



Identification of Photoperiod-Induced LncRNAs and mRNAs in Pituitary Pars Tuberalis of Sheep

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The pituitary pars tuberalis (PT) is the regulating center of seasonal reproduction, which can sense the melatonin signal and eventually cause downstream changes of GnRH secretion through TSH β . Recently, lncRNAs have been identified in animal reproductive-related tissues, and they play important roles in reproductive regulation. Therefore, in this study, we expect to identify photoperiod-induced lncRNAs and genes in pituitary PT of sheep by comparison of expression profiles between short photoperiod (SP) and long photoperiod (LP). Through RNA-Seq, a total of 55,472 lncRNAs were identified in pituitary PT of Sunite ewes. The number of differentially expressed (DE) genes and lncRNAs between SP and LP increased gradually with the extension of LP (from LP7 to LP42). The notable LP-induced candidate genes included *EYA3*, *TSHB*, *SIX1*, *DCT*, *VMO1*, *AREG*, *SUV39H2*, and *EZH2*, and SP-induced genes involved *ENSOARG00000012585*, *CHGA*, *FOS*, *SOCS3*, and *TH*. In enriched pathways for DE genes and lncRNA target genes between SP and LP, the reproduction- and circadian-related pathways were highlighted. In addition, the interactome analysis of lncRNAs and their targets implied that *MSTRG.209166* and its *trans*-target *TSHB*, *MSTRG.288068* and its *cis*-target *SIX1*, and *ENSOARG00000026131* and its *cis*-target *TH* might participate in regulation of seasonal reproduction. Together, these results will help to determine important photoperiod-induced lncRNAs and genes and give us some new insights into the epigenetic regulation of seasonal reproduction in sheep.

Keywords: lncRNAs, mRNAs, photoperiod, sheep, pituitary pars tuberalis

INTRODUCTION

The reproductive activity of some animal species inhabiting the temperate zone is limited in specific seasons in order to maximize the survival possibility of their offspring. According to the different seasons of breeding, these animals are categorized as long photoperiod (LP) breeders and short photoperiod (SP) breeders (1, 2). Among them, sheep belongs to the SP breeder (3); that is, they are mostly bred in autumn and winter. For example, Sunite sheep in China exhibit obvious seasonal reproductive behavior throughout the year, i.e., estrus from August to March of the next year and anestrus from April to July (2, 4).

Seasonal reproduction is an important factor limiting the production efficiency of the sheep industry; therefore, research on the molecular basis of seasonal reproduction of sheep is indispensable for possible artificial regulation of this trait in the future. However, the molecular mechanism and regulatory network of seasonal reproduction are not very clear until now.

Pituitary pars tuberalis (PT) plays an important role of transmission center in the seasonal reproduction of animals. The pineal gland first converts external photoperiod signals into biological signals (nocturnal melatonin secretion), which causes photoperiod-induced signals (*EYA3*, *TSHB*, etc.) alternation in PT. Thus, the thyrotrophin secreted by the anterior pituitary will increase in long days, which acts on TSH receptor-expressing cells in the adjacent mediobasal hypothalamus, leading to type III thyroid hormone deiodinase (DIO3) switch to type II thyroid hormone deiodinase (DIO2). DIO2 regulates the thyroid hormone in the hypothalamus, controlling the activity of the hypothalamus–pituitary–gonad axis to exhibit the summer phenotypes by direct (GnRH neuron) or indirect (*KISS1/RFRP* system) ways (5, 6). Besides, the photoperiod-induced gene expression in PT is mainly affected by photoperiod, independently of the TH status (6). However, molecular changes related to seasonal reproduction in medio-basal hypothalamus are regulated not only by photoperiod but also by TH. Therefore, PT is the best ideal tissue for seasonal reproduction analyses. So far, several important genes involved in the regulation of seasonal reproduction have been discovered in PT. For example, LP-induced genes (e.g., *EYA3* and *TSHB*) and an SP-induced gene (*CHGA*) were revealed in PT of sheep (3, 7, 8). Their expression levels had a remarkable change in different photoperiods. In recent years, long non-coding RNAs (lncRNAs) are considered as key regulators of gene expression because they play crucial roles in transcriptional and post-transcriptional regulation. In sheep, many lncRNAs have been identified in reproduction-related tissues, such as ovary, uterus, and germ cells (9–12). Moreover, lncRNAs play important roles in many aspects of sheep reproductive regulation, such as fecundity (12, 13), gonadal development (14), and sex hormone response (15). However, the functions of lncRNAs in animal PT on seasonal reproduction are unknown. Therefore, one purpose of this research is to seek the new photoperiod-induced genes and lncRNAs in PT of sheep by transcriptome sequencing. Another objective is to explore the potential relationship between genes and lncRNAs by target prediction and joint analysis of their co-expression in PT of sheep. These results will give us some new insights into the epigenetic regulation of seasonal reproduction in sheep.

METHODS

Ethical Statement

All the animals were authorized by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS; Beijing, China). In addition, ethical approval of animal survival was given by the animal ethics committee of IAS-CAAS (No. IAS2018-3, April 10, 2018).

Animal and Tissue Acquisition

Experiments were conducted on 12 adult Sunite ewes (2–3 years old; weight 30–40 kg), which were selected from a farm in Urat Middle Banner (40° 75′ north latitude), Bayan Nur City, Inner Mongolia Autonomous Region, China, and maintained in a farm in the Tianjin Institute of Animal Sciences, Tianjin (39° 13′ north latitude), China. All ewes were raised under the same conditions, with free access to water and feed. Construction of ovariectomized (OVX) and estradiol-implanted sheep model and light control experiment were previously described in detail (2, 12). Ewes were ovariectomized and estradiol-implanted (E_2 , Sigma Chemical Co., St. Louis, MO, USA) to maintain plasma estradiol levels of 3–5 pg/ml (6, 16) in October, 2016, according to the model developed by Karsch et al. (1984) (17). This OVX + E_2 model normalizes the level of circulating E_2 (12), which uncovers the well-documented central seasonal shift in the negative feedback action of E_2 on gonadotropin secretion (18). After the surgery, the ewes recovered for 30 days before artificial light control. Then, in November 2016, all ewes were brought indoors and submitted to a light program simulating the outdoor photoperiodic condition by time-control switch. Firstly, all ewes were kept in artificial SP with lights on during 10:30–18:30 (SP, 8:16 h light/dark) for 21 days and switched to LP with lights on during 06:30–22:30 (LP, 16:8 h light/dark) for 42 days, with free access to water and food. Ewes were euthanized [intravenous pentobarbital (100 mg/kg)] at ZT4 [4 h after lights on] of SP21, LP7, LP21, and LP42 (8, 19–21). Consistent with the specific location described by Wood et al. (7) and Lomet et al. (6), the PT tissue (Figure 1) of each ewe was immediately collected and stored at -80°C for total RNA extraction.

RNA Extraction, Library Construction, and Sequencing

Pituitary PT tissues were used for RNA extraction with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Examination involving the integrality and quality of the isolated RNA was performed via electrophoresis and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

The rRNA was depleted from 3 μg of total RNA using Ribo-Zero™ Gold Kits (Epicentre, Madison, WI, USA). Sequencing libraries of the 12 samples (SP21, $n = 3$; LP7, $n = 3$; LP21, $n = 3$; LP42, $n = 3$) were generated using NEB Next Ultra Directional RNA LibraryPrep Kit for Illumina (NEB, Ipswich, MA, USA) according to the manufacturer's instructions, and index codes were used to label the sequences of each sample. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform (Illumina, San Diego, CA, USA). Raw data of the performed RNA-seq have been recorded in the SRA public database (Accession number of BioProject: PRJNA680667).

