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Expression profiles of differentially expressed genes affecting fecundity in goat ovarian tissues

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ABSTRACT. Although RNA-Seq is an effective method for identifying and exploring novel functional genes in mammals, it has rarely been applied to study fertility-related genes in the goat. In this study, RNA-Seq was used to screen the estrus ovaries of uniparous and multiparous Anhui white goats (AWGs). In total, 15,890 genes were identified and 2201 of these were found to be differentially expressed between the genetic libraries from uniparous and multiparous goats. Compared to the uniparous library, 1583 genes were up-regulated and 618 genes were down-regulated in the multiparous library. The *FER1L4* gene showed the level of highest up-regulation in the multiparous library, while *SRD5A2* expression showed the greatest down-regulation.

In order to determine the functions of *FER1L4* and *SRD5A2* in goats, the expression profiles of the two genes in different tissues from AWGs and Boer goats at diestrus were analyzed by quantitative PCR. *FER1L4* and *SRD5A2* showed tissue specific expression patterns and were highly expressed in ovaries from both AWGs and Boer goats. *FER1L4* was more highly expressed in ovaries from multiparous than uniparous AWGs. In contrast, *SRD5A2* was expressed at a lower level in multiparous AWGs. These results indicated that *FER1L4* and *SRD5A2* may be associated with the high fecundity of AWGs.

Key words: Goat; Ovary; Fecundity; Differentially expressed genes; Tissue expression profile

INTRODUCTION

Litter size is one of the most important economic traits in goat production and varies among individuals and breeds. Fecundity has a direct impact on goat production efficiency and is a result of interactions among multiple genes and the environment. There has been some progress in characterizing the major genes involved in animal fecundity, such as identification of *BMPR-IB* (Mulsant et al., 2001; Davis et al., 2002), *BMP15* (Galloway et al., 2000; Zhang et al., 2009), *GDF9* (Hanrahan et al., 2004), and *PRLR* (Kmieć et al., 2006). However, information on the identity and roles of fertility-related genes is still relatively limited in the goat. In general, litter sizes are low in goats with one or two kids being normal (Sun et al., 2010). The increasing demand for goat meat has contributed to the rapid development of the goat industry in recent years, and improvements to breeding methods have become increasingly important.

The Anhui white goat (AWG) shows precocious puberty compared to other goat breeds, has relatively high fertility, and the skin can be used to produce high quality leather goods. AWG ewes are fertile all year round and can produce kids twice in one year or three times in two years. The AWG breed produces a relatively high number of kids, with average production of 2.3 lambs per litter. This breed is therefore an ideal model for studying goat breeding traits. Here, we have concentrated on the ovary as this has a very significant influence on the kidding rate.

The RNA-Seq method is now widely used to investigate the transcriptome as it allows both mapping and quantification of expressed sequences. For example, it has been used to detect differentially expressed genes in mice (Mortazavi et al., 2008), cattle (Huang and Khatib, 2010), humans (Hackett et al., 2012), and pigs (Gunawan et al., 2013). However, relatively little study has been made of fecundity-related genes in goat. In this study, we screened for differentially expressed genes between ovaries from uniparous and multiparous AWGs. This analysis identified *FER1L4* and *SRD5A2* as the two genes showing the highest level of differential expression. The expression profiles of these genes were compared in different tissues from AWGs in estrus and Boer goats in diestrus by real-time PCR to investigate their function. This study provides a molecular basis for new breeding approaches in this commercially important animal species.

MATERIAL AND METHODS

Experimental materials

The AWGs and Boer goats used in this study were obtained from Boda Livestock

Technology Development Co., Ltd. of Hefei (Anhui, China). Six goats in estrus, including three uniparous and three multiparous AWGs, and three Boer goats in diestrus were selected randomly based on their breeding records in the previous five years. All experimental animals were of similar age and appearance. The animals were housed under the same conditions and fed the same diets until slaughter. After slaughter, the ovaries, heart, live, spleen , lung, kidney, uterus, oviduct, muscle (Longissimus), and intestine were obtained and immediately snap-frozen in liquid nitrogen, and stored at -80°C until total RNA extraction. All the experimental procedures with AWGs in the present study were given prior approval by the ethics committee of Anhui Agricultural University, Anhui, China, under permit No. AHAU20101025.

