Differential expression of Toll-like receptors in goat dominant and nondominant follicles

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ABSTRACT. The mechanism of dominant follicle selection is unclear because of its physiological complexity. However, some studies have reported that the immune system plays an important role in reproductive physiology. The objective of the current study was to investigate the differential expression of Toll-like receptors (*TLRs*) in the dominant (DFs) and nondominant follicles (NFs), and to determine the correlation between the expression of *TLRs* and the related genes, such as *WNT4* and *FOXL2*. In this comparative study, the expression

levels of *TLR*s, *WNT4*, and *FOXL2* genes of DFs and NFs were obtained from three Dazu black goats were estimated using the real-time PCR. Our results showed no significant difference in the expression of seven *TLRs* (excluding *TLR2*, *TLR5*, and *TLR8*), *WNT4*, and *FOXL2* between the DFs and NFs. In addition, the mRNA expression levels of *WNT4* significantly correlated with the relative expression of *TLR6* (r = 0.949739, $P < 0.01$); however, no significant expression of the *TLR* genes was found to be associated with *FOXL2* mRNA expression. Our results support the fact that *TLRs* are not involved in the process of dominant follicle selection; however, *TLR6* might play a role in the development of follicles by interacting with *WNT4*.

Key words: Toll-like receptors; Goat; Fecundity; *WNT4*; *FOXL2*; Dominant follicle selection

INTRODUCTION

Toll-like receptors (TLRs) play an important role in pathogen recognition through the broadly specific innate immune system (Brubaker et al., 2015). In addition, their functional mechanism in immune system mainly depends on some specific genes such as *MyD88*, *CD40*, and *NF-kB* (Raja et al., 2011). A previous study has reported the presence of ten family members of TLRs in several mammals, including goat (Raja et al., 2011). However, to date, the TLRs have been thought not only to be the key mediators of the innate immune system, but also important players in the female reproduction, and their expression levels vary in different phases of the menstrual cycle (Aflatoonian et al., 2007; Aflatoonian and Fazeli, 2008; Zandieh et al., 2016).

Mammalian follicular development is a complex process, including the recruitment of the primitive follicles, development of preantral follicles, and selection, growth and maturation of antral follicle or follicle atresia. Follicle selection is a key step to determine the number of ovulated oocytes, and oocyte growth is an important index for dominant follicle selection. Some studies have reported the absence of immune cells (Bromfield and Sheldon, 2011) as well as some TLR genes, such as *TLR4* (Hoshino et al., 1999), in the ovarian follicles. However, ovulation itself is regarded as a sterile inflammatory process involving the innate immune system (Spanel-Borowski, 2011) and some TLRs, such as TLR2 and TLR4, play a role in sperm capacitation and oocyte fertilization (Shimada et al., 2008). Until now, the mechanism of follicle selection is unclear due to its physiological complexity, and it is also not clear whether the TLRs could impact this process during follicle development.

Furthermore, the ovarian development requires the female-specific functions mediated by the WNT4 (wingless-related MMTV integration site 4), and FOXL2 (forkhead box L2) proteins, which establish fetal granulosa cells and regulate folliculogenesis (Tomizuka et al., 2008; Hirano et al., 2016). Another study attributed the putative mechanism of FOXL2-mediated ovarian development to the regulation of ERβ-dependent gene expression in the early-phase follicular development (Hirano et al., 2016). In addition, *WNT4* repression leads to the premature activation of follicles prior to formation of testis-like cords (Maatouk et al., 2013). Therefore, these two genes, *WNT4*, and *FOXL2*, play an important role in the development of the follicle. Thus, it is critical to understand the mechanism of follicle selection by studying the involvement and regulation of TLRs, WNT4, and FOXL2 in goat follicular selection.

MATERIAL AND METHODS

Animals

Experimental procedures used in this study were approved by the Committee on the Ethics of Animal Experiments of the Southwest University [No. (2007) 3] and the Animal Protection Law of China. Ovaries of three 1-1.2-year-old Dazu black goats were collected at Southwest University in China. Their follicles were stripped from ovaries in 37°C saline immediately after removal. The follicles of 3 female individuals were divided into dominant (diameter > 5 mm) and nondominant follicles (3 mm < diameter < 5 mm) using the standard protocol (Medan et al., 2005).