Reference Genome Mapping and Transcriptome Assembly

Raw data in fastq format were processed through in-house perl scripts. In this step, clean reads were obtained by removing

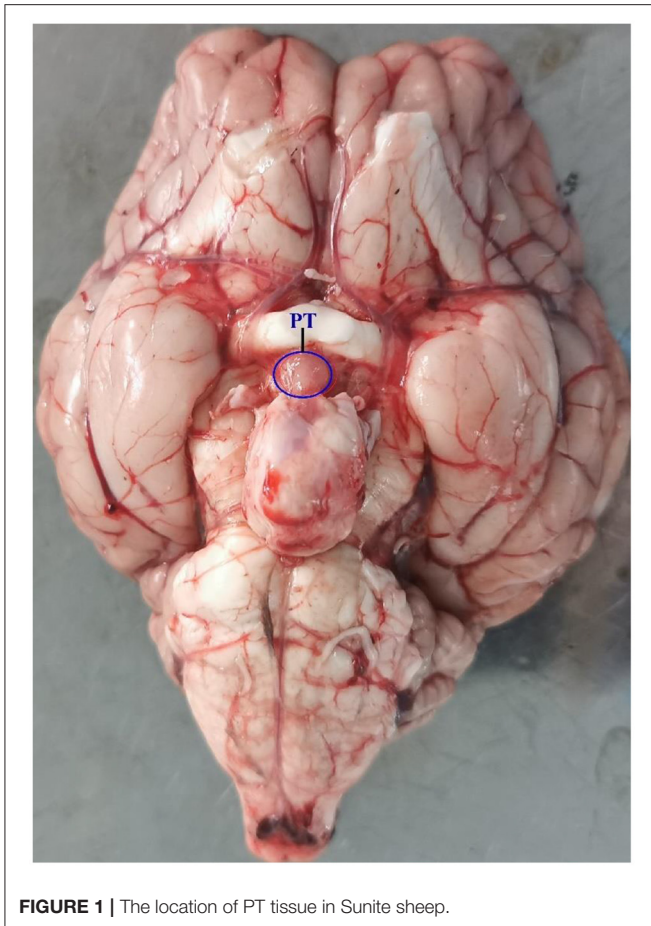


FIGURE 1 | The location of PT tissue in Sunite sheep.

reads with adapter contamination, reads that contained poly-N, and low-quality reads from raw data. Simultaneously, the Q20, Q30, and GC contents of the clean data were calculated. All downstream analysis was based on high-quality clean data. HISAT2 (22) was used to align clean reads of each sample to the sheep reference genome *Oar_v3.1*. StringTie (23) was used for transcriptome assembly and reconstruction. Thus, known lncRNA and mRNA transcripts were identified, and the position of transcripts was obtained.

lncRNA Identification and Differentially Expression Analysis

Novel lncRNAs with more than two exons and lengths of more than 200 nt were predicted by CNCI (24), CPC (25), PFAM (26), and CPAT (27) software after transcriptome assembly. The fragments per kilobase per million mapped reads [FPKM (28)] values were calculated to represent the expression of genes and lncRNAs. To determine the effect of photoperiod on genes and lncRNAs expression in PT of sheep, the expression of genes and lncRNAs at every time point of LP (LP7, LP 21, or LP 42) was compared with the SP21 group using DESeq, i.e., SP 21 vs. LP 7, SP 21 vs. LP 21, and SP 21 vs. LP 42. In addition, $p < 0.05$ and $|\text{Fold change}| > 1$ was considered standard of differential expression between the SP and LP.

GO and KEGG Pathway Enrichment Analysis of Differentially Expressed Genes and lncRNAs

Gene Ontology (GO) enrichment analysis of differentially expressed genes or lncRNA target genes was implemented by the Goseq R package, in which gene length bias was corrected (29). GO classifies functions into three groups: cellular components, molecular functions, and biological processes. The KEGG biological pathways database (<http://www.genome.jp>) is a central public database for understanding high-level functions and regulatory network research. Enrichment analysis was performed on each pathway in KEGG using a hypergeometric test. According to significant threshold (p -value: 0.05), the genes were screened and enriched for the pathways. Next, the significance of the pathway enrichment analysis was corrected by FDR, and the corrected p -value (q -value) was obtained.

Construction of Integral lncRNA–mRNA Interaction Networks

The primary role of lncRNAs, which are a type of non-coding RNA, is to regulate their target genes by cis-regulating nearby protein-coding genes and trans-regulating distal protein-coding genes. Here, protein coding genes with a distance of < 100 Kb were assumed to be the cis-target genes, and Pearson correlation coefficients with the lncRNAs of > 0.95 were assumed to represent the trans-target genes (30).

To further reveal the potential roles of lncRNAs that are involved in modulating the reproductive process, integral interaction networks containing lncRNAs and their corresponding target genes were built using Cytoscape software (31) in each comparison (SP 21 vs. LP 7, SP 21 vs. LP 21, and SP 21 vs. LP 42), which include cis- and trans-forms of regulation.

RNA-seq Data Validation

EYA3, *TSHB*, *CHGA*, *MSTRG.209166*, *MSTRG.22781*, and *MSTRG.42035* were selected to validate the accuracy of RNA sequencing *via* the reverse-transcription quantitative polymerase chain reaction (RT-qPCR). *GAPDH* was used as an internal reference to normalize target gene expression. All primers used in the RT-qPCR are shown in **Supplementary Table 1**. cDNA was used to perform RT-qPCR after reverse transcription from total RNA to cDNA. The RT-qPCR reaction conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The data obtained from RT-qPCR reaction were then calculated using the $2^{-\Delta\Delta C_t}$ method (32, 33) and processed by SPSS 19.0 with a one-way analysis of variance. The results are presented as means \pm standard deviation. Furthermore, $p < 0.05$ was regarded as statistically significant.

RESULTS

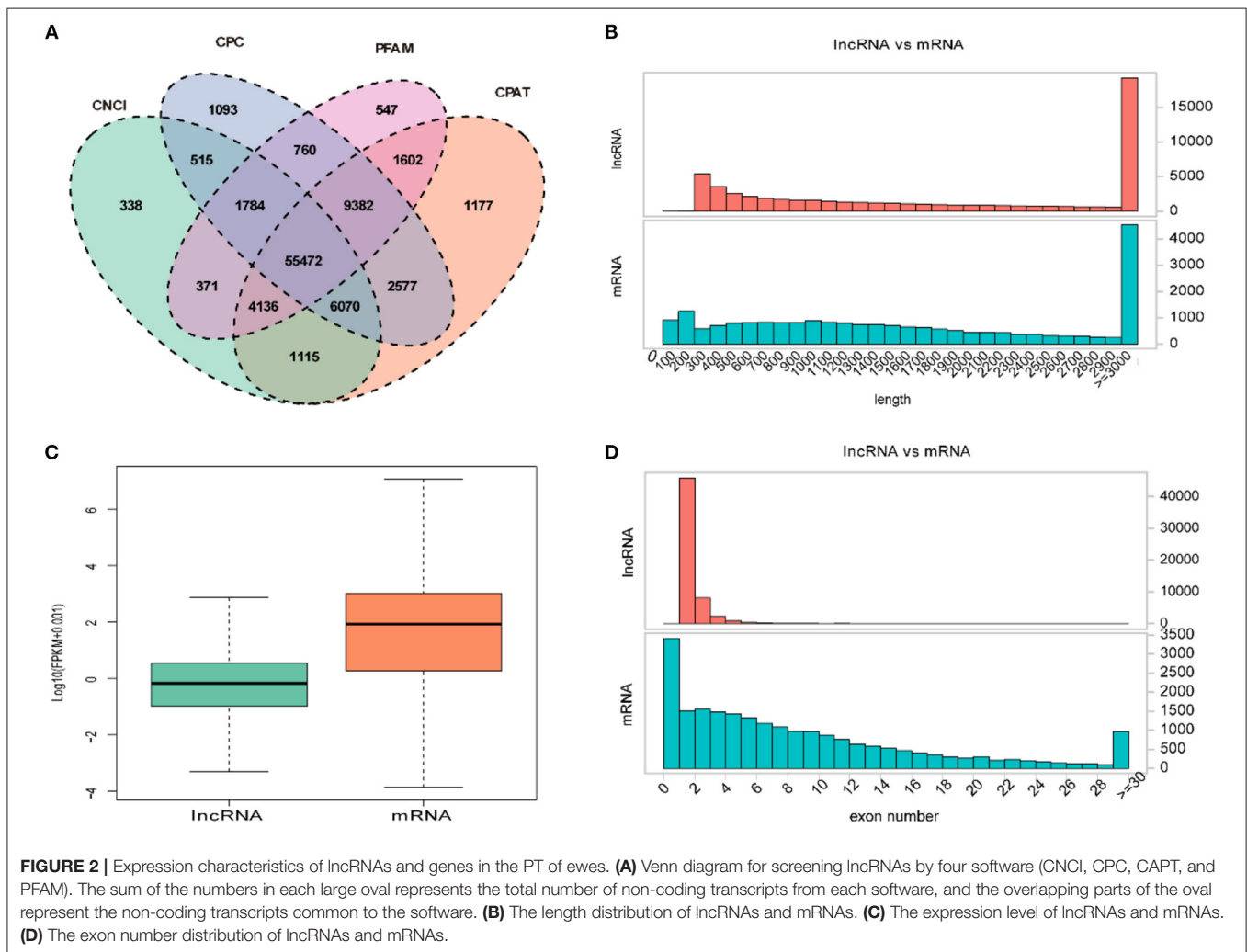
Summary of Transcriptome Sequencing Data

