged to the "MAPK signaling pathway", corresponding to the GO term "protein phosphorylation". The findings should further our understanding on gene regulation during testicular development and sperm formation.

Author Contributions: Methodology, Y.Z. and X.C.; software, W.T.; investigation, Y.R. and T.J.; resources, X.C.; validation, data curation, X.C.; writing—manuscript drafting, Y.Z.; writing—manuscript revision, X.T., W.G. and K.F.; All authors have read and agreed to the published version of the manuscript.

Funding: The current study was supported by the National Natural Science Foundation of China (No. 32260835), the Science and Technology Project of Guizhou Province (Oian Kehe Foundation-ZK [(2021)] Genera 151), and the Guizhou High Level Innovative Talents Project (Oian Kehe Platform Talents [(2022)] 021-1).

Institutional Review Board Statement: The animals used in our study were consented by their owners. All procedures involving animals were approved and authorized by Guizhou University. The laboratory animal castration protocol for this study was approved by the Laboratory Animal Ethics of Guizhou University (No. EAE-GZU-2021-P024, Guiyang, China; 30 March 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data from sequencing are available at NCBI under BioProject ID: PRJNA879963.

Acknowledgments: The authors are thankful to Fuxing Husbandry Co., Ltd. (Guizhou, China) for providing the experimental Qianbei Ma goats.

Conflicts of Interest: The authors declare that they have no competing interests.

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