

Expression and localization of PIWI proteins in testis and ovary of domestic sheep

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Abstract: The piRNA-PIWI protein complex plays crucial roles in safeguarding the genome against inordinate transposon mobilization and regulation of embryonic development. A previous study indicated the presence of piRNA in sheep reproductive organs. However, the tissue distribution and cellular localization of PIWI proteins in sheep remains unclear. Therefore the present study aimed to explore the expression profiles of mRNAs of mammalian PIWI proteins (PIWIL1, PIWIL2, PIWIL4 and AGO3) in 9 tissues derived from adult male and female sheep. Results showed the expression of *PIWIL1*, *PIWIL2*, and *PIWIL4* was significantly higher in the testis and ovary than in the other tissues. Immunohistochemistry analysis of testes indicated that each of the 4 proteins had specific cellular localizations, and some of the localizations were different from those of other species. All the proteins were mainly localized in the primary spermatocytes, suggesting that they are crucial for silencing of transposon to guarantee the integrity of the gamete genome during early stage of spermatogenesis. In the ovaries, the PIWI proteins were mainly localized in oocytes from antral follicles and leukocytes in ovarian blood. Our results provide insights to better understand the functions of PIWI proteins during spermatogenesis, oogenesis and immune defence in sheep.

Keywords: PIWI proteins; reproduction; localization; testis; ovary

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PIWI proteins can specifically bind PIWI-interacting RNAs (piRNAs) to form an RNA-induced silencing complex (RISC). RISC plays crucial roles in safeguarding the genome against inordinate transposon mobilization in model organisms such as *Drosophila*, *C. elegans* and zebrafish (Batista et al. 2008; Meister 2013; Wang et al. 2014; Han et al. 2015, 2017). PIWI proteins have been found to be essential in gonadal and embryonic development (Deng and Lin 2002; Houwing et al. 2007). The absence or abnormality of PIWI proteins may result in disorders of spermatogenesis or folliculogenesis (Thomson and Lin 2009; Pillai and Chuma 2012; Ding et al. 2013; Goh et al. 2015). PIWI proteins are therefore essential in maintaining functions related to animal fertility.

Three kinds of PIWI proteins have been reported from the study of *Drosophila* germline cells, viz AGO3, PIWI and AUB (Gunawardane et al. 2007; Brennecke et al. 2007; Luteijn and Ketting 2013). AGO3 is required to cleave transposon RNAs, which results in slicing of piRNA precursors and formation of additional primary piRNAs. Additionally, AGO3 is also necessary for mammals. For example, AGO3 was detected in non-reproductive tissues and cells from humans (Wang et al. 2012) or pigs (Krawczynski et al. 2015), and it was essential for RISC formation. The other 3 PIWI proteins, MIWI (or named PIWIL1), MILI (PIWIL2) and MIWI2 (PIWIL4) were commonly found in germline cells from mammals such as marmoset (Hirano et al. 2014) and mice (Toth et al. 2016). Therefore, those 4 PIWI proteins (AGO3, PIWIL1, PIWIL2 and PIWIL4) could be preferential candidates in sheep studies.

Our previous study showed the presence of piRNA in reproductive organs of domestic sheep and a total of 166,164 candidate piRNAs were predicted in sheep ovaries (Di et al. 2014). Recent studies in humans and mouse have shown that piRNAs are also expressed in non-reproductive tissues, but their functions remain a subject of investigation (Tosar et al. 2018; Perera et al. 2019). Based on our findings and those of other studies, we hypothesized that PIWI proteins occur in a wide array of sheep tissues but mostly in gonads and that each protein acts at a specific time period. To test our hypothesis and provide clues to further reveal the functions of PIWI proteins, we investigated the tissue distribution and cell localization of PIWI proteins in adult male and female sheep.

