GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Expression of the luteinizing hormone receptor (*LHR*) gene in ovine non-gonadal tissues during estrous cycle

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Genet. Mol. Res. 11 (4): 3766-3780 (2012)
Received March 14, 2012
Accepted June 12, 2012
Published October 15, 2012
DOI http://dx.doi.org/10.4238/2012.October.15.8

ABSTRACT. Luteinizing hormone (LH) is an important glycoprotein hormone that regulates gonadal function in mammals and in turn regulates physiological status changes during the estrous cycle. The function of LH is mediated by luteinizing hormone receptor (LHR). In order to examine the expression patterns of the *LHR* gene in non-gonadal tissues during the 4 phases of the ovine estrous cycle, tissues from healthy non-pregnant adult Hu sheep were examined according to the estrous cycle for normal ovaries using real-time fluorescence quantitative PCR and ELISA methods with *GAPDH* as the reference gene. *LHR* mRNA expression levels were significantly correlated with protein concentrations and the *LHR* gene was abundantly expressed in olfactory bulb, hypothalamus, rumen, small intestine, kidney, and uterine tissues. When comparing the expression levels of *LHR* during the 4 estrous phases in particular tissues, the results showed that *LHR* expression levels were significantly different and relatively lower at the estrous stage in a number of non-gonadal tissues. The trends of change in *LHR* expression levels were highly significantly correlated between hypothalamus and rectum, hypophysis and oviduct, ileum and

uterus, and among jejunum, olfactory bulb, and kidney ($P < 0.01$), and there was also significant correlation between duodenum and oviduct, hypothalamus and medulla oblongata, jejunum and uterus, omasum and abomasum, and reticulum and colon ($P < 0.05$). These results indicate that the ovine *LHR* gene (or *LH*) might control important mechanisms in non-gonadal tissues and that the level of *LH* activity in some tissues may be influenced by hormonal status during the estrous cycle.

Key words: Luteinizing hormone receptor (LHR); ELISA; qRT-PCR; Non-gonadal tissues

INTRODUCTION

Luteinizing hormone (LH) is an important glycoprotein hormone that regulates gonadal function in mammals. Its function is mediated by the luteinizing hormone receptor (LHR), which belongs to the G protein-coupled receptor family (Ascoli et al., 2002). It is well known that LHR is widely expressed in gonadal cells. However, the presence of this receptor has also been reported in several other non-gonadal tissues (Rao and Lei, 2007). Previous research on LHR in cattle (Sun et al., 1997; Shemesh et al., 2001), pig (Ziecik et al., 1986; Gawronska et al., 1999; Wasowicz and Ziecik, 2000), goat (Shen et al., 2009), mouse (Zhang et al., 2001), rabbit, and rat (Sawitzke and Odell, 1991) has shown that the *LHR* gene is expressed in a variety of non-gonadal tissues and organs, including oviduct, cervix, urinary bladder, placenta, fetal membranes, umbilical cord, brain, pineal gland, spinal cord, pituitary gland, breast, skin, adrenal gland, blood vessels in target tissues, cells of the immune system, bone, heart, stomach, spleen, kidney, muscle, liver, and intestine (Vischer and Bogerd, 2003; Kwok et al., 2005; Rao and Lei, 2007; Ziecik et al., 2007; Chen et al., 2010). This suggests that *LH* is involved in the regulation of function of other organs. Hu sheep originate from the Taihu Lake area of China, which covers the Provinces of Jiangsu and Zhejiang, and the vicinity of Shanghai. The breed is known for its exceptional lamb skin, early sexual maturity, and high fecundity (200-250%) (Yue, 1996; Wang et al., 2000). To the best of our knowledge, there have been no previous studies of the expression of *LHR* in different tissues of sheep. Therefore, the objective of this study was to investigate in detail the tissue-specific expression patterns of *LHR* in sheep during the estrous cycle using real-time quantitative RT-PCR and ELISA methods. This study has attempted to help broaden our knowledge of the function of LH in non-gonadal tissues.

MATERIAL AND METHODS

Animals and treatments

Healthy multiparous (2-3 years) Hu sheep were from the Hu Sheep Stock Seed Farm, Zhejiang Province, China. The average body weight was 35-40 kg. All animals were handled in accordance with the Guidelines for Care and Use of Experimental Animals (Nanjing Agricultural University). Before sampling, animals in different estrous phases were injected intramuscularly with 0.1 mg cloprostenol sodium (PG-Cl; SuZhou Sumu Animal Medical Industry Co. Ltd., Jiangsu, China). Two days after the injection, all sheep entered estrus. When the sheep

entered a second natural estrus, healthy animals were selected at estrus (day 0), metestrus (day 3), diestrus (day 10), and proestrus (day 14) for sampling. The onset of estrus was confirmed by a teaser ram, vaginal examination, ovarian status (Figure 1), and behavior, with this time point assigned as day 0, and one animal was slaughtered. Metestrous, diestrous, and proestrous animals were then slaughtered at days 3, 10, and 14, respectively. All sheep were slaughtered after fasting for 2 h. The liver, pancreas, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, colon, rectum, hypothalamus, hypophysis, medulla oblongata, olfactory bulb, uterus, oviduct (isthmus), and kidney were sampled, immediately frozen in liquid nitrogen, and then stored at -80°C until total RNA and protein extraction.