Main reagents

We used the following kits: RNAiso Plus tissue extraction kit (Takara, Japan); Prime Script™ RT reagent kit with gDNA Eraser for reverse transcription: (Takara); FastStart Universal SYBR Green Master kit (ROX) for quantitative fluorescence analysis: (Roche, USA). We also used chloroform, isopropanol, and absolute ethyl alcohol from Wuxi Zhanwang Chemical Reagent Co. Ltd, China.

Library preparation and sequencing

Ovaries of three groups of goats, namely uniparous AWG, multiparous AWG, and Boer goat, were each ground to powder in liquid nitrogen. Total RNA was extracted from each sample using TRIzol (Takara) according to the manufacturer instruction. Equimolar quantities of RNA from each sample were combined into one pool.

Total RNAs from all samples was pooled prior to library preparation. The mRNA was first extracted from the total RNA using oligo (dT) magnetic beads and sheared into short fragments of about 200 bp. These fragmented mRNAs were then used as templates for cDNA synthesis. The cDNAs were PCR amplified to complete the library. An Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used to analyze the sample library. The cDNA library was sequenced using an Illumina HiSeq™ 2000 platform.

Sequence analysis

The quality of the raw sequence reads was checked and contaminating reads were removed, for example, low quality reads, adaptor reads, reads with 5' primer contaminants, reads without a 3' primer, reads without the insert tag, reads with poly(A), and reads shorter than 18 nt. The clean reads were mapped to goat reference gene sequences (http://goat.kiz.ac.cn/GGD/download.htm) using SOAPaligner/SOAP2 (Li et al., 2009), allowing up to two base mismatches. The gene expression level was then calculated using the Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al., 2008). The statistical significance of the differential expression of each gene was determined using the P-value (Benjamini et al., 2001). The FDR (false discovery rate) ≤ 0.001 and |log2Ratio| ≥ 1 were used as the threshold to identify differentially expressed genes.

Real-time PCR

cDNA was extracted using Prime ScriptTM RT reagent Kit with gDNA Eraser (Takara)

according to the manufacturer instruction. The real-time (q-)PCR was carried out using an ABI StepOnePlus™ Real-Time PCR System (ABI, USA) with a SYBR Green qPCR Mix to examine the expression profile of *FER1L4* and *SRD5A2* genes in different tissues. β-Actin was used as the internal control. The gene primers are listed in Table 1. The reaction mixture contained 7.5 µL Rox, 0.9 µL each upstream and downstream primer, 1.5 µL cDNA, and 5.1 µL ddH_2O. The amplification conditions were an initial denaturation step at 95°C for 10 min, followed by 95°C for 15 min, and 40 cycles at 60°C for 10 min. All reactions were performed in triplicate. The melting curve of each gene was measured after amplification using SYBR Green with a fluorescein setting. Relative quantification was estimated using the comparative CT method, and relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Ishida-Takagishi et al., 2012).

Table 1. Primer sequences used to amplify target genes.

Gene name	Primer sequences
FER1L4	F: 5'-CCGGCCTGGATACCTCTCTAT-3'
	R: 5'-CAGTCGGCCTTGGAAACAAA-3'
SRD5A2	F: 5'-GCTCAGGAAGCCTGGAGAAAT-3'
	R: 5'-GGCATAGCCGATCCATTCAA-3'
β-actin	F: 5'-GTCATTGAGAGCAATGCCAG-3'
	R: 5'-GTGTTCCTACCCCCAATGTG-3'

RESULTS

Analysis of RNA-Seq libraries

Two RNA-Seq libraries were constructed from uniparous and multiparous samples and each generated over 5.9 million raw reads that were sufficient for the quantitative analysis of gene expression. After filtering out adaptor sequences, i.e., regions containing N sequences, and low quality sequences, each RNA-Seq library contained over 5.8 million clean reads; the uniparous and multiparous libraries gave 98.60 and 98.16% clean reads, respectively (Figure 1), demonstrating that both libraries were high-quality.