MATERIAL AND METHODS

Animal sampling

Six tissues (cerebrum, cerebellum, heart, liver, spleen, kidney) were collected from 3 adult rams and ewes of the Small Tail Han sheep breed. Simultaneously, testis, epididymis, and vas deferens (spermatiduct) from the rams and ovary, uterine body and oviduct from the ewes were also collected. All the tissue samples were preserved in liquid nitrogen prior to the quantitative real-time PCR (qPCR) analysis. In addition, testes and ovaries in follicular phase were collected from other 3 adult rams and ewes, respectively, and fixed with 4.0% paraformaldehyde for the immunohistochemistry analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted from each tissue using the RNeasy Pure Tissue Kit (Qiagen, Beijing, P. R. China) with DNase treatment. The concentration and purity of the extracted RNA were assessed with Nanodrop 2000. The extracted RNA has an A260/280 value between 1.8 and 2.0 and its concentration is higher than 100 ng/ μ L. The RNA was stored at -80°C prior to analysis. The extracted RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the PrimeScriptTM RT Reagent Kit (TaKaRa, Dalian, P. R. China).

Primer design

The qPCR primers (Table 1) were designed, using Primer 3.0 software (Untergasser et al. 2012), based on the sheep *PIWIL1* (XM_027980234.1), *PIWIL2* (XM_027964351.1), *PIWIL4* (XM_027978990.1), *AGO3* (XM_027967800.1) and β -actin (NM_001009784.3) mRNA sequences available at the Genbank.

Quantitative real-time PCR (qPCR)

qPCR was carried out using the Roche Light Cycler[®] 480II (Roche, Beijing, P. R. China) with QuantiNova SYBR Green PCR Kit (TaKaRa, Dalian, P. R. China). Standard curves were established

Table 1. Details of primer sequences that were used in qPCR

Gene	Primer sequence (5'-3')	Accession no. of reference seq	Location of primer by exon (-th)	Product size (bp)	Amplification efficiency (%)
<i>PIWIL1</i>	F: GTGGACGACAGAGAGGAACA R: ACGACCTCCTCTCTCTGCTA	XM_027980234.1	6 to 7	101	92.7
<i>PIWIL2</i>	F: TCCCTGAGCTTTCCTTCATGA R: ATTATGGTGCTGTTTGGGGC	XM_027964351.1	5 to 6	110	92.6
<i>PIWIL4</i>	F: CGTCATGGATTTGTCTGTCTGT R: TCCTGGGGCACGTCTAAATT	XM_027978990.1	4 to 5	123	99.9
<i>AGO3</i>	F: GCCAATCAGCACTAACCCCTG R: AGGGTGGTCGTATCCTTCTG	XM_027967800.1	8 to 9	118	96.3
<i>β-actin</i>	F: CCAACCGTGAGAAGATGACC R: CCCGAGGCGTACAGGGACAG	NM_001009784.3	3 to 4	87	100.0

to verify the efficiencies of primer amplification. The amplification efficiency ranged between 90% and 105%, and the melting curves showed a single sharp peak. The amplification reaction was performed in 20 µl final reaction volume made up of 10 µl SYBR Green Premix Ex Taq II (TaKaRa, Dalian, P. R. China), 0.8 µl each primer, 2 µl cDNA and 6.4 µl RNase-Free ddH₂O. The reaction profiles were initial denaturation at 95 °C for 5 s, followed by 40 cycles of denaturation at 95 °C for 5 s and at 60 °C for 30 s, and a final step of 4 °C forever.

Immunohistochemistry (IHC) analysis

Testes and ovaries in follicular phase from 3 adult rams and ewes, respectively, were fixed with 4.0% paraformaldehyde at 4 °C for 48 hours. They were then dehydrated with gradient alcohol and embedded in optimal cutting temperature compound. They were frozen at –80 °C until section processing. Serial 5 µm sections were obtained and spread out on glass slides treated with APES. For IHC analysis, the tissue slice sections were processed by washing in 1 × PBS followed by goat serum blocked for 1 hour. They were then incubated overnight at 4 °C with Anti-PIWIL1 (ab12337; Abcam, UK), Anti-PIWIL2 (ab85084; Abcam, UK), Anti-PIWIL4 (ab111714; Abcam, UK) and Anti-AGO3 (ab3593; Abcam, UK) antibodies at a dilution factor of 1 : 100. The slices were incubated at room temperature for 20 minutes. After washing the slices thrice with 1 × PBS, secondary antibody (ab6802; Abcam, UK) was applied at a dilution factor of 1 : 500 and then

incubated at room temperature for 1 hour. All slices were washed with 1 × PBS and stained with diaminobenzidine (DAB) for 5 min, after that haematoxylin was applied to counterstain for 5 minutes. The slices were analyzed using light microscopy.