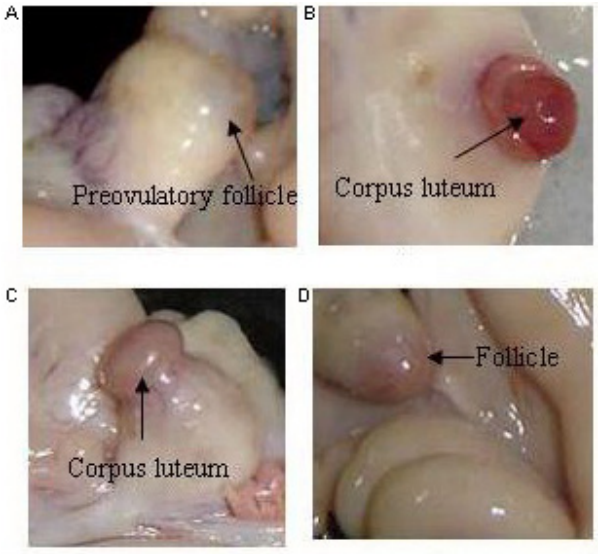

Figure 1. The ovarian status during four estrous phases in Hu sheep. **A.** Estrous (day 0) phase; **B.** metestrous (day 3) phase; **C.** diestrous (day 10) phase; **D.** proestrous (day 14) phase.

Total RNA extraction and qRT-PCR

Total RNA was extracted from tissues using a Trizol reagent total RNA extraction kit (Invitrogen, CA, USA) according to manufacturer instructions. All RNA samples had an optical density OD_{260}:OD_{280} ratio of between 1.8 and 2.0, and integrity of total RNA was verified by denaturing agarose gel electrophoresis, indicating pure RNA isolates. Reverse transcription was performed using a PrimeScript RT reagent kit (TaKaRa, Dalian, China), according to the manufacturer protocol, and the resulting cDNA was used as a template for real-time quantitative PCR. The housekeeping gene *GAPDH* was used as a reference gene. Primer sequences for the *GAPDH* (forward: 5'-TCCTGCACCACCAACTGCTT-3'; reverse: 5'-GCAGGTCAGATCCACAACGG-3') and *LHR* genes (forward: 5'-CAGTTGATGCCCAAA CCAAA-3'; reverse: 5'-GGCAAATGCTGACCTTCATG-3') were designed based on cDNA sequences for sheep obtained from GenBank (*LHR*: L36329.1; *GAPDH*: NM_001190390.1). All HPLC-grade oligonucleotides were produced by Invitrogen.

Real-time quantitative PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems) with SYBR Green fluorescent labels. Samples (final volume of 25 µL) were run in triplicate and contained the following: 0.5 µL SYBR Green (Invitrogen), 2.5 µL 10X buffer, 2 µL $MgCl_2$, 2 µL 10 mM of each dNTP, 0.3 µL 50 pM/µL of each primer, 0.3 µL *Taq* (TaKaRa), 15.1 µL dH_2O, and 2 µL cDNA template. Amplification was conducted using the following protocol: initial denaturation phase at 95°C for 2 min, and then 40 cycles at 94°C for 10 s for denaturation, then for a further 10 s for annealing (*GAPDH*: 61°C; *LHR*: 58°C), and at 72°C for 40 s for the extension step. Melting curve analyses were performed after real-time PCRs to monitor PCR product purity (Figure 2).

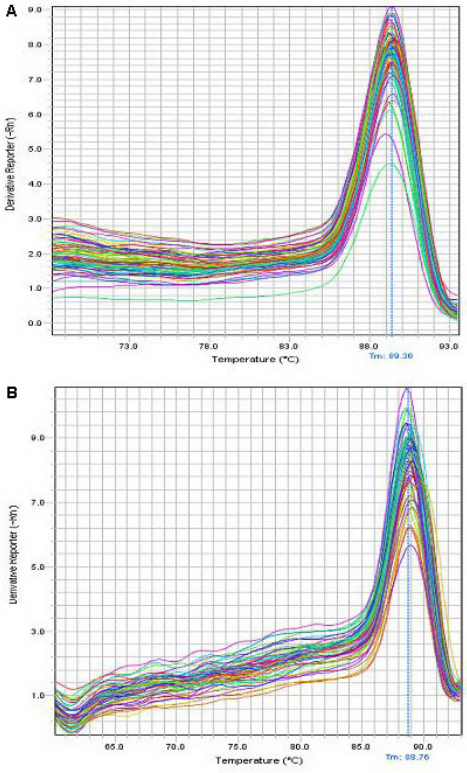

Figure 2. Melting curves for *LHR* (**A**) and *GAPDH* (**B**) genes.

Protein extraction from tissues and ELISA

Protein in frozen tissues was obtained by sonicating small pieces of tissue in 1 mL immuno-precipitation assay buffer (50 mM Tris, 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS). Tissue homogenates were centrifuged and supernatant was used for ELISA analysis. Total protein samples were diluted to 0.5 mg/mL. Standards or samples were tested using the LHR ELISA Kit (Groundwork Biotechnology Diagnosticate Ltd., USA, Catalogue No. L063-57), according to the manufacturer protocol, and the OD of plates was read at 450 nm using a microtiter plate reader.