Methods and data analysis

Total cellular RNA was extracted from the isolated follicles using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer's protocol. The RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA was synthesized by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The samples used in the quantitative real-time PCR (Q-PCR) analyses were the same as those used in the sequencing experiments. The primers of the candidate genes are shown in Table 1. The Q-PCR was performed in triplicate in a 20-μL reaction volume comprising 10 μL 2X SYBR® Select Master Mix (Life Technologies, Carslbad, CA, USA), 6.4 μL H_2O, 0.8 μL each forward and reverse primer (10 pmol/μL), and 2 μL cDNA (~16 ng). The reaction was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The cycle threshold (Ct) values were normalized to the control gene (GAPDH). The relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Each experiment was independently repeated three times, and each sample was evaluated in triplicate. Using the *t*-test in the SPSS18 software, a P value less than 0.05 was considered significant. The assessment of genes was correlated using *r*, with the following statistical formula:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2}} \quad \text{(Equation 1)}$$

where x_i and y_i are the corresponding values of the data of two groups, respectively, and the $\bar{x}$ and $\bar{y}$ are the mean values of the data of two groups. The correlation coefficient (r) was calculated to determine the correlation between the two variables and their relative directions.

RESULTS

The quality of Q-PCR was determined by agarose gel electrophoresis as shown in Figure 1. A high PCR-effect and appropriate fragment length of the amplicon of each candidate gene, except *TLR2*, *TLR5*, and *TLR8*, were observed on the agarose gel. Therefore, we excluded these three genes from our study. The relative mRNA expression of the TLR genes, measured by Q-PCR, are shown in Figure 2. The results showed no significant difference in the relative mRNA expression of *TLRs*, *WNT4*, and *FOXL2* between dominant and nondominant follicles.

Table 1. Information of Q-PCR primer.

Candidate genes	Primer	Primer sequences	SZ or RF	Tm (°C)
FOXL2	FOXL2-F	CCGGCATCTACCAGTACATTATAGC	Boulanger et al. (2014)	60
	FOXL2-R	GCACTCGTTGAGGCTGAGGT		
WNT4	WNT4-F	CTCCCTCACGAAGACAAGGT	151	60
	WNT4-R	CATCCACATCAGAGCTTGCC		
TLR1	TLR1-F	CTG CCCATATGCCAAGAG TT	159	60
	TLR1-R	GGCATCTTCTCTTTCCCCAT		
TLR2	TLR2-F	CTGTGTGCGTCTTCCTCAGA	228	62
	TLR2-R	TCAGGGAGCAGAGTAACCAGA		
TLR3	TLR3-F	TCTTTTCGGGACTGTTGA CC	224	62
	TLR3-R	AAATCCCCCATCCAAGGTAG		
TLR4	TLR4-F	GGTTTCCACAAAAGCCGTAA	137	62
	TLR4-R	AGGACGATGAAGATGATGCC		
TLR5	TLR5-F	TCAATGGGA GCCAGATTTTC	198	62
	TLR5-R	CCTTCAGCTCCTGGAGTGTC		
TLR6	TLR6-F	CGACATTGAAGGCACTGAAA	148	62
	TLR6-R	TCCTGAGGACAAAGCATGTG		
TLR7	TLR7-F	TCTCCAAGGTGCTTTCCAGT	166	62
	TLR7-R	CCACCAGACAAACCACACAG		
TLR8	TLR8-F	TCACACGGGTAACGAATGAA	143	62
	TLR8-R	TTTGAGGTTGAGAAATGCCC		
TLR9	TLR9-F	CTCTCCTTGGACTGCTTTGG	204	62
	TLR9-R	CACTGCACTCTGCACCTTGT		
TLR10	TLR10-F	TCACCTGACATCTTTGCGAG	187	60
	TLR10-R	TCGGAATGGATTTCTTCCTG		
GAPDH	GAPDH-F	CGACTTCAACAGCGACACTCAC	119	60
	GAPDH-R	CCCTGTTGCTGTAGCCGAATTC		

SZ is the size (bp) of candidate gene; RF is the reference for the primer pair; F is the forward primer; and R is the reverse primer.