Statistical analysis

Data were collected and analyzed using the LightCycler[®] 480 software. *β-actin* was used as a reference gene. The 2^{-ΔΔCt} algorithm (Schmittgen and Livak 2008) was used to calculate the relative expression of the target genes. An average value was calculated and the relative RQ plots were generated for each tissue and gene. Differences in expression levels were tested for their significance using ANOVA, and graphical presentation of data was performed using Prism GraphPad (v6.01; www.graphpad.com).

RESULTS

Tissue expressions of *PIWIL1*, *PIWIL2*, *PIWIL4* and *AGO3* genes in sheep

qPCR analysis showed that *PIWIL1*, *PIWIL2*, *PIWIL4* and *AGO3* were expressed in almost all somatic tissues of both sexes (Figures 1 and 2). In rams, the expression levels of *PIWIL1*, *PIWIL2*, and *PIWIL4* were significantly higher ($P < 0.05$) in testis than in other reproductive (epididymis and vas deferens) and somatic (cerebrum, cerebel-

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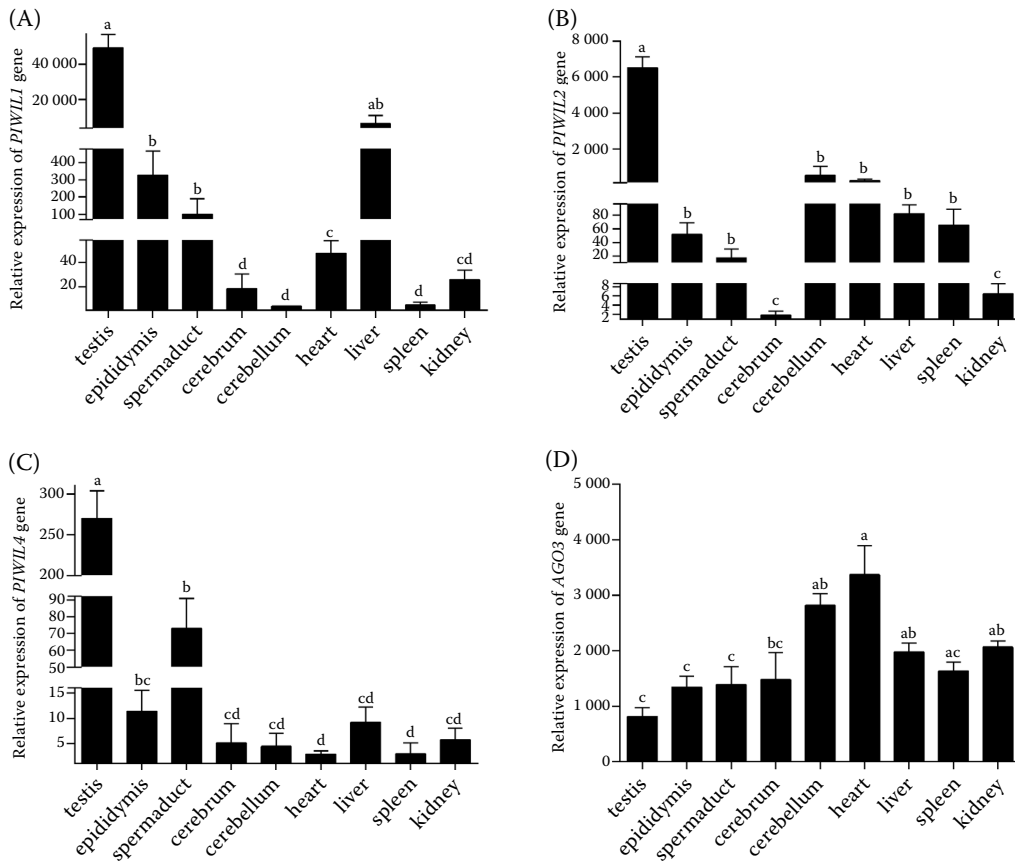