To identify differentially expressed lncRNAs and genes between SP and LP, RNA libraries of different photoperiods were constructed. After removing low-quality sequences, a total of

TABLE 1 | Summary of raw reads after quality control and mapping to the reference genome.

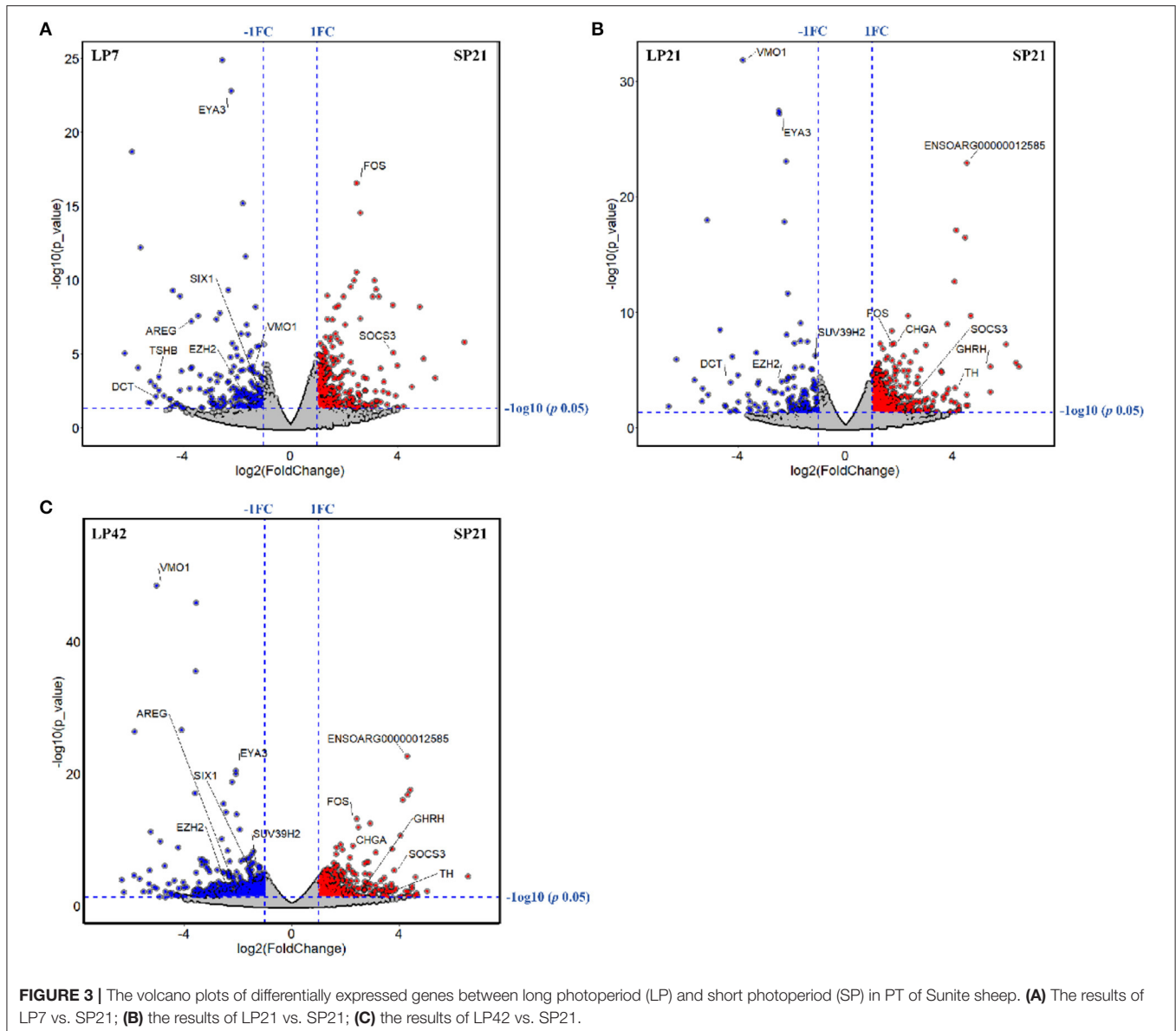
Photoperiod	Sample	Raw reads	Clean reads	Clean reads rate (%)	Q30 (%)	Mapped reads	Mapping rate (%)
SP21	SP2101	125,660,438	122,442,196	97.44	96.91	115,418,878	94.26
	SP2102	125,942,826	122,243,686	97.06	95.9	114,892,152	93.99
	SP2103	132,973,810	129,237,768	97.19	95.94	121,797,173	94.24
LP7	LP701	113,017,724	109,422,884	96.82	94.45	102,336,864	93.52
	LP702	120,666,156	116,544,984	96.58	94.44	108,968,477	93.50
	LP703	120,732,238	117,373,382	97.22	96.09	110,710,654	94.32
LP21	LP2101	109,242,794	106,213,878	97.23	93.56	98,166,189	92.42
	LP2102	123,453,896	119,838,276	97.07	96.16	113,659,315	94.84
	LP2103	122,212,386	116,562,600	95.38	92.52	108,893,984	93.42
LP42	LP4201	112,488,634	109,485,312	97.33	93.83	101,355,097	92.57
	LP4202	108,789,602	105,051,072	96.56	94.51	98,080,693	93.36
	LP4203	123,811,728	120,508,540	97.33	95.94	113,375,002	94.08

SP, short photoperiod; LP, long photoperiod; Q30, the rate of bases whose quality is >30 Phred value in clean reads.



1,394,924,578 clean reads with >92.52% of Q30 were obtained after sequencing all 12 libraries. Approximately 92–95% of the reads were successfully aligned to the *Ovis aries* reference genome (*Oar_v3.1*) (Table 1).

Overall, a total of 55,472 lncRNAs were identified in PT of 12 ewes using four programs (CNCI, CPC, PFAM, and CAPT) (Figure 2A). The length distribution of lncRNAs was consistent with that of protein-coding gene (Figure 2B). Of them, the



transcripts of lncRNAs and mRNAs with lengths of more than 3,000 bp accounted for the majority, and its number in lncRNAs was significantly greater than that in mRNAs (**Figure 2B**). However, transcript levels of lncRNAs were lower than those of mRNAs in the PT of Sunite ewes (**Figure 2C**). Most of the lncRNAs have only two or three exons, whereas mRNAs contain a wide range of exons from 2 to 30 (**Figure 2D**).

Photoperiod-Induced Factor Analysis Through Differentially Expressed Genes and lncRNAs

The number of differentially expressed (DE) genes between SP and LP increased gradually with the extension of LP (from LP7 to LP42). Specifically, there were 499 DE mRNAs in SP21 vs.