Statistical analysis

The qRT-PCR results are presented as the gene expression of the target gene (*LHR*) relative to that of the housekeeping gene (*GAPDH*), and the values for the target gene (*LHR*) relative to the liver value were determined by the expression $2^{-\Delta\Delta CT}$. All data were analyzed by one-way ANOVA of least significant difference (LSD) *post hoc* test. The correlations between the data from real-time PCR and ELISA were analyzed by two-tailed Pearson's correlation. Statistical analyses were performed using the SPSS 13.0 statistical software package. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Expression levels of *LHR* mRNA in various tissues of Hu sheep

LHR mRNA expression levels were measured by real-time qRT-PCR in samples from liver, pancreas, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, colon, rectum, hypothalamus, hypophysis, medulla oblongata, olfactory bulb, uterus, oviduct, and kidney. *LHR* expression was normalized to endogenous control *GAPDH*. *LHR* expression in the liver served as calibrator. *LHR* gene expression levels are achieved using the $2^{-\Delta\Delta CT}$ method of quantification. The relative expression levels of *LHR* in various tissues analyzed by one-way ANOVA showed that the gene was expressed at significantly higher levels in the olfactory bulb, hypothalamus, rumen, kidney, and uterine tissues, while it was at the lowest level in liver. The *LHR* expression levels in the digestive system tissues were also abundant, especially in rumen, and those in small intestine tissues were higher than in large intestine tissues (Table 1).

Table 1. Expression profile of *LHR* mRNA in various tissues from Hu sheep.

Non-gonadal organ tissues	*LHR* mRNA
Brain	
Hypophysis	2.50 ± 2.61^{af}
Medulla oblongata	3.71 ± 4.89^{abf}
Olfactory bulb	12.79 ± 23.55^{c}
Hypothalamus	6.75 ± 9.51^{abc}
Digestive system organs	
Rumen	11.99 ± 17.89^{bcd}
Reticulum	2.36 ± 2.74^{af}
Omasum	2.47 ± 2.32^{af}
Abomasum	3.35 ± 3.88^{af}
Duodenum	4.66 ± 7.34^{adef}
Jejunum	7.02 ± 9.12^{ac}
Ileum	8.20 ± 11.38^{ac}
Cecum	1.98 ± 1.83^{af}
Colon	3.69 ± 4.63^{adf}
Rectum	3.64 ± 3.41^{af}
Pancreas	4.15 ± 4.01^{adf}
Liver	1.00 ± 0.00^{a}
Genitourinary organs	
Kidney	12.74 ± 23.10^{ce}
Oviduct	4.13 ± 6.86^{adf}
Uterus	9.66 ± 16.48^{fc}

Groups marked with the same superscript letters are not statistically different. $P < 0.05$ is denoted with different superscript lower case letters.

Expression levels of *LHR* mRNA in brain tissues of Hu sheep at different estrous stages

LHR mRNA expression levels were prominent at different estrous stages in the hypophysis, medulla oblongata, olfactory bulb, and hypothalamus of the Hu sheep. Among the changes in expression levels of *LHR* in different estrous stages, the results showed that at the estrous stage *LHR* expression in the medulla oblongata was greatest, with a level 8.21-fold higher than the lowest level in the hypothalamus, while at the metestrous stage, expression in hypothalamus tissue was the highest, 251.95, which was also the highest measurement of all tissues in the study, with level 78.73-fold greater than the lowest level in the olfactory bulb. During diestrus, expression in the olfactory bulb was the highest (135.61) with a level 31.83-fold higher than the lowest level in the medulla oblongata. Finally, the expression level in hypophysis was the highest in the proestrous stage (Figure 3).

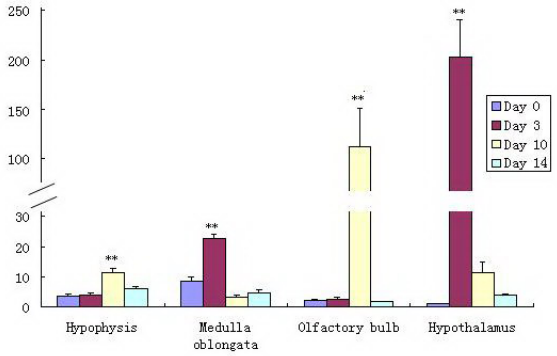

Figure 3. Expression profile of *LHR* mRNA in the brain tissues of Hu sheep at estrous (day 0), metestrous (day 3), diestrous (day 10), and proestrous (day 14) phases. *LHR* gene expression is normalized to endogenous control *GAPDH*, and the values for the target gene (*LHR*) relative to the liver value of the estrous phase were determined by the expression $2^{-\Delta\Delta CT}$. The relative expression level of *LHR* in variant tissues is shown as the mean of three measurements and analyzed by one-way ANOVA. $P < 0.01$ is denoted with asterisks above each bar in one tissue.

When comparing the levels over 4 estrous phases in one tissue, the result showed that *LHR* mRNA expression levels in hypophysis and olfactory bulb were detected significantly higher at the diestrous stage, particularly in the olfactory bulb, the *LHR* mRNA expression level at the diestrous stage was approximately 42-62-fold higher than in the other phases. *LHR* expression levels in the medulla oblongata and hypothalamus were detected significantly higher at the metestrous stage. In the hypothalamus, the *LHR* mRNA expression level at the metestrous stage was extremely high, approximately 18-189-fold higher than at other phases (Figure 3).