Figure 1. Expression of *PIWIL1*, *PIWIL2*, *PIWIL4*, and *AGO3* genes in different tissues from rams

Different lowercase letters indicate a significant difference ($P < 0.05$) in gene expression between different tissues

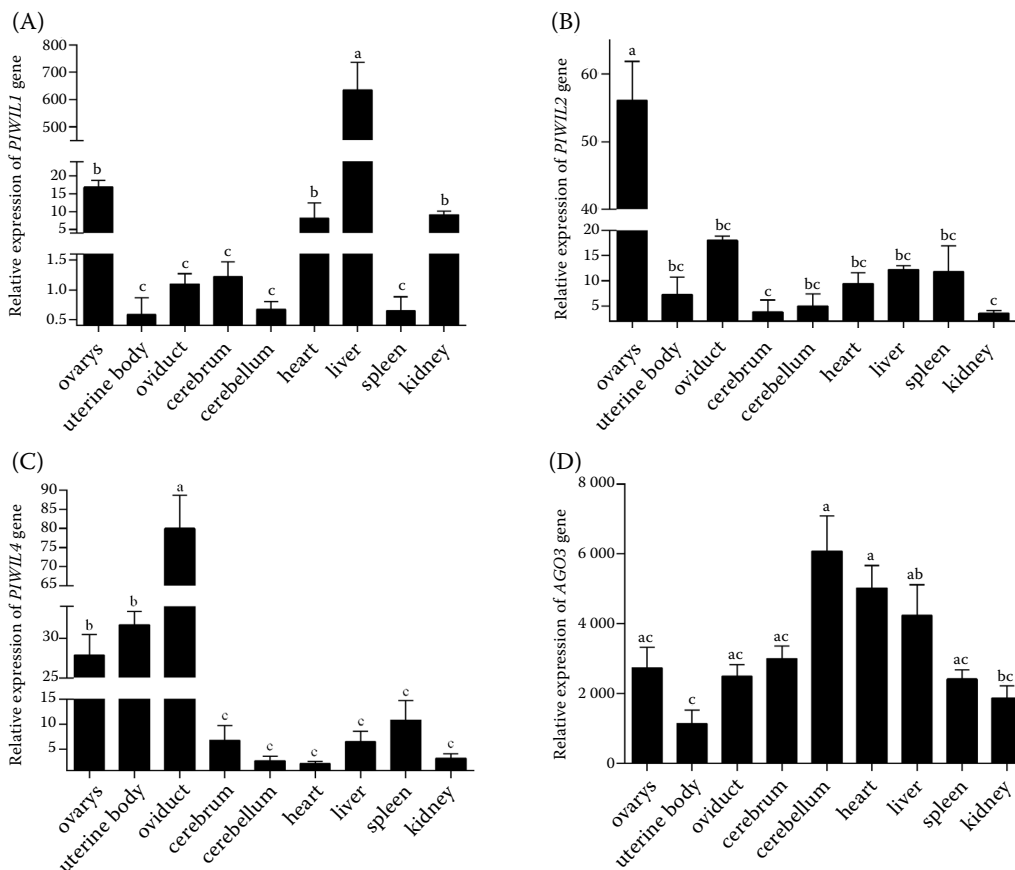


Figure 2. Expression of *PIWIL1*, *PIWIL2*, *PIWIL4*, and *AGO3* genes in different tissues from ewes

Different lowercase letters indicate a significant difference ($P < 0.05$) in gene expression between different tissues

lum, heart, spleen and kidney) tissues (Figure 1A, B and C). In ewes, *PIWIL1* and *PIWIL2* had higher expression levels ($P < 0.05$) in ovary than in the other reproductive (uterine body and oviduct) and somatic (cerebrum, cerebellum and spleen) tissues (Figure 2A and B). *PIWIL1* was also highly expressed in liver in both sexes. Although *AGO3* was widely expressed in diverse tissues, no significant differences were observed in its level of expression between most of the tissues (Figure 1D and 2D).