LP7, in which *FOS*, *SOCS3*, *EYA3*, *TSHB*, *SIX1*, *DCT*, *VMO1*, *AREG*, and *EZH2* were related to reproduction (**Figure 3A**). In SP21 vs. LP21, 625 DE mRNAs were detected, including *CHGA*, *FOS*, *SOCS3*, *GHRH*, *TH*, *EYA3*, *TSHB*, *DCT*, *VMO1*, *SUV39H2*, and *EZH2* that were associated with reproduction (**Figure 3B**). In addition, 874 DE mRNAs were screened between the SP21 and LP42 group. Of them, the genes related to reproduction included *CHGA*, *FOS*, *SOCS3*, *GHRH*, *TH*, *EYA3*, *TSHB*, *SIX1*, *VMO1*, *AREG*, and *EZH2* (**Figure 3C**). The top 10 DE mRNAs in each contrast group are shown in **Table 2**. Among these genes, there are specific differentially expressed genes (*FOS*, *TMEM200A*, *MLIP*, *PMP2*, *PRX*, and *SPTBN5* and seven novel genes) that are different from previous reports (6, 7). Besides, for the top genes, it is worth paying attention to those genes that appear in the above contrast groups simultaneously,

TABLE 2 | The top 10 differentially expressed mRNAs in three comparisons between LP and SP.

Comparisons	Gene	p-value	Log2foldchange	Up/Down	Gene name
LP7 vs. SP21	ENSOARG00000014847	1.29E-25	-2.531934183	Up	ENSOARG00000014847
	ENSOARG00000003071	1.55E-23	-2.209131416	Up	EYA3
	ENSOARG00000002985	2.10E-19	-5.881442781	Up	ENSOARG00000002985
	ENSOARG00000001783	2.83E-17	2.460057284	Down	FOS
	ENSOARG00000017954	6.56E-16	-1.77693187	Up	ACSL4
	ENSOARG00000012585	2.89E-15	2.610496836	Down	ENSOARG00000012585
	ENSOARG00000015485	6.34E-13	-5.568818193	Up	DQA
	ENSOARG00000020015	2.54E-12	-1.658620427	Up	TMEM200A
	ENSOARG00000000857	3.00E-11	2.448160683	Down	ENSOARG00000000857
	ENSOARG00000003776	1.04E-10	3.130953431	Down	ENSOARG00000003776
LP21 vs. SP21	ENSOARG00000007112	1.47E-32	-3.819122926	Up	VMO1
	ENSOARG00000005662	9.30E-29	-9.037863894	Up	ENSOARG00000005662
	ENSOARG00000017954	3.92E-28	-2.474582452	Up	ACSL4
	ENSOARG00000003071	6.86E-28	-2.454903078	Up	EYA3
	ENSOARG00000008195	8.26E-24	-2.196263967	Up	CYP4V2
	ENSOARG00000012585	1.20E-23	4.510495819	Down	ENSOARG00000012585
	ENSOARG00000018081	1.59E-23	-8.570717405	Up	BPIFC
	ENSOARG00000008250	1.05E-18	-5.150050353	Up	KLKB1
	ENSOARG00000014847	1.50E-18	-2.278049468	Up	ENSOARG00000014847
	ENSOARG00000009143	7.84E-18	4.11635763	Down	ENSOARG00000009143
LP42 vs. SP21	ENSOARG00000009116	2.03E-56	-7.230483397	Up	PMP2
	ENSOARG00000007112	2.98E-49	-5.030524117	Up	VMO1
	ENSOARG00000017954	1.45E-46	-3.565896967	Up	ACSL4
	ENSOARG00000006792	2.83E-43	-7.98187898	Up	MLIP
	ENSOARG00000006630	3.02E-36	-3.574968045	Up	PRX
	ENSOARG00000020448	2.35E-27	-4.099272015	Up	SPTBN5
	ENSOARG00000008250	4.24E-27	-5.842338208	Up	KLKB1
	ENSOARG00000018081	1.34E-23	-8.659205306	Up	BPIFC
	ENSOARG00000012585	2.18E-23	4.291991999	Down	ENSOARG00000012585
	ENSOARG00000003071	3.48E-21	-2.091523071	Up	EYA3

and they are the most likely photoperiodic-induced factors in the regulation of seasonal reproduction. To sum up, the notable LP-induced genes included *EYA3*, *TSHB*, *VMO1*, *DCT*, and *EZH2*. SP-induced genes involved *ENSOARG00000012585*, *CHGA*, *FOS*, *SOCS3*, and *TH*. For lncRNAs, the number of DE lncRNAs was also increasing with the extension of the LP. Specifically, 1,887, 2,038, and 2,614 DE lncRNAs were detected in the SP21 vs. LP7 group (**Supplementary Table 5**), SP21 vs. LP21 group (**Supplementary Table 6**), and SP21 vs. LP42 group (**Supplementary Table 7**), respectively. These DE lncRNAs may be involved in the regulation of the expression of important genes for seasonal reproductive trait. Besides, our results had some common overlap DE mRNAs with the reports of Wood et al. (2015) and Lomet et al. (2017) (6, 7). The detailed information is shown in **Supplementary Table 8**.

GO Annotation and KEGG Enrichment Analysis of DE Genes and lncRNAs

GO and KEGG analyses were conducted for the differentially expressed mRNAs and target genes of differentially expressed

lncRNAs. The top 10 enriched GO terms for lncRNAs and mRNAs of SP21 vs. LP7 (blue), SP21 vs. LP21 (orange), and SP21 vs. LP42 (green) are shown in **Figures 4A,B** (data in **Supplementary Tables 9–11** for lncRNAs and **Supplementary Tables 12–14** for mRNAs). The following GO terms are noteworthy, including neuron terms (such as neuron part, axon, synapse part, and response to stimulus), receptor terms (such as cell surface receptor signaling pathway and receptor complex), regulation of apoptosis process, regulation of cell death, regulation of cell proliferation, and metabolic process (such as regulation of metabolic process and positive regulation of phosphorus metabolic process). In order to further focus on the function of differentially expressed genes between SP and LP, we constructed a simplified network of related statistically significant GO terms (**Figure 4C**) for differentially expressed genes overlapping between SP and three of LP points using the Cytoscape add-on ClueGO (34, 35). The results emphasized enrichment for cell proliferation and differentiation, cell response and pathway, cell death and regulation, and the cytokine process, which might play an important role in the