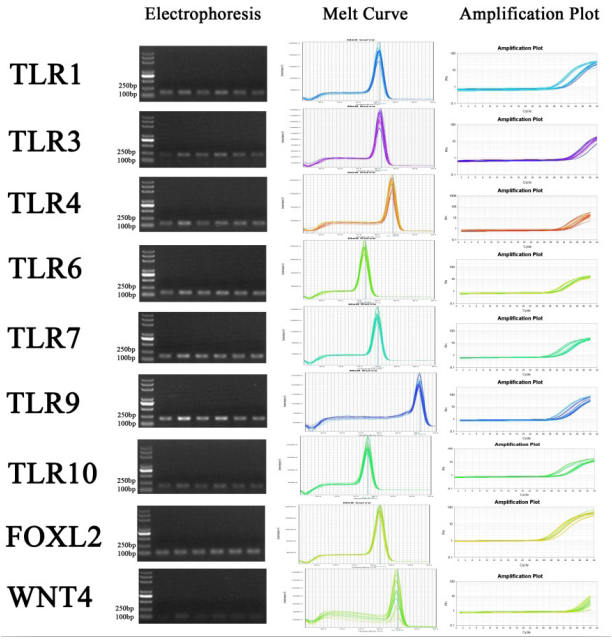

Figure 1. Relative mRNA expression patterns of TLRs, WNT4, and FOXL2 in dominant and nondominant follicles in goat using Q-PCR.

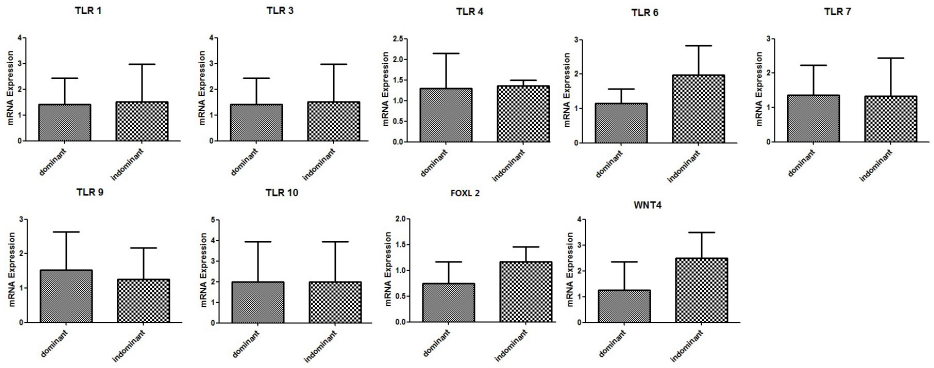

Figure 2. Real-time Q-PCR validation of the expression of TLRs, WNT4, and FOXL2 in dominant and nondominant goat follicles. The abundance of target genes was normalized relative to the abundance of the GAPDH gene. Bars in each panel represent the mean ± standard error (sample number = 3, and 3 parallel repetitions per sample).

Correlation between mRNA expressions of the TLR genes with those of the *WNT4* and *FOXL2* genes are shown in Table 2. Among TLRs, a non-significant negative correlation was observed between *TLR3* and *TLR7* (r = -0.00375), between *TLR3* and *TLR9* (r = -0.03518). The relative mRNA expression level of the *TLR1* gene was significantly correlated with those of *TLR7* (r = 0.992134, P < 0.01) and *TLR9* (r = 0.931833, P = 0.01). The relative mRNA expression level of *TLR10* was positively associated with those of *TLR1* (r = 0.976384, P < 0.01), *TLR7* (r = 0.990495, P < 0.01), and *TLR9* (r = 0.981517, P < 0.01). In addition, the relative mRNA expression level of *TLR7* was positively associated with that of *TLR9* (r = 0.964066, P < 0.01). The correlation analysis between the relative mRNA expression of TLR genes and *WNT4* and *FOXL2* genes showed that the high levels of the relative mRNA expression of the *WNT4* gene were correlated with those of the *FOXL2* (r = 0.885591, P = 0.02) and *TLR6* (r = 0.949739, P < 0.01). However, the mRNA expression of no TLR gene was significantly associated with that of the *FOXL2* gene.

Table 2. Correlation analysis between TLRs and WNT4 and FOXL2.

	FoxL2	TLR10	TLR1	TLR3	TLR4	TLR6	TLR7	TLR9	WNT4
FOXL2	1								
TLR10	+0.491528	1							
TLR1	+0.51741	+0.976384**	1						
TLR3	+0.164768	+0.046931	-0.02672	1					
TLR4	+0.489107	+0.638114	+0.504745	+0.009249	1				
TLR6	+0.830143	+0.635346	+0.630347	+0.597080	+0.410306	1			
TLR7	+0.505915	+0.990495**	+0.992134**	-0.00375	+0.548934	+0.630456	1		
TLR9	+0.411253	+0.981517**	+0.931833**	-0.03518	+0.691826	+0.527048	+0.964066**	1	
WNT4	+0.885591*	+0.701023	+0.671865	+0.440016	+0.650055	+0.949739*	+0.6731	+0.617487	1

The absolute value of the correlation coefficient closer to 1 indicates greater correlation, and the value closer to 0 indicates lower correlation; "+" indicates positive correlation between genes, and "-" indicates negative correlation. *P < 0.05, and **P < 0.01 using the *t*-test.