Localization of *PIWIL1*, *PIWIL2*, *PIWIL4* and *AGO3* proteins in testis and ovary of sheep

Based on the qPCR results from the current and previous studies on other species (Zhou et al. 2010; Lim et al. 2013; Wang et al. 2015; Gainetdinov et al. 2017), testis and ovary tissues were selected for fur-

ther analyses determining the specific cell types in which *PIWIL1*, *PIWIL2*, *PIWIL4*, and *AGO3* proteins were expressed (Figures 3 and 4). The IHC analysis of testis showed that *PIWIL1* was highly expressed in primary spermatocytes, but it was weakly expressed in spermatogonia, secondary spermatocytes and spermatids. *PIWIL2* was mainly localized in spermatogonia and primary spermatocytes but it was weakly expressed in secondary spermatocytes. *PIWIL4* was weakly expressed in primary spermatocytes. *AGO3* was mainly expressed in primary spermatocytes, while a small amount was localized in spermatogonia and spermatids (Figure 3).

The IHC analysis of ovary showed that all the *PIWI* proteins were expressed in oocytes, with a higher level being observed in oocytes from antral follicles compared to small non-ovulatory ones (Figure 4A). In addition, we surprisingly found that all the *PIWI* proteins were also localized in leukocytes in ovarian blood (Figure 4B).