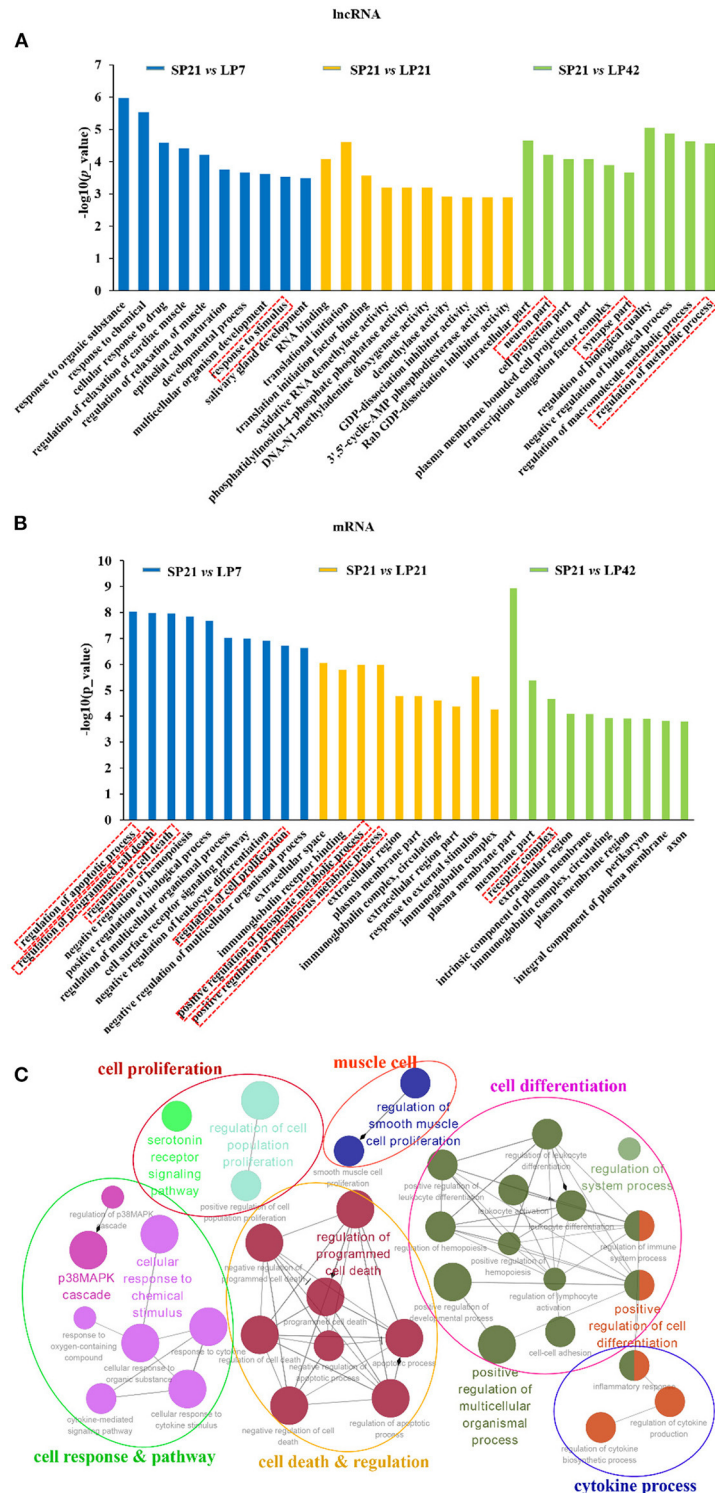
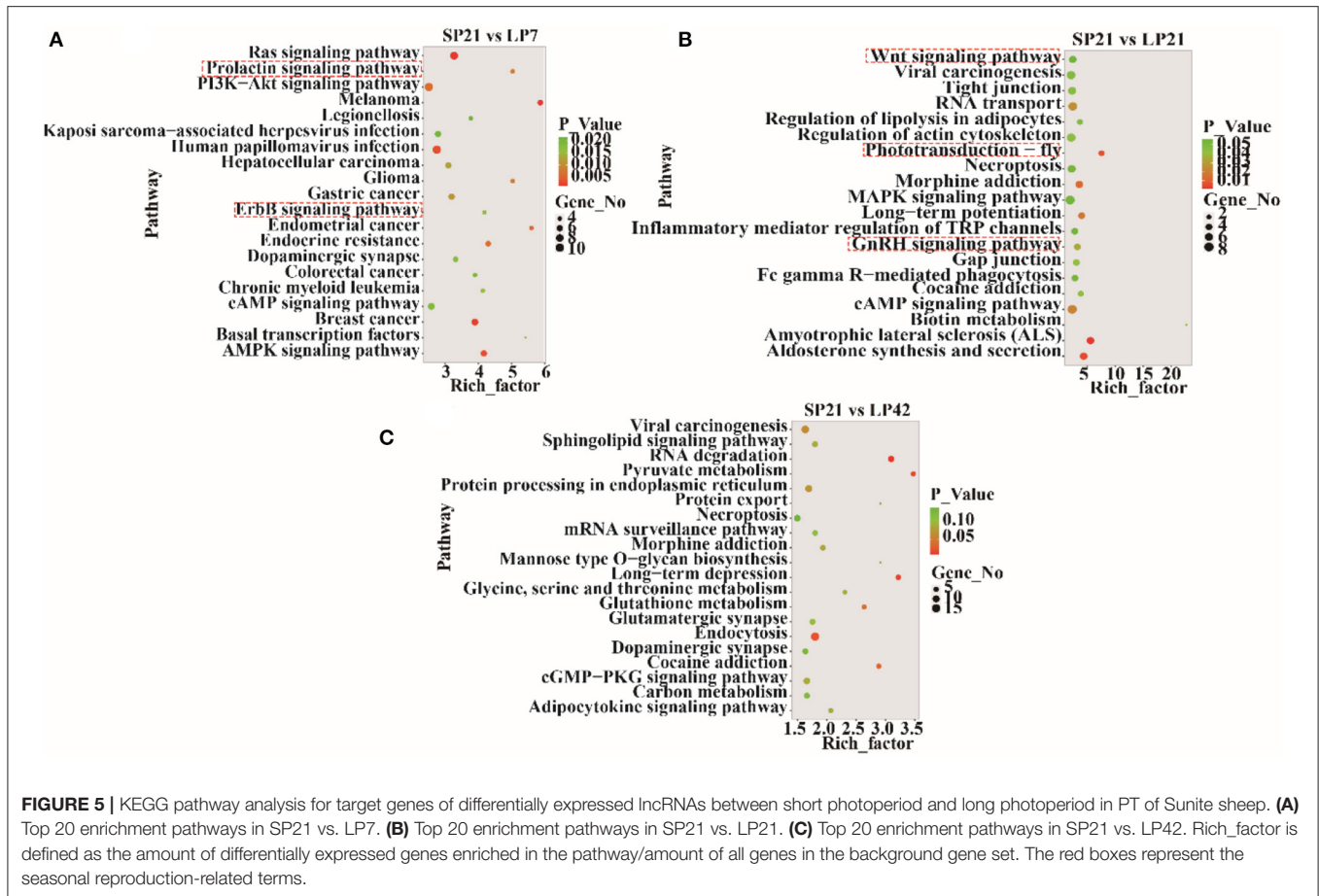


FIGURE 4 | Top enriched Gene Ontology (GO) terms of differentially expressed lncRNAs and genes between short photoperiod and long photoperiod in PT of Sunite sheep. **(A)** The top GO terms of target genes of differentially expressed lncRNAs; **(B)** the top GO terms of differentially expressed mRNAs. The GO terms in blue are from SP21 vs. LP7; those in orange are from SP21 vs. LP21, and those in green are from SP21 vs. LP42. **(C)** A simplified network of related statistically significant GO terms ($p < 0.01$) using the Cytoscape add-on ClueGO (34, 35). The network of overlap DE genes among SP21 vs. LP7, LP21, and LP42 are shown. The filled colored circles (nodes) represent each statistically significant parent GO term. The lines (edges) between the nodes show that there are overlapping genes within each term. The colored ovals group these parent GO terms into more generic functional descriptions. The red boxes represent the seasonal reproduction-related terms.



regulation of seasonal breeding in sheep at the level of the PT/MBH (36, 37).