DISCUSSION

Consistent with the recent reports of TLR expression in humans, high levels of the mRNA expression of *TLR1*, *TLR3*, *TLR4*, *TLR6*, *TLR7*, *TLR9*, and *TLR10* genes were found

in follicles (Taghavi et al., 2014). Furthermore, this finding is in agreement with the study of Bromfield and Sheldon (2011), which showed that the TLRs involved in the development of other female reproductive tissues, such as bovine granulosa cells, initiate an innate immune response to lipopolysaccharide (LPS) via the TLR4 pathway, leading to the inflammation and perturbation of meiotic competence. The expressions of *TLR2* and *TLR4* genes were upregulated in response to LPS in the uterine horn and uterine body, as well as *TLR4* expressed in the ovary of rabbit (Chen et al., 2014). The abnormal expression of TLRs might adversely affect the oocyte quality and fertility rate, and it might also lead to the lower embryo quality in women with polycystic ovary syndrome (PCOS) (Gu et al., 2016). However, in this study, no significant differences in mRNA expression of the TLR genes were observed between the dominant (DFs) and nondominant follicles (NFs). To date, differential expression of the TLRs related to the dominant follicular selection process has not been reported in vertebrates. On one hand, our results suggest that the TLRs do not play a key role in this biological process. On the other hand, the widespread expression of TLRs in different developmental stages of follicles was consistent with the fact that the autoimmune system plays a critical role in the reproductive functioning in both males and females (Sominsky et al., 2013).

Originally, the *FOXL2* gene was considered to play a role in the sex determination and generation of sex-reversal phenotype in goat (Auguste et al., 2011; Boulanger et al., 2014). In addition, previous studies have shown that a somatic missense mutation in the *FOXL2* gene, which is expressed in the ovary, have a high correlation to granulosa cell tumors (Schmidt et al., 2004; Gustin et al., 2016). The *FOXL2* mutant has also been found to affect the hormone production, apoptosis, and proliferation *in vitro* (Leung et al., 2016). However, more evidence is required to support the fact that *FOXL2* is involved in some pathway related to the regulation of follicle development (Georges et al., 2013; Jin et al., 2016). Here, the high mRNA expression levels of the TLR genes in the DFs and NFs are consistent with the above findings. Furthermore, no significant difference in the expression of TLR genes was observed between these two developmental stages of follicles, and the correlation between TLRs and *FOXL2* indicated no interaction between them to regulate the process of dominant follicular selection.

Previous studies have demonstrated that the *WNT4* gene was expressed in the ovary and is involved in gonadal development (Meng et al., 2015). It is an irreplaceable signal gene for the development of the ovary; the expression of a *WNT4* knock-out gene in gonads led to the sexually dimorphic functions (Naillat et al., 2015). Thus, owing to the high levels of *WNT4* mRNA expression in the DFs and NFs, and no significant difference between the follicles of the two groups, WNT4 could be considered an important factor involved in follicular cell development (Wu et al., 2015), but not in the process of dominant follicle selection.

According to the classification based on the cellular location of TLRs, the TLR6 is located within the cell membrane (Taghavi et al., 2014). At the same time, high levels of the *TLR6* mRNA expression have been found on the surface epithelium of human ovaries (Zhou et al., 2009). Besides, the *TLR6* gene expression was significantly higher in patients with poor ovarian response in comparison to the normal women (Taghavi et al., 2014). Interestingly, the mRNA expression levels of *TLR6* positively associated with those of the *WNT4* gene. It could be inferred that the interaction between TLR6 and WNT4 proteins is involved in the normal physiological development of follicles.

The aim of this study was to discover the differential expression of the TLRs in the DFs and NFs, and to investigate the correlation between TLRs and *WNT4* and *FOXL2* using Q-PCR. Our results showed no significant difference in the relative expression of TLRs,

WNT4, and FOXL2 between the DFs and NFs, indicating that the TLRs were not involved in the dominant selection process. In addition, a significant correlation was observed in the relative mRNA expression levels of *WNT4* and *TLR6* ($r = 0.949739$, $P < 0.01$), indicating that *TLR6* might play a role in the development of follicles by interacting with *WNT4*. We acknowledge that our study design was not optimal because of the small sample size and a specific breed of animals. However, despite these limitations, our results have enhanced our understanding of the regulation of TLRs, *WNT4* and *FOXL2* in goat follicular selection.

Conflicts of interest

The authors declare no conflict of interest.

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