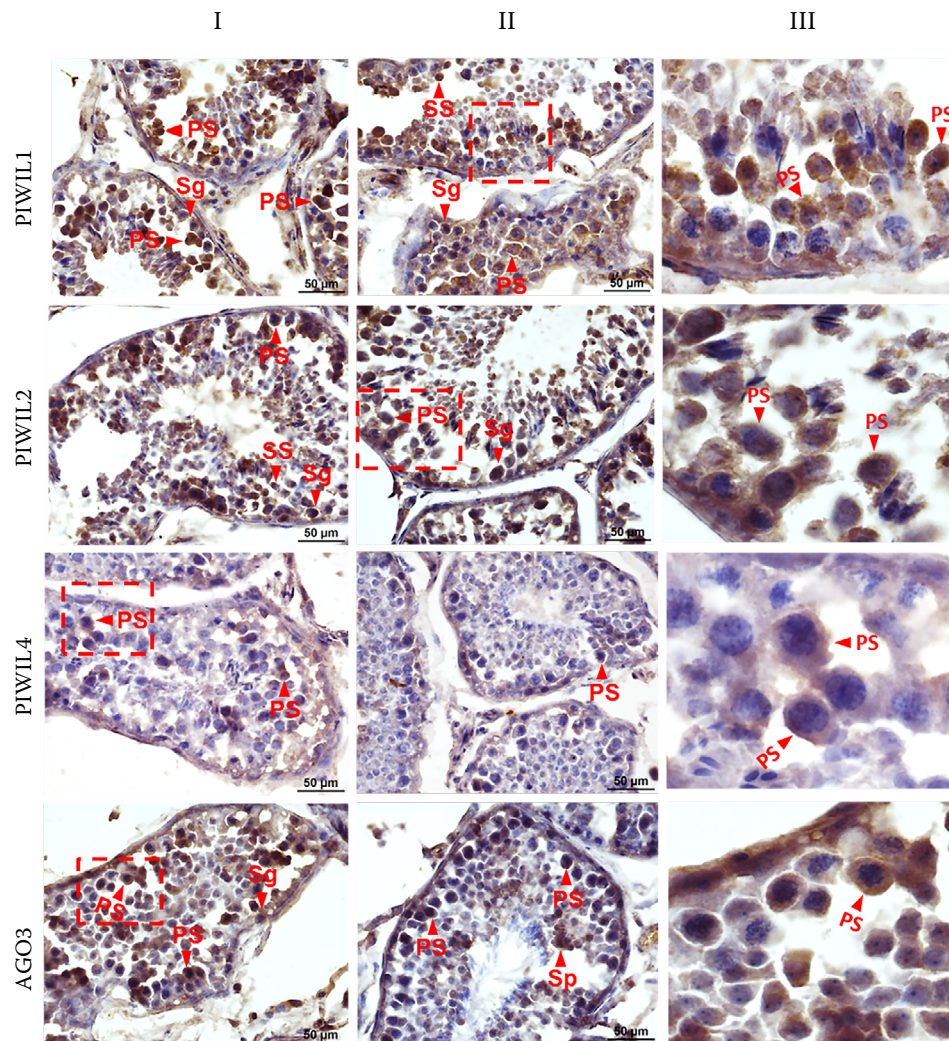


Figure 3. Localization of *PIWIL1*, *PIWIL2*, *PIWIL4* and *AGO3* proteins in the testis of sheep. The direction of the red arrowheads represents the cell types localized by these proteins. Plate III shows an enlarged view of the contents of the box in Plates I or II

PS = primary spermatocytes; Sg = spermatogonia; Sp = spermatids; SS = secondary spermatocytes

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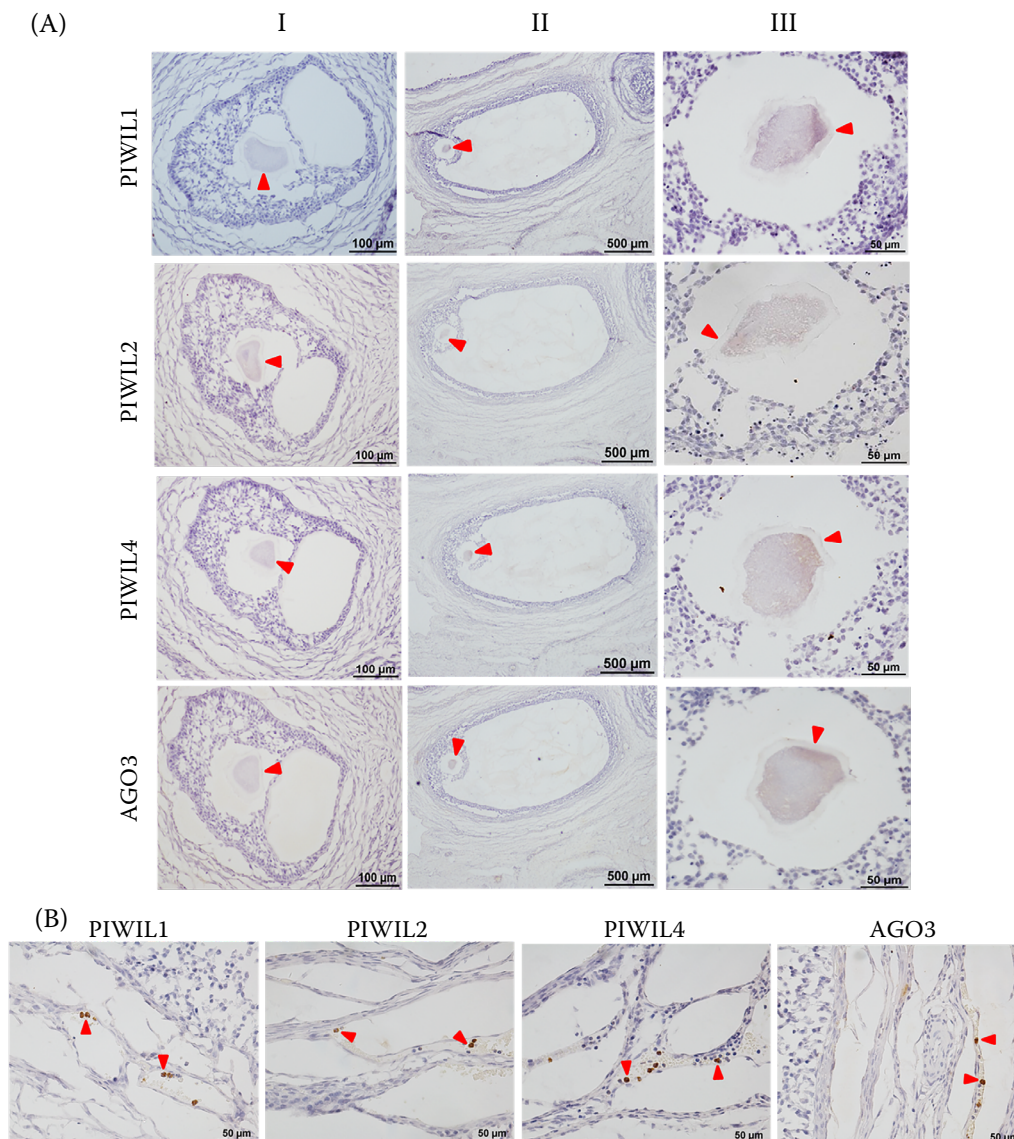