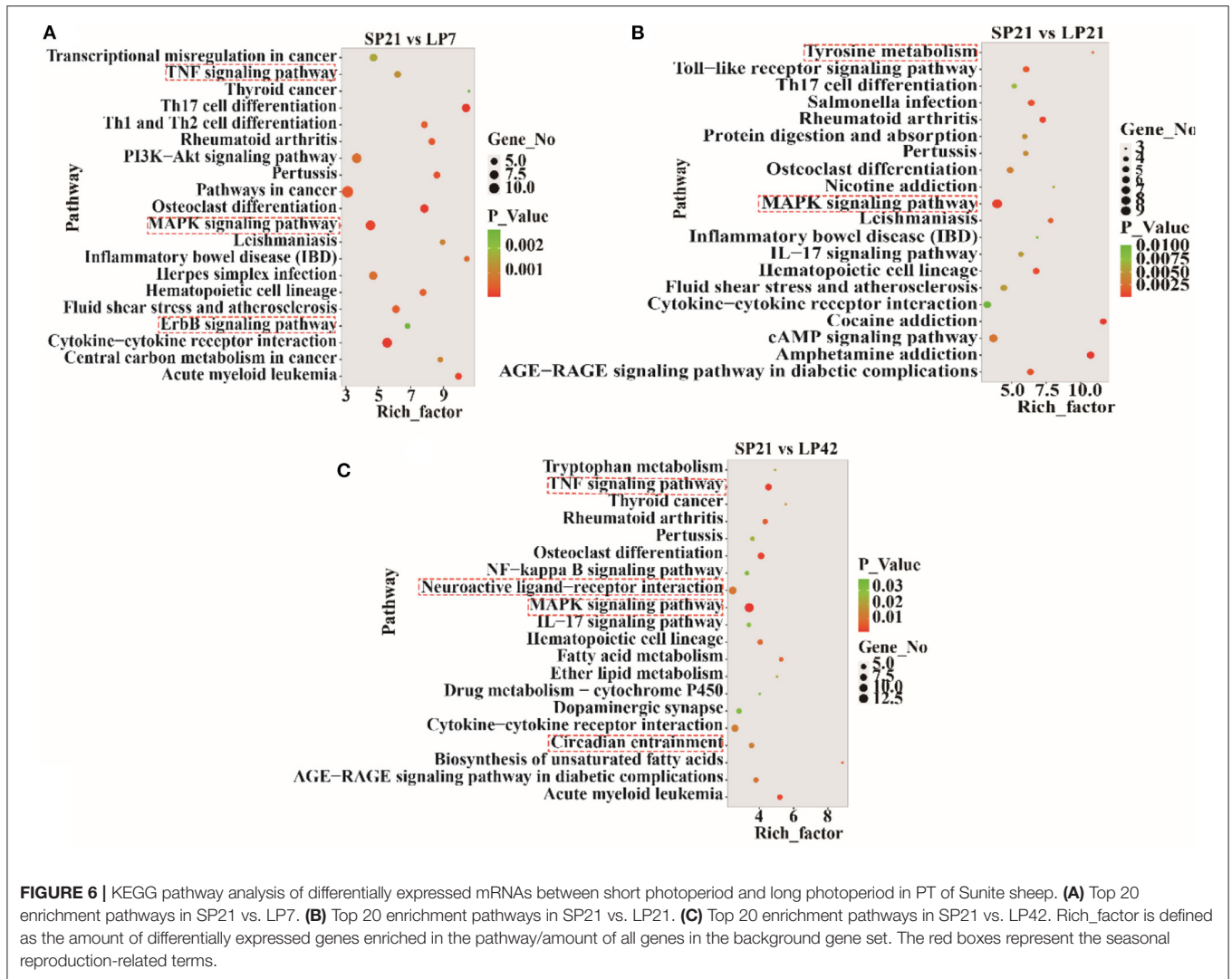
The top 20 enriched KEGG pathways for target genes of differentially expressed lncRNAs and pathways for differentially expressed mRNAs are shown in **Figures 5, 6**, respectively. The most interesting pathways included reproduction-related pathways (Prolactin signaling pathway, ErbB signaling pathway, Wnt signaling pathway, MAPK signaling pathway, and GnRH signaling pathway in **Figure 5**; ErbB signaling pathway and MAPK signaling pathway in **Figure 6**), circadian related pathways (phototransduction-fly in **Figure 5**; circadian entrainment in **Figure 6**), and neuroactive ligand-receptor interaction, TNF signaling pathway, tyrosine metabolism, and neuroactive ligand-receptor interaction pathway (detailed data in **Supplementary Tables 15–17** for lncRNAs and **Supplementary Tables 18–20** for mRNAs). Here, we also combined the overlap DE genes from three comparisons and assigned KEGG terms to create a simplified network of related statistically significant KEGG terms (34, 35) (**Figure 7**). **Figure 7** also showed that many differentially expressed genes between SP and LP are enriched in pathways related to reproduction, such as signaling (prolactin signaling pathway, cAMP signaling pathway, and JAK-STAT signaling pathway), neuron, neurotransmitters, and cell process.

lncRNA-mRNA Network Construction

The differentially expressed lncRNAs and their target genes were selected to construct the lncRNA-mRNA network with co-expression information. In the network of SP21 vs. LP7, 23 differentially expressed lncRNAs and 21 target genes were included, among which the *trans* relationship between *MSTRG.209166* and *TSHB* was hinted (**Figure 8A**, **Supplementary Table 21**). In SP21 vs. LP21, 29 differentially expressed lncRNAs and 26 target genes were selected to construct network. Notably, *MSTRG.209166* was also found to *trans*-regulate *TSHB* and *MSTRG.235014* *cis*-regulated *DDC*, because the two genes were important for seasonal reproduction (**Figure 8B**, **Supplementary Table 22**). In SP21 vs. LP42, 102 differentially expressed lncRNAs and 91 target genes formed a more sophisticated network. For several key genes of seasonal reproduction, several target combinations between lncRNAs and them were highlighted. For example, they implied that *MSTRG.209166* *trans*-regulated *TSHB*, *MSTRG.288068* *cis*-regulated *SIX1*, *MSTRG.272793* *cis*-regulated *KIT*, and *ENSOARG00000026131* *cis*-regulated *TH* (**Figure 8C**, **Supplementary Table 23**).

Gene Expression Validation

A total of six genes, namely, three mRNAs (*EYA3*, *TSHB*, and *CHGA*) related to seasonal reproduction and three random



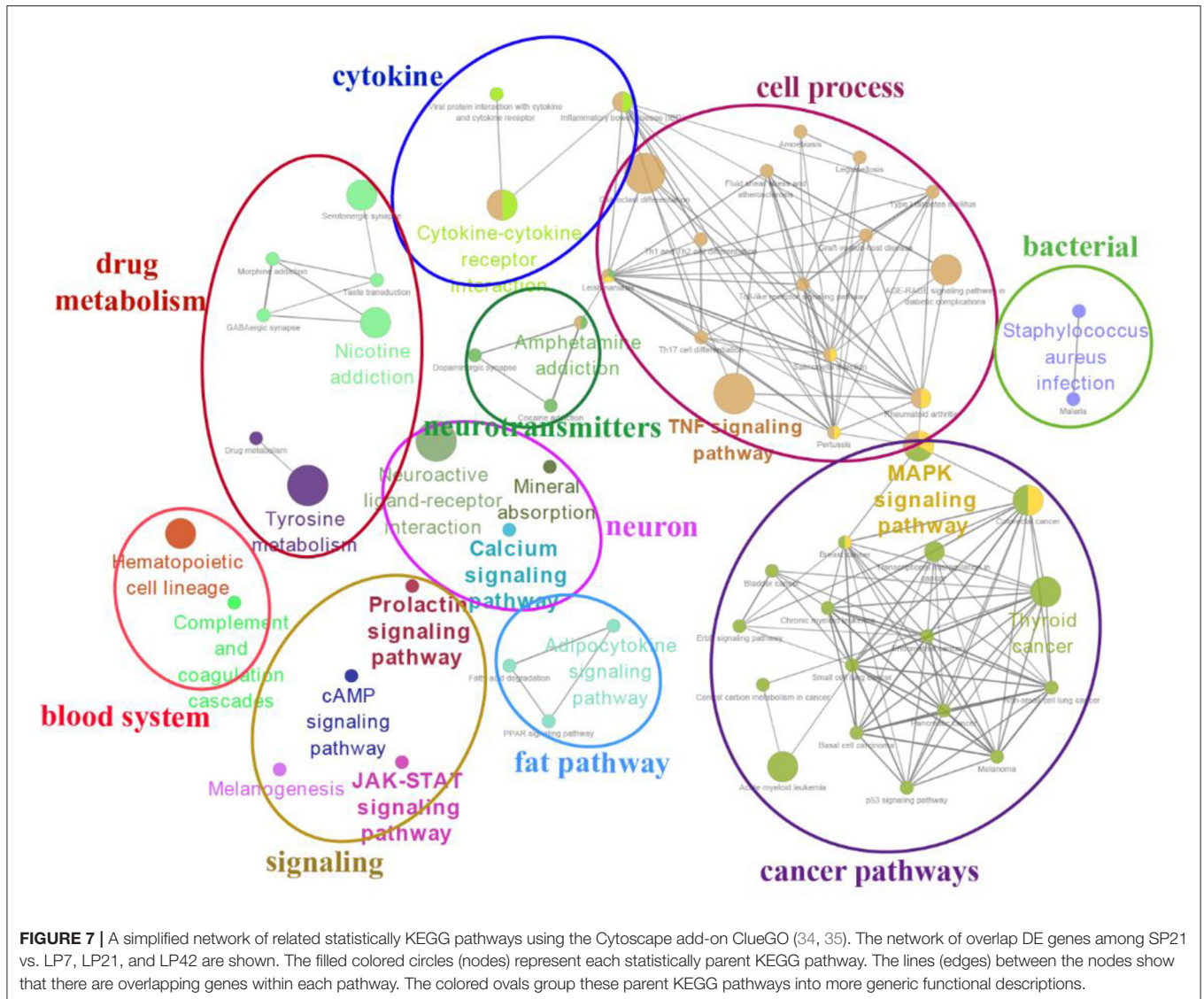
lncRNAs (*MSTRG.290436*, *MSTRG.22781*, and *MSTRG.17707*), were selected for RT-qPCR verification. The results indicated that there is a similar expression pattern between RNA-Seq and RT-qPCR data (Figure 9).