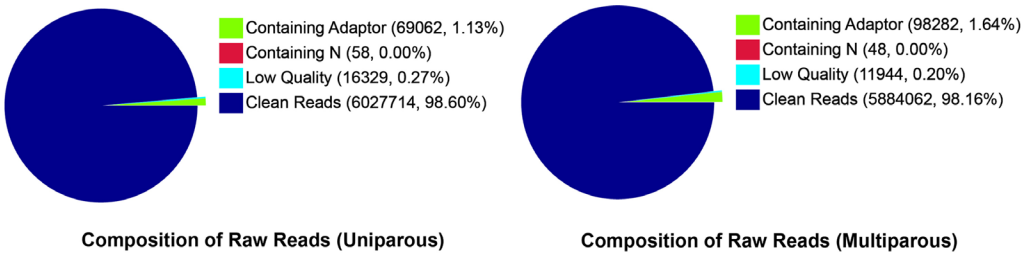

Figure 1. Composition of total raw reads from the uniparous and multiparous libraries.

Screening differentially expressed genes

In total, 15,890 genes were detected and 2201 genes showed differential expression between the uniparous and multiparous libraries. Compared to the uniparous library, 1583 genes

were up-regulated and 618 genes were down-regulated in the multiparous library with an FDR ≤ 0.001 and a |log2Ratio| ≥ 1 as standard (Figure 2). Of these differentially expressed genes, *FER1L4* showed the greatest up-regulation and *SRD5A2* the greatest down-regulation in the multiparous library. The 10 genes showing the largest differential expression effects are listed in Table 2.

Table 2. Ten genes showing the highest levels of differential expression.

Gene	log2 ratio (multiparous/uniparous)	P value	FDR	Up/down-regulation (multiparous/uniparous)
FER1L4	10.49393799	2.24E-47	1.94E-45	Up
GVINP1	10.47220442	1.50E-08	1.42E-07	Up
GVIN1	10.26986667	1.20E-07	1.00E-06	Up
EVI2A	9.914612892	0.000122	0.000596714	Up
EYA2	9.489224544	4.78E-07	3.66E-06	Up
SRD5A2	-14.22914539	3.42E-74	5.54E-72	Down
SST	-13.25260436	2.15E-19	5.65E-18	Down
IBSP	-13.20576698	2.68E-48	2.43E-46	Down
BTN1A1	-13.1823193	6.07E-33	3.30E-31	Down
CSRP3	-12.1761523	1.76E-15	3.41E-14	Down

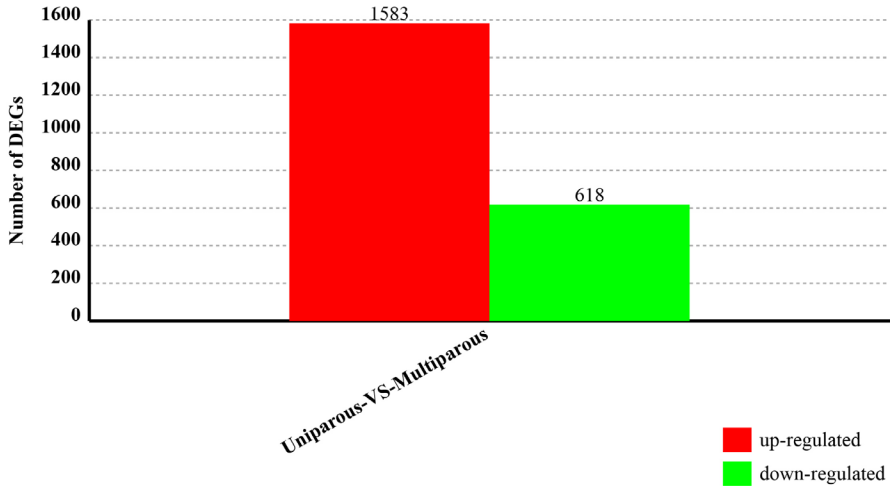

Figure 2. The number of differentially expressed genes between uniparous and multiparous samples. The red represents upregulated genes in the multiparous library, green represents genes downregulated in the multiparous library.