Expression levels of *LHR* mRNA in the digestive system of Hu sheep in different estrous stages

LHR mRNA expression levels in the digestive system were changed prominently at the different estrous stages (Figure 4). Among the ruminant stomach tissues, *LHR* mRNA expression levels were detected significantly higher at the metestrous and diestrous stages in the rumen and at the proestrous stage in the reticulum, while in omasum and abomasums, *LHR* expression levels were significantly lowest at the estrous stage. During the metestrous and diestrous phases, *LHR* mRNA

expression level in the rumen was detected as the highest (72.78 and 108.86), it was 29.59- and 13.57-fold higher than the lowest level in the reticulum, while during proestrous phase, the *LHR* mRNA expression level in the reticulum was 7.94-fold higher than the lowest level in the rumen. Trend results showed that, in rumen, the expression levels increased from proestrus to the highest level in diestrus, and the levels during metestrus and diestrus were higher than those in the proestrous and estrous stages. In the reticulum, the expression levels in estrus and diestrus were similar, and are the highest during proestrous phases. In omasum, except during the estrous phase, the expression levels were relatively high and the levels in the diestrous and proestrous phases were similar. In abomasum, the *LHR* mRNA expression levels decreased from the metestrous to the estrous phase.

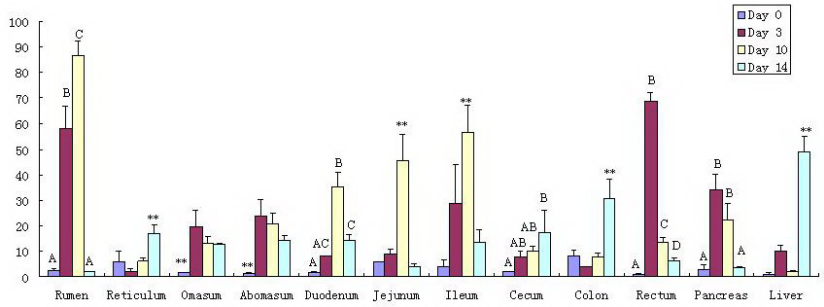

Figure 4. Expression profile of *LHR* mRNA expression levels in the digestive system at estrous (day 0), metestrous (day 3), diestrous (day 10), and proestrous (day 14) phases. *LHR* gene expression is normalized to endogenous control *GAPDH*, and the values for the target gene (*LHR*) relative to the liver value of the estrous phase were determined by the expression $2^{-\Delta\Delta CT}$. The relative expression level of *LHR* in variant tissues is shown as the mean of three measurements and analyzed by one-way ANOVA. $P < 0.01$ is denoted with asterisks or different capital letters above each bar in one tissue. Bars marked with the same letters are not statistically different.

In small intestine tissues (including duodenum, jejunum, and ileum) during the estrous phase, the *LHR* mRNA expression level in the duodenum was the lowest, while in jejunum, *LHR* expression was the highest. During the metestrous and diestrous phases, *LHR* expression levels in ileum were the highest. During proestrus phase, the expression levels in duodenum and ileum were higher than that in the jejunum. The average expression levels of *LHR* mRNA in the ileum (31.23) were relatively higher than that in the duodenum (18.54) and jejunum (18.50) among small intestine tissues. Overall, *LHR* mRNA expression levels in the small intestine tissues were detected significantly higher at the diestrous stage, and had the same changing trend, i.e., the expression levels were increased from the estrous stage to the diestrous stage then decreased after the diestrous stage.

In large intestine tissues (including cecum, colon, and rectum), the results showed that in the estrous and proestrous phases, *LHR* expression levels were the lowest in the rectum, while in the metestrous and diestrous stages, the expression levels were the lowest in the colon. The average expression levels of *LHR* mRNA in rectum (28.26) were higher than that in the cecum (11.02) and the colon (15.69) among large intestine tissues. *LHR* mRNA expression levels tended to increase from the estrous stage in the cecum, decline from metestrus in the rectum, while in the colon, the expression levels were the highest in the proestrous phase and the lowest in the metestrous phase.

In the pancreas, *LHR* expression was significantly higher in the luteal phase (metes-

trus and diestrus) than that in the follicular phase (proestrus and estrus). In the liver, *LHR* expression levels were the highest in the proestrous phase, while the levels were relatively lower in the estrous and diestrous stages.

Expression levels of *LHR* mRNA in the genitourinary system of Hu sheep in different estrous stages

The *LHR* mRNA expression levels were significantly higher in the diestrous stage and lowest in the estrous stage in the kidney, oviduct, and uterus across the estrous cycle. The expression levels tended to increase from estrus, and after diestrus the levels began to decrease (Figure 5).