DISCUSSION

Seasonal reproduction is a result of adaptation of animal reproductive activities to environmental changes, which is essential for breeding success and survival of future generations. However, the detailed molecular mechanism of animal seasonal reproduction has not been fully revealed. So far, several key genes for seasonal reproduction have been found in animals, including *EYA3* gene in Japanese quail (38) and sheep (8), the vasoactive intestinal peptide (*VIP*) gene in Yangzhou goose (39), *TSHB* in sheep (8), *TSHB* and pituitary adenylate cyclase activating polypeptide (*PACAP*) in mice (19, 40), and *CHGA* and Tachykinin 1 (*TAC1*) in sheep (3, 7), which are mainly expressed in the pituitary PT. The pituitary PT is an important regulatory

center for seasonal reproductive traits. Therefore, it was used as the target tissue in this study and the first objective of this study is to screen new candidate photoperiod-induced genes in PT of sheep.

Combined with the results of gene differential expression, GO, and KEGG pathway enrichment analyses, some important candidate genes were screened out. They included *CHGA*, *FOS*, *SOCS3*, *EYA3*, *TSHB*, *SIX1*, *GHRH*, *DCT*, *TH*, *VMO1*, *AREG*, *SUV39H2*, and *EZH2*, which were mainly involved in the following pathways: reproduction-related pathways, TNF signaling pathway, circadian entrainment, and tyrosine metabolism. Specifically, LP-induced genes included *EYA3*, *VMO1*, *TSHB*, *SIX1*, *GHRH*, *DCT*, *TH*, *VMO1*, *AREG*, *SUV39H2*, and *EZH2* and SP-induced genes involved *ENSOARG00000012585*, *CHGA*, *FOS*, *SOCS3*, and *TH*. Wood et al. (7) reported *CHGA* as a SP-activator, which had a significantly high expression at SP compared with LP in PT of rams. Our results in PT of ewes also further confirm that *CHGA* is a SP-induced gene. For *FOS*, *SOCS3*, and *ENSOARG00000012585*,



they may be considered as new candidate SP-induced genes in PT, whose function in PT need further analysis. The suprachiasmatic nuclei of the hypothalamus (SCN) are the master circadian clock in mammals. The role of *FOS* and *SOCS3* in SCN has been reported in long-day breeding animals (redheaded bunting, rat, and hamster), which are enriched in the TNF signaling pathway and circadian entrainment. Their expression patterns under SP and LP are opposite to those of short-day breeding animals (41–43). For example, *FOS* is predominantly expressed in the SCN of redheaded buntings under LP conditions (42). Moreover, our results showed that *FOS* was highly expressed in pituitary PT of sheep under SP. Photoperiod also modulated *SOCS3* gene expression and maintained its expression in a high level in the SCN of hamsters during LP compared with SP (44). Then, *SOCS3* conveys seasonal changes into leptin sensitivity in the Siberian hamster (44).

So far, several LP-induced genes have been revealed. *EYA3* and *TSHB*, as LP-induced genes, displayed a marked increase

from SP to LP in PT of sheep (3, 7, 45). Similarly, in this study, *EYA3* and *TSHB* were LP-induced and went up at LP compared with SP in PT of ewes, further certifying the findings of previous studies (8, 15, 46). In both sheep and mouse, *EYA3* and its partner *SIX1* synergistically act as upstream inducers of the *TSHB* transcription to induce the expression of *TSHB* (8, 19). Furthermore, *TSHB* acts locally on *TSHR*-expressing cells in the adjacent basal hypothalamus, leading to altered expression of *DIO2* (5, 6, 47). Then, *DIO2* can regulate the secretion of TH in the basal hypothalamus and further modulate the transition of reproductive status through the spatial structure of GnRH neurons (7). When the GnRH neurons were wrapped by ependymal cells, little GnRH were released into pituitary, which caused the decrease of LH and FSH secretion, and eventually sheep entered the state of anestrus (7).

Tyrosine metabolism can mediate the biological effects of many hormones and cytokines on reproduction, immunity, and cell growth (48, 49). In this study, the DE *DCT* was enriched