Expression profile of *FER1L4* in different tissues from AWG

The expression profiles of *FER1L4* and *SRD5A2* were analyzed in different tissues of AWGs and Boer goats by q-PCR. The *FER1L4* gene showed obvious tissue specificity (Figure 3). In uniparous AWGs, *FER1L4* was most highly expressed in the small intestine, followed by kidney, spleen, lung, muscle, and ovary, but expression in the heart, liver, uterus, and oviduct was at very low levels. In multiparous AWGs, *FER1L4* was most highly expressed in the ovary, followed by lung, but was expressed at very low levels in other tissues. The level of expression of *FER1L4* was higher in the ovary of multiparous AWGs than uniparous animals.

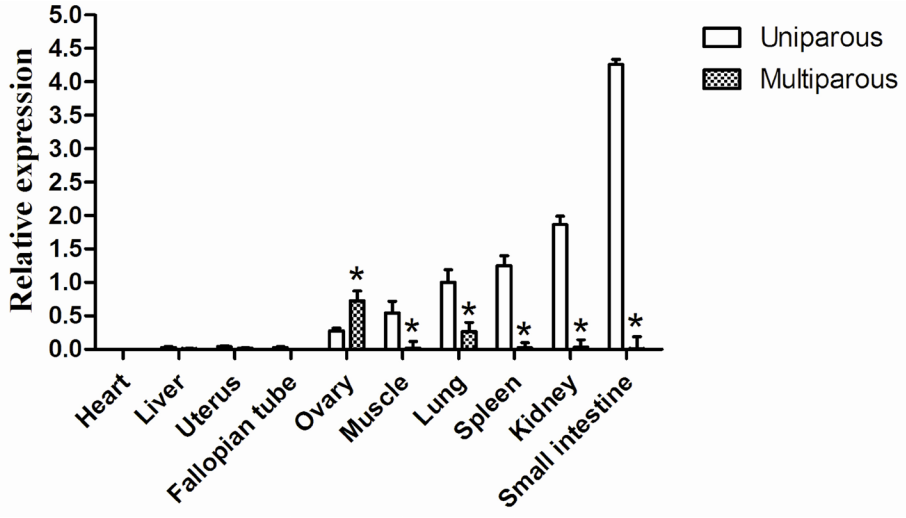

Figure 3. Expression profile of *FER1L4* in different tissues of uniparous and multiparous Anhui White Goat. Asterisk: P ≤ 0.05.

Expression profile of *SRD5A2* in different tissues from AWG

Expression of *SRD5A2* showed clear tissue specificity (Figure 4). In uniparous AWGs, *SRD5A2* was highly expressed in the liver, followed by kidney, muscle, and ovary, and showed lowest expression in the uterus and oviduct, with effectively no expression in other tissues. In multiparous AWGs, *SRD5A2* was most highly expressed in the uterus, followed by small intestine and ovary, but showed little evidence of expression in other tissues. The level of *SRD5A2* expression was lower in the ovary of multiparous AWGs than in uniparous animals.