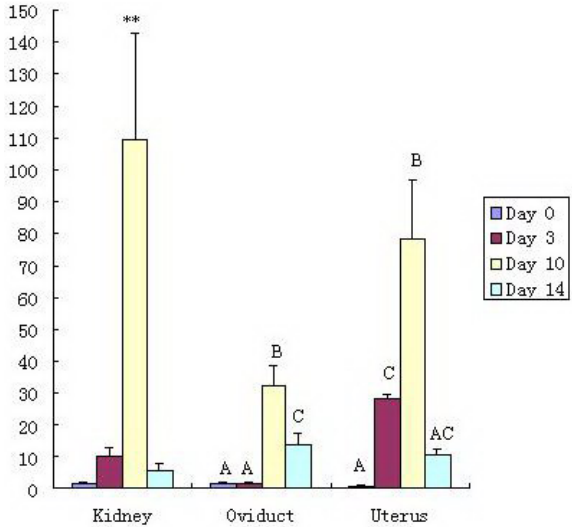

Figure 5. Expression profile of *LHR* mRNA expression levels in the genitourinary system at estrous (day 0), metestrous (day 3), diestrous (day 10), and proestrous (day 14) phases. *LHR* gene expression is normalized to endogenous control *GAPDH*, and the values for the target gene (*LHR*) relative to the liver value of the estrous phase were determined by the expression $2^{-\Delta\Delta CT}$. The relative expression level of *LHR* in variant tissues is shown as the mean of three measurements and analyzed by one-way ANOVA. $P \leq 0.01$ is denoted with asterisks or different capital letters above each bar in one tissue. Bars marked with the same letters are not statistically different.

Correlation of *LHR* mRNA expression levels among four estrous stages in different non-gonadal tissues of Hu sheep

For understanding the correlation of *LHR* mRNA expression levels changing trends, the values at 4 estrous stages in non-gonadal tissues were analyzed using two-tailed Pearson's correlation (Table 2). The results showed a highly significant correlation with *LHR* mRNA expression between hypophysis and oviduct, hypothalamus and rectum, olfactory bulb and jejunum, olfactory bulb and kidney, jejunum and kidney, ileum and uterus ($P < 0.01$), and there were also significant correlations between hypophysis and duodenum, oviduct and duodenum, jejunum and uterus, kidney and uterus, hypothalamus and medulla oblongata, omasum and abomasums, and reticulum and colon ($P < 0.05$).

Table 2. Results of correlation analysis of *LHR* mRNA expression levels among four estrous stages in different tissues of Hu sheep.

	Hypophysis	Medulla oblongata	Olfactory bulb	Hypothalamus	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	Pancreas	Liver	Kidney	Oviduct	Uterus
Hypophysis																			
Pearson's correlation																			
Sig. (2-tailed)																			
N																			
Medulla oblongata																			
Pearson's correlation	-0.628																		
Sig. (2-tailed)	0.372																		
N	4																		
Olfactory bulb																			
Pearson's correlation	0.947	-0.481																	
Sig. (2-tailed)	0.053	0.519																	
N	4	4																	
Hypothalamus																			
Pearson's correlation	-0.402	0.956*	-0.289																
Sig. (2-tailed)	0.598	0.044	0.711																
N	4	4	4																
Rumen																			
Pearson's correlation	0.659	0.153	0.785	0.365															
Sig. (2-tailed)	0.341	0.847	0.215	0.635															
N	4	4	4	4															
Reticulum																			
Pearson's correlation	0.192	-0.637	-0.121	-0.573	-0.495														
Sig. (2-tailed)	0.808	0.363	0.879	0.427	0.505														
N	4	4	4	4	4														
Omasum																			
Pearson's correlation	0.189	0.484	0.121	0.712	0.572	-0.041													
Sig. (2-tailed)	0.811	0.516	0.879	0.288	0.428	0.959													
N	4	4	4	4	4	4													
Abomasum																			
Pearson's correlation	0.408	0.356	0.370	0.614	0.752	-0.108	0.966*												
Sig. (2-tailed)	0.592	0.644	0.630	0.386	0.248	0.892	0.034												
N	4	4	4	4	4	4	4												
Duodenum																			
Pearson's correlation	0.988*	-0.525	0.929	-0.273	0.724	0.172	0.336	0.541											
Sig. (2-tailed)	0.012	0.475	0.071	0.727	0.276	0.828	0.664	0.459											
N	4	4	4	4	4	4	4	4											
Jejunum																			
Pearson's correlation	0.919	-0.394	0.995**	-0.202	0.838	-0.207	0.167	0.415	0.910										
Sig. (2-tailed)	0.081	0.606	0.005	0.798	0.162	0.793	0.833	0.585	0.090										
N	4	4	4	4	4	4	4	4	4										
Ileum																			
Pearson's correlation	0.865	-0.158	0.915	0.088	0.945	-0.220	0.498	0.705	0.909	0.938									
Sig. (2-tailed)	0.135	0.842	0.085	0.912	0.055	0.780	0.502	0.295	0.091	0.062									
N	4	4	4	4	4	4	4	4	4	4									
Cecum																			
Pearson's correlation	0.407	-0.354	0.109	-0.147	0.002	0.816	0.525	0.486	0.466	0.059	0.217								
Sig. (2-tailed)	0.593	0.646	0.891	0.853	0.998	0.184	0.475	0.514	0.534	0.941	0.783								
N	4	4	4	4	4	4	4	4	4	4	4								
Colon																			
Pearson's correlation	0.043	-0.532	-0.271	-0.493	-0.589	0.988*	-0.035	-0.140	0.030	-0.353	-0.345	0.787							
Sig. (2-tailed)	0.957	0.468	0.729	0.507	0.411	0.012	0.965	0.860	0.970	0.647	0.655	0.213							
N	4	4	4	4	4	4	4	4	4	4	4	4							
Rectum																			
Pearson's correlation	-0.293	0.916	-0.189	0.993**	0.458	-0.554	0.778	0.700	-0.158	-0.102	0.198	-0.080	-0.490						
Sig. (2-tailed)	0.707	0.084	0.811	0.007	0.542	0.446	0.222	0.300	0.842	0.898	0.802	0.920	0.510						
N	4	4	4	4	4	4	4	4	4	4	4	4	4						

Continued on next page

Table 2. Continued.