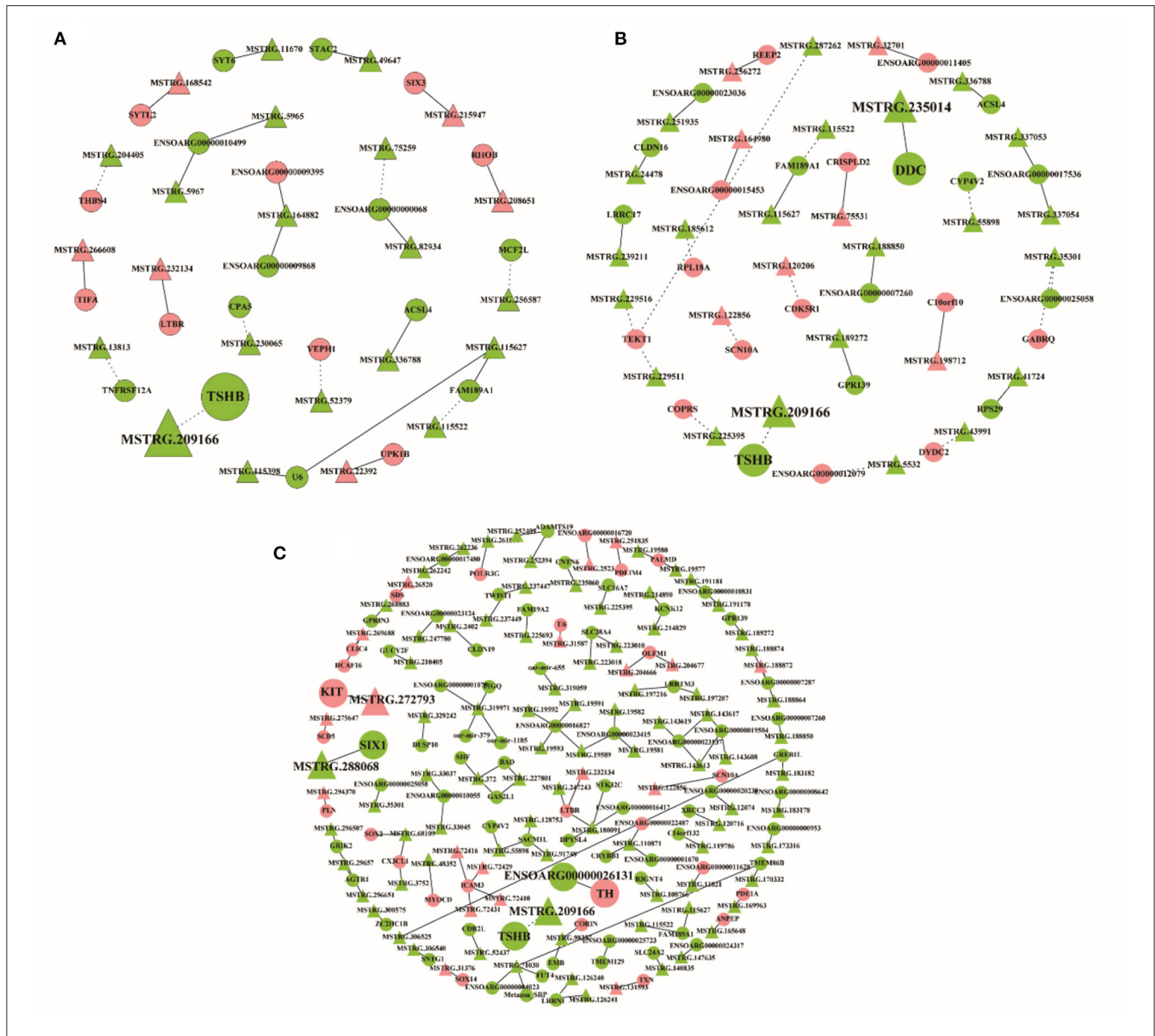
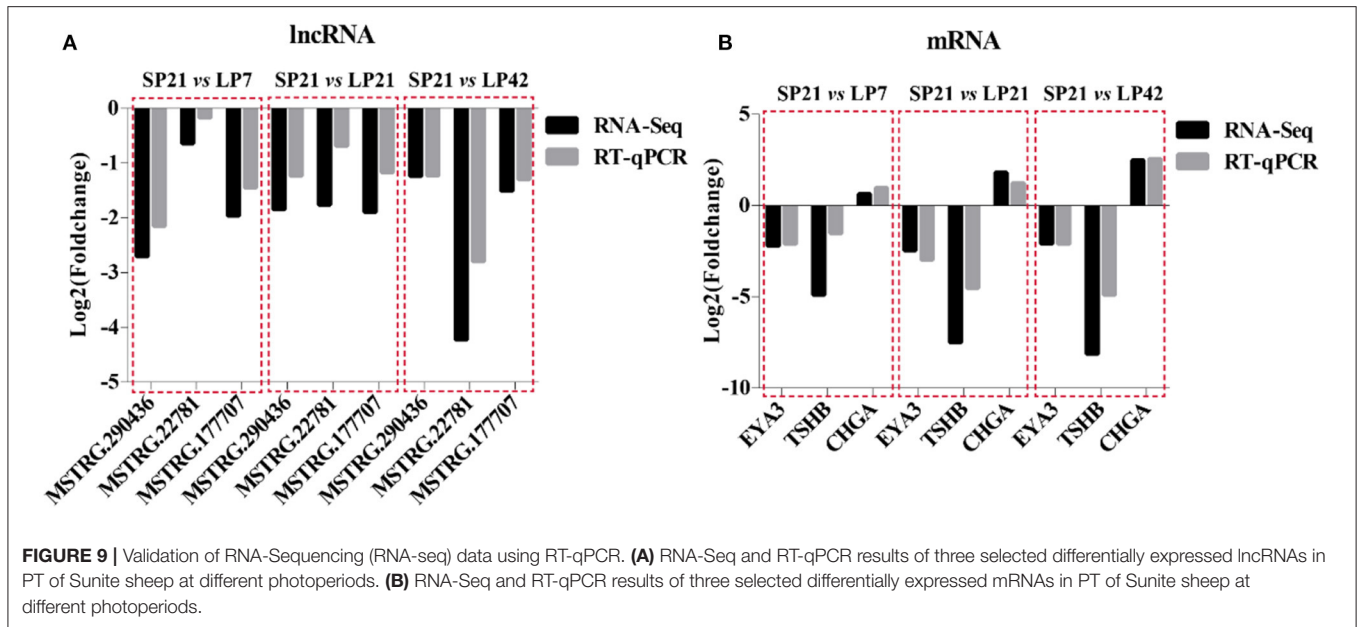


FIGURE 8 | The interaction networks of lncRNAs and their corresponding target gene. **(A)** The networks for SP21 vs. LP7; **(B)** the networks for SP21 vs. LP21; **(C)** the networks for SP21 vs. LP42. The dashed and solid lines represent trans- and cis-regulation functions, respectively. Red and green represent up- and downregulation, respectively. Circles and triangles represent mRNAs and lncRNAs, respectively.

in the tyrosine metabolism pathway. *DCT* was found as a novel long-day marker, whose expression levels in tanycytes lining the infra-lateral walls and floor of the ovine third ventricle showed marked increase with the extension of the photoperiod (6, 45). Similarly, in this study, *DCT* was significantly upregulated during LP compared with SP in PT of sheep. These results suggested that *DCT* expression was crucial for initiation of anestrus in sheep. Previous research has shown that *TH* can modulate *DCT* expression (46). *DCT* is a part of, or is driven by, the circannual clock in sheep. Therefore, these characteristics—LP induction and circannual changes in expression—place *DCT* in a list of key

genes involved in seasonal timing, which had included *TSHB*, *EYA3*, *DIO2*, and *DIO3* (45, 46).

In this study, the expression level of *VMO1*, *AREG*, *SUV39H2*, and *EZH2* was also significantly higher during LP than those at SP in PT of sheep. As secreted factors, *VMO1* and *AREG* were found to be acutely induced by LP in MBH of Ile-de-France ewes (6). In polyovular species, the LH-driven signaling can promote oocyte maturation and cumulus expansion by *AREG*. In addition, murine data indicated that LH binding to LHCGR in mural granulosa cells will up-regulate *AREG* (50). However, the function of *AREG* gene in PT is still unknown,



and it may be considered as a candidate LP-induced gene to analyze. For two histone methyltransferases genes, *SUV39H2* and *EZH2*, the results about seasonal changes of their expression in the PT are consistent with the hypothesis that epigenetic changes in PT cells are involved in circannual timing (6). Besides, the acute LP responsiveness of most of these markers (*TSHB*, *VMO1*, *EZH2*, and *EYA3*) is also in accordance with the findings from Dardente et al. (45) and Lomet et al. (6) in Ile-de-France ewes.

In recent years, studies have indicated that lncRNAs play important roles in many aspects of sheep reproductive regulation, such as fecundity (12, 13), gonadal development (14), and sex hormone response (15). As epigenetic regulators, lncRNAs can regulate the expression of reproduction-related genes. Thus, the other objective of this study is to predict lncRNAs that target key genes for seasonal reproduction in PT of sheep. Firstly, our result showed that the sequence length and exon number of mRNAs and lncRNAs in sheep pituitary have different patterns with those in hypothalamus of sheep (3,448 nt and 2.5 exons) (13). This implies that lncRNAs have tissue-specific characteristics. Then, through lncRNA–mRNA network construction, several notable target combinations between lncRNAs and key genes for seasonal reproduction were predicted. They specifically included the *trans* relationship between *MSTRG.209166* and *TSHB* and *cis* relationships between *MSTRG.235014* and *DDC*, *MSTRG.272793* and *KIT*, and *ENSOARG0000026131* and *TH*. Importantly, the *trans* relationship between *MSTRG.209166* and *TSHB* was shared among all of the comparison groups (SP vs. LP), which suggested that *MSTRG.209166* might play an important role in seasonal reproduction by regulating the expression of *TSHB*. *TSHB* is an important hub in the pathway of seasonal reproduction, so this study provides a new molecular object

(*MSTRG.209166*) for the follow-up epigenetic regulation study of seasonal reproduction in sheep. In addition, the several candidate lncRNAs mentioned above (e.g., *MSTRG.235014*, *MSTRG.272793*, and *ENSOARG0000026131*) are also worthy of in-depth analysis.

CONCLUSION

In summary, our study provided a genome-wide view of lncRNA and mRNA expression profiling in pituitary PT of sheep during LPs and SPs. Several new candidate photoperiod-induced genes and lncRNAs targeting key genes of seasonal reproduction were predicted in PT of sheep. These results will provide new clues for understanding the molecular regulation of seasonal reproduction in sheep.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject; PRJNA680667.

ETHICS STATEMENT

The animal study was reviewed and approved by The Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS; Beijing, China) (No. IAS2018-3,10 April 2018). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

QX and MC: design of experiment and analysis for PT of sheep. QL and XH: construction of the OVX+E2 sheep model and light control experiment. RD: Experimental guidance and writing. XZ, JZ, and XG: light control experiment and collection of samples.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2021.644474/full#supplementary-material>

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