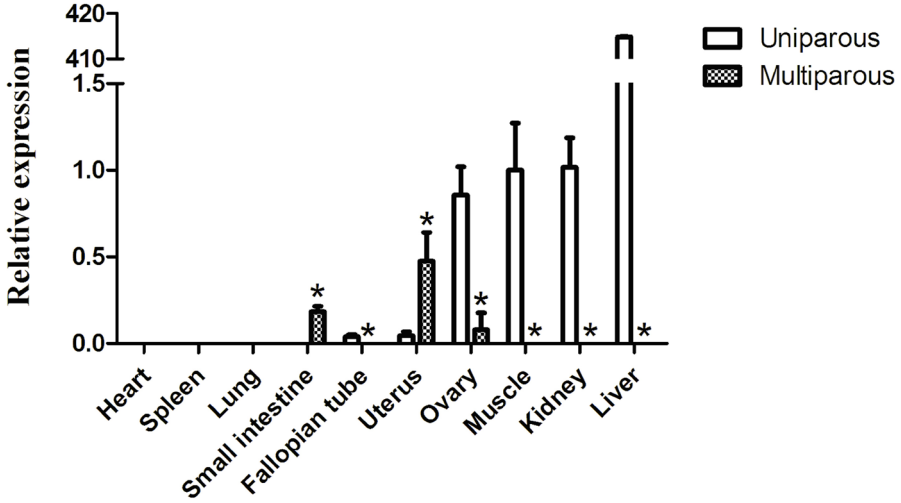

Figure 4. Expression profile of *SRD5A2* in different tissues of uniparous and multiparous Anhui White Goats. Asterisk: P ≤ 0.05.

Expression profile of *FER1L4* in different tissues from Boer Goat

FER1L4 showed clear tissue specificity in expression in different tissues from Boer goats (Figure 5). The highest level of expression was found in the oviduct, followed by spleen, small intestine, liver, ovary, and muscle, with little evidence of expression in other tissues. Boer goats showed higher *FER1L4* expression in the ovary compared to AWGs.

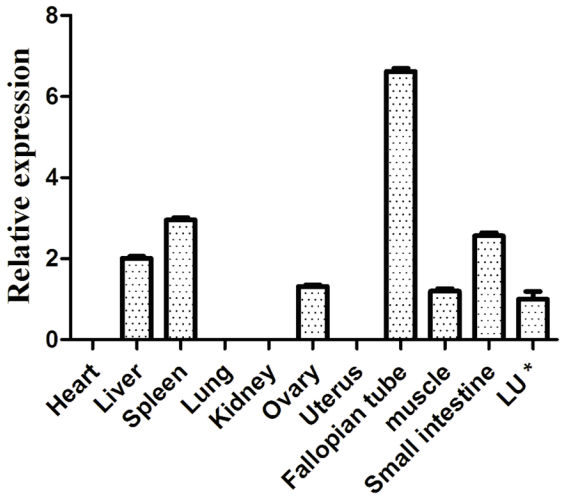

Figure 5. Expression profile of *FER1L4* in different tissues of Boer goats. Asterisk: Ovary of uniparous Anhui White Goat.

Expression profile of *SRD5A2* in different tissues from Boer goats

SRD5A2 also showed clear tissue specificity in Boer goats (Figure 6). Expression of *SRD5A2* was highest in liver, followed by spleen, ovary, and muscle, but expression was difficult to detect in other tissues. Expression of *SRD5A2* was higher in the ovary of Boer goats compared to AWGs.

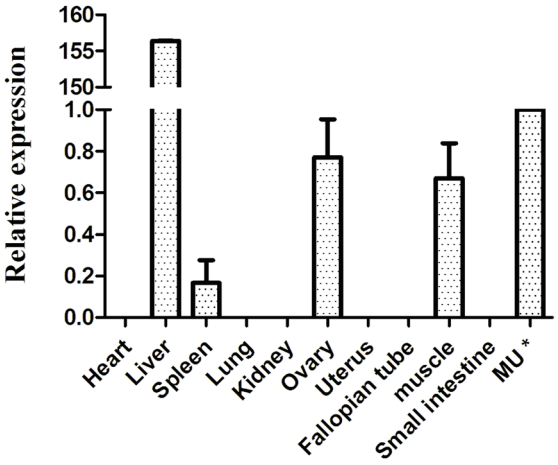

Figure 6. Expression profile of *SRD5A2* in different tissues of Boer goats. Asterisk: Ovary of uniparous Anhui White Goat.

DISCUSSION

Screening and analyzing of differentially expressed genes

In total, 2201 genes showed differential expression between uniparous and multiparous AWGs; this is a higher number than found in similar studies elsewhere (Gao et al., 2013). Although other studies have provided reference genomes of cattle and other close relatives of the domestic goat (Geng et al., 2013), these are of limited value to goat breeders due to species differences. However, the recently available goat genome sequence (published online 23 December 2012) has enabled the current analysis to be performed directly in the goat (Dong et al., 2013). The results here showed that more than 80% of the reads could mapped to the reference goat genome and the number of unmapped reads was significantly decreased. The effectiveness and accuracy of these results have increased dramatically and offer new information related to gene expression profiles of uniparous and multiparous goats. This data will provide more accurate and reliable information for follow-up studies.