	Hypophysis	Medulla oblongata	Olfactory bulb	Hypothalamus	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	Pancreas	Liver	Kidney	Oviduct	Uterus
Pancreas																			
Pearson's correlation	0.143	0.680	0.282	0.837	0.815	-0.631	0.792	0.836	0.264	0.366	0.614	-0.069	-0.637	0.888					
Sig. (2-tailed)	0.857	0.320	0.718	0.163	0.185	0.369	0.208	0.164	0.736	0.634	0.386	0.931	0.363	0.112					
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4					
Liver																			
Pearson's correlation	-0.079	-0.244	-0.393	-0.176	-0.503	0.902	0.250	0.099	-0.043	-0.452	-0.329	0.851	0.943	-0.171	-0.387				
Sig. (2-tailed)	0.921	0.756	0.607	0.824	0.497	0.098	0.750	0.901	0.957	0.548	0.671	0.149	0.057	0.829	0.613				
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4				
Kidney																			
Pearson's correlation	0.947	-0.438	0.998**	-0.234	0.819	-0.132	0.185	0.430	0.939	0.997**	0.939	0.135	-0.279	-0.131	0.336	-0.380			
Sig. (2-tailed)	0.053	0.562	0.002	0.766	0.181	0.868	0.815	0.570	0.061	0.003	0.061	0.865	0.721	0.869	0.664	0.620			
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4			
Oviduct																			
Pearson's correlation	0.996**	-0.674	0.917	-0.448	0.599	0.277	0.177	0.386	0.982*	0.882	0.826	0.468	0.129	-0.340	0.081	0.001	0.916		
Sig. (2-tailed)	0.004	0.326	0.083	0.552	0.401	0.723	0.823	0.614	0.018	0.118	0.174	0.532	0.871	0.660	0.919	0.999	0.084		
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		
Uterus																			
Pearson's correlation	0.887	-0.213	0.944	0.023	0.932	-0.222	0.427	0.646	0.919	0.963*	0.997**	0.181	-0.352	0.132	0.565	-0.363	0.963	0.847	
Sig. (2-tailed)	0.113	0.787	0.056	0.977	0.068	0.778	0.573	0.354	0.081	0.037	0.003	0.819	0.648	0.868	0.435	0.637	0.037	0.153	
N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

Measurement of tissue LHR protein expression levels by ELISA

An ELISA was developed to verify LHR protein expression in different tissues of Hu sheep. The correlation between LHR protein concentration and mRNA expression level in various tissues was analyzed using two-tailed Pearson's correlation. The results showed that the *LHR* mRNA expression levels of tissues were all significantly correlated with protein concentration ($P < 0.01$) in the 4 estrous stages (Figure 6).

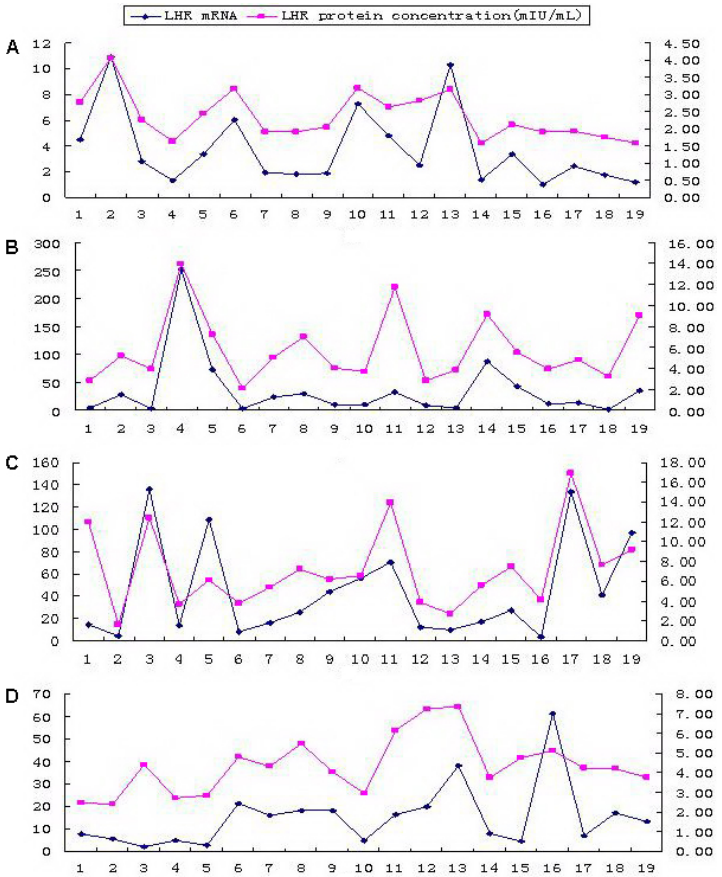

Figure 6. *LHR* mRNA expression levels (qRT-PCR) and LHR protein concentration (ELISA) in Hu sheep tissues during (**A**) estrous (day 0), (**B**) metestrous (day 3), (**C**) diestrous (day 10), and (**D**) proestrous (day 14) phases. The correlations between the data from real-time PCR and ELISA were analyzed by two-tailed Pearson's correlation. The correlation value of estrus (A) was 0.908, metestrus (B) was 0.752, diestrus (C) was 0.726, and proestrus (D) was 0.571, which showed that *LHR* mRNA expression levels were all significantly correlated with protein concentration ($P < 0.01$).

DISCUSSION

The mammalian estrous cycle is divided into 4 phases: proestrus, estrus, metestrus, and diestrus, or 2 phases: follicular (proestrus and estrus) and luteal (metestrus and diestrus),

according to the different morphological types of ovary (follicular or luteal formation) caused by gonadotropic hormones. LH is one of the important glycoprotein hormones that participates in regulating physiological status change in mammals and its expression changes during the estrous cycle. The activity of LH is mediated by the *LHR*, and therefore the expression levels of *LHR* can indicate the intensity of LH function in one organ. To the best of our knowledge, this is the first study to evaluate *LHR* expression levels in tissues from whole Hu sheep (ruminant) and to clarify trends in *LHR* expression level changes in non-gonadal organs in sheep during the estrous cycle.

Previous studies on *LHR* in cattle (Sun et al., 1997; Shemesh et al. 2001), pig (Ziecik, 1986; Gawronska, 1999; Wasowicz and Ziecik, 2000), goat (Shen et al., 2009), mouse (Zhang et al., 2001), rabbit, and rat (Sawitzke and Odell, 1991) showed that *LHR* is expressed in a wide variety of non-gonadal tissues and organs, such as oviduct, cervix, urinary bladder, placenta, fetal membranes, umbilical cord, brain, pineal gland, spinal cord, pituitary gland, breast, skin, adrenal gland, blood vessels in target tissues, cells of the immune system, bone, heart, stomach, spleen, kidney, muscle, liver, and intestine (Rao and Lei, 2007; Ziecik et al., 2007; Vischer and Bogerd, 2003; Kwok et al., 2005; Chen et al., 2010). In the present study, *LHR* mRNA and protein were detected in non-gonadal tissues from Hu sheep using real-time quantitative PCR and ELISA. The result demonstrated that *LHR* participates in the functions of these non-gonadal organs. The significant correlation between *LHR* mRNA expression levels and protein concentrations ($P < 0.01$) implies that *LHR* mRNA level could be taken as an index of LHR protein concentration in tissues. *LHR* expression was prominent in certain tissues at various times among the 4 estrous stages, indicating the intensity of LH function intensity in these tissue changes with specific physiological situations.

This research first revealed the correlation of change trends of *LHR* expression levels among different non-gonadal tissues at 4 estrous stages using two-tailed Pearson's correlation. In animal reproductive activities, the hypothalamus synthesizes and secretes certain neurohormones, such as GnRH, which in turn stimulate or inhibit the secretion of pituitary hormones, such as LH and FSH (follicle-stimulating hormone), and then collaboratively controls follicular development and the estrous cycle. In the present study, *LHR* expression levels in the hypothalamus and hypophysis were significantly different over the 4 estrous phases, but the change trends were not similar. *LHR* expression peaked at metestrus and then declined until the estrous stage, while in hypophysis, *LHR* expression peaked at diestrus. The trend in the expression changes in hypothalamus was analyzed and was significantly correlation with that in rectum and medulla oblongata.

The oviducts are organs that support gamete transport, maturation, fertilization, early embryonic growth, and development as well as their timely transport for implantation in the uterus. Previous studies on *LHR* expression in other animals showed that the most noticeable immunostaining was seen in the mucosal epithelium, and staining of the epithelium of the ampulla was more intense for *LHR* than in the epithelium of the isthmus. Staining of the myosalpinx was less intense than that of the mucosal layer. *In vitro*, LH modulates spontaneous contraction of the porcine oviduct, causing its relaxation, particularly during the preovulatory stage of the estrous cycle (Gawronska et al., 1999). The *LHR* transcript was detected in pig oviduct during the early luteal and follicular phase of the estrous cycle (Gawronska et al., 2000). In women, tubal-receptor concentrations vary during the menstrual cycle, the Fallopian tubes contain more *LHR* during the secretary phase than during the proliferative phase (Lei et

al., 1993). *LHR* mRNA expression levels in the oviduct of Jining gray goat and Yimeng black goat in the 4 estrous cycle phases were detected differently, which were the highest in the infundibulum during estrus, and in the ampulla and isthmus during proestrus (Li et al., 2009; Wang et al., 2011). Similar to previous studies, the present research showed that *LHR* expression levels in Hu sheep oviduct (isthmus) were different during all 4 phases of the estrous cycle, abundantly higher at the proestrous stage and relatively lower at estrus and metestrus, but *LHR* expression was the highest at diestrus. The changes in receptor quantity indicate that LH might directly regulate tubal function.