Function of *FER1L4* and *SRD5A2*

Currently, the function of *FER1L4* is unknown. This gene is a recently discovered LncRNA and belongs to the FER1-like protein family (Song et al., 2013). An expression study showed that *FER1L4* was significantly down-regulated in gastric cancer tissues (Song et al., 2013). The available evidence suggests *FER1L4* might play a crucial role in human gastric cancer and be a new biomarker for clinical prognosis (Liu et al., 2014). However, the *FER1L4* gene has been shown to be located in the cell membrane in the cellular component of GO categories. This suggests that *FER1L4* may be involved in cell internal and external information exchange mechanisms. We found a high level of *FER1L4* expression here suggesting that this gene may be a key candidate gene associated with high fertility in the goat.

Steroid 5α-reductase (SRD5A), including type-1 and type-2 5α-reductase, catalyzes testosterone (T) to dihydrotestosterone (DHT), and plays an important role in sex differentiation and androgen-mediated physiological processes. *SRD5A2* is located on chromosome 2p23 and has five exons and four introns. It is mainly expressed in the reproductive system and prostate tissue (Ellsworth et al., 1995), has a high affinity for steroid substrates, and participates in the reproductive system and prostate tissue development (Yokoi et al., 1998). *SRD5A2* is also closely related with fertilization (Kang et al., 2014), sexual abnormality (Kostyrko et al., 1994), prostate disease (Kaefer et al., 1996; Akalu et al., 1999), polycystic ovary syndrome (Goodarzi et al., 2006), and cancer (Akalu et al., 1999; Li et al., 2013). Studies of human *SRD5A2* deficiency pedigrees showed that male patients displayed pseudo-hermaphroditism, while female patients had reduced fertility (Hochberg et al., 1996). These characteristics are the opposite of the results in the present study. This discrepancy may be due to differences in post-transcriptional translation or species differences.

Relationship between litter size and *FER1L4* and *SRD5A2* in AWGs

We found that *FER1L4* and *SRD5A2* were highly expressed in the ovaries of estrus AWGs, and that the level of expression of *FER1L4* was higher in multiparous than uniparous ovaries. *SRD5A2* behaved in a contrasting manner and was expressed at a lower level.

Expression of *FER1L4* and *SRD5A2* was at high levels in the ovaries of Boer goats; this finding is not consistent with the expression level in the estrous ovaries of uniparous and multiparous AWGs. Possibly, this result may have been because the tested ewes were at different stages of the estrous cycle or because of species differences. This study is the first to show a relationship between *FER1L4* and *SRD5A2* genes and kidding numbers.

Tissue specific expression of *FER1L4* and *SRD5A2*

The expression of *FER1L4* and *SRD5A2* showed obvious tissue specificity in both AWGs and Boer goats. *FER1L4* was expressed in the ovaries of AWGs and Boer goats, but showed little evidence of expression in heart, kidney, and uterus. *SRD5A2* was expressed in the ovaries of AWGs and Boer goats, but showed little evidence of expression in the heart, lung, uterus, and small intestine; it also showed specifically high expression in the liver of uniparous AWGs and in Boer goats.

The above results indicate that *FER1L4* and *SRD5A2* genes were both expressed in the ovaries of AWGs and Boer goats, and highly expressed in the liver, suggesting they may also be relevant to goat fecundity. Our observations also indicate their function may not be limited to specific breeds, and that they may also participate in a variety of other physiological activities such as digestion and detoxification.

CONCLUSION

On the basis of screening for differential gene expression in the ovaries of uniparous and multiparous AWGs, we identified *FER1L4* and *SRD5A2* genes as the two genes showing highest differential expression between uniparous and multiparous AWGs. Our preliminary analysis examined the relationship between these genes and the high fertility in goats. This analysis indicated that both genes may be associated with high fecundity in AWG, but their relationship to fertility in Boer goats is not clear. Further research will be necessary to elucidate this relationship.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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