LHR expression in the myometrium is generally higher during the progesterone-dominated instead of the estrogen-dominated phase of the cycle and is also active in pregnancy in humans and pigs and in the postpartum and postmenopausal periods in women (Ziecik et al., 1986; Reshef et al., 1990; Zuo et al., 1994). The highest concentration of LH receptors in the bovine endometrial tissue was observed on days 15-17 of the estrous cycle and declined afterwards to a nadir (Shemesh et al., 2001). Higher concentrations of LH/hCG receptors during the luteal phase (Ziecik et al., 1995), and the expression level of *LHR* mRNA in the uterus decreased on days 0 and 4, and increased on day 12 in guinea pigs (Jiang et al., 2011). *LHR* mRNA expression was different in the uterus of Jining gray goat during the 4 estrous cycle phases, being lowest in estrus, highest in diestrus, and intermediate in proestrus (Li et al., 2009). In the present study, *LHR* was detected in the uterus of Hu sheep at its highest level at the diestrous stage, and generally increasing from estrus and declining after the diestrous stage, indicating that *LHR* was expressed more in the luteal phase than in the follicular phase. This result is consistent with the previous finding in goat (Shen et al., 2009), pig (Rzucidlo et al., 1998), cattle (Shemesh et al., 2001), and guinea pigs (Jiang et al., 2011). The physiological significance of expression of *LHR* is suggested because LH induces COX-2 and increases prostaglandin release in the uterine vein. The maximal stimulation of the receptor and its mRNA at proestrus-estrus may serve to increase the amount of prostanoids reaching the regressing corpus luteum either directly by increasing prostanoid production or indirectly by increasing the blood flow to ovary (Shemesh et al., 1997).

In digestive tissues, significant differences in *LHR* expression levels were detected at the 4 estrous phases. The Hu sheep is a ruminant with four stomachs: rumen, reticulum, omasum, and abomasum. *LHR* expression levels detected in rumen were relatively higher in the luteal phase (metestrous and diestrous stages) than in the follicular phase (estrous and proestrous stages), while in the omasum and abomasum *LHR* expression was abundant except at estrous stage, and the *LHR* expression change trends were significantly correlated between omasum and abomasum. In the intestines, *LHR* expression throughout the estrous cycle in the small intestine (duodenum, jejunum and ileum) was similar, all at the highest level at diestrous stage and with levels increasing from estrus and declining after the diestrous phase. After analysis with two-tailed Pearson correlation, the result showed that *LHR* expression change trends were not significantly correlated among small intestine tissues, but there was significant correlation among duodenum, oviduct, and hypophysis, among jejunum, olfactory bulb, kidney, and uterus, and between ileum and uterus. In the large intestine (cecum, colon and rectum), trends in *LHR* expression changes were not similar. *LHR* expression levels were the highest at proestrous stage in the cecum and colon tissues and at metestrous stage in the rectum. The trend in change of *LHR* expression levels in the colon was significantly correlated with that in reticulum, and expression levels in the rectum were strongly significantly correlated with that in the hypothalamus.

Overall, in estrus, under regulation by gonadotropic hormones, ovarian follicles are maturing and estrogen secretions exert their largest influence. At this time, the female exhibits sexual behavior signaled by visible physiological changes. In this study, *LHR* gene expression levels were significantly lower in a number of non-gonadal tissues at the estrous stage (day 0). The relatively lower LH's function intensity may have influenced the function of digestive system, because appetite, feed intake, and the number of ruminates of Hu sheep were decreased at estrous stage. In metestrus, the signs of estrogen stimulation subside and the corpus luteum starts to form, and in this period, *LHR* expression levels were increased in several tissues, including medulla oblongata, hypothalamus, ruminant stomach (except reticulum), intestine tissues (except colon), pancreas, liver, kidney, and uterus. This indicated that a number of non-gonadal tissue functions increased or recovered gradually with decreasing concentration of estrogen or with corpus luteum formation. When the corpus luteum is formed, the estrous phase of animal enters the diestrous phase, and in this period the corpus luteum actively produces progesterone. The highest relative *LHR* expression levels were detected in non-gonadal tissues, such as the hypophysis, olfactory bulb, rumen, small intestine, kidney, oviduct, and uterus. In proestrous phase, one or several follicles of the ovary start to grow under the influence of FSH, LH, and estrogen. *LHR* expression levels were declining at this point except in few tissues. The result indicated that *LHR* expression or LH function intensity in a number of non-gonadal tissues may be positively correlated with progesterone concentration or negatively correlated with estrogen concentration. Whether the functions of these tissues are influenced with reproductive hormones needs to be elucidated in a further study, and the regulatory mechanism of *LHR* expression in different tissues also remains to be investigated, which will mainly focus on histological study.

CONCLUSION

This study demonstrates that *LHR* mRNA expression level is significantly correlated with protein concentration in the tissues of Hu sheep, and *LHR* expression levels are significantly different during phases of the estrous cycle. The expression change trends in some non-gonadal tissues are significantly correlated with those in the hypothalamus, hypophysis and oviduct, or uterus. These results indicate that *LHR* is an important adjustment factor for the function of several ovine non-gonadal tissues and its function intensity depends on the hormonal status during the phases of the estrous cycle.

ACKNOWLEDGMENTS

Research supported by the National Mutton Sheep Industry Technology System in China (#CARS-39) and the Natural Science Foundation of Jiangsu Province in China (#BK2010356).

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