Figure 4. Localization of PIWIL1, PIWIL2, PIWIL4 and AGO3 proteins in (A) follicles and (B) blood vessel in the ovary of sheep. The direction of the red arrowheads represents the cell types localized by these proteins. Plates I and II in (A) show small non-ovulatory follicles and antral follicles in follicular phase, respectively. Plate III in (A) highlights the oocyte in Plate II

DISCUSSION

Results in this study indicated that PIWI proteins were widely expressed in diverse tissues of sheep. This implies they likely play multiple essential functions except roles in gametes. Recent studies have detected piRNAs in multiple somatic tissues in mammals such as humans and mice (Tosar et al. 2018; Perera et al. 2019). We have also shown that PIWI proteins were present in multiple non-reproductive tissues in sheep. For instance, *PIWIL1* was highly expressed in liver. Expression of PIWI proteins in non-reproduc-

tive tissues may be involved in epigenetic regulation (Lin and Yin 2008), the mechanisms of which require further investigations. Unexpectedly, *AGO3* was highly expressed in all detected tissues. This suggests that *AGO3* most probably plays an essential role in basic physiological functions in different tissues.

PIWI proteins are mainly expressed in sheep gonads. Moreover, each PIWI protein is expressed at specific time periods, which differ between species, during sperm and oocyte development.

Expression of *PIWIL1* was detected in the gonads of multiple species, although the type of cells in which

it was expressed varies among species. For instance, *PIWIL1* signal was observed in spermatogonia, spermatocytes, oogonia and oocytes of adult turbot (Wang et al. 2018). In humans, *PIWIL1* was detected specifically in pachytene spermatocytes, but not in spermatogonia or Sertoli cells (Hempfling et al. 2017). piRNAs associate with *PIWIL1* in a single compact granule, known as satellite body in pachytene-stage spermatocytes and chromatoid body in haploid round spermatids in mice (Siomi et al. 2011). Once bound by the translational regulatory protein PABPC1, piRNA-*PIWIL1* complex, which is crucial for mRNA metabolism and protein translation during spermatogenesis, forms in chromosomal regions (Kimura et al. 2009; Xu et al. 2014). It was observed that different domains of *PIWIL1* interact with various partners and participate in translational regulation (Wu et al. 2018). In recent years, the expression of *PIWIL1*, which tends to vary with age, has also been detected in gonad tissues of domestic animals. For instance, the transcript abundance of *PIWIL1* was 3-fold higher in the testes of adult pigs than of neonatal ones (Kowalczykiewicz et al. 2012). Additionally, *PIWIL1* was detected specifically in the germline cells in mature seminiferous tubules in bovine testis, and its expression level was higher in mature sperm than in embryonic stage (Russell et al. 2016). Similarly, the expression level of *PIWIL1* is significantly increased in yak testis during sexual maturity (Gong et al. 2018). The higher expression of mRNA and *PIWIL1* protein was observed in ovine testis and ovary, and the protein was localized in all male germline cell types and mature oocyte. The results lay a foundation to further verify the role of *PIWI* in spermatogenesis, from spermatogonia to spermatid stages and oogenesis (Zhao et al. 2011). Its high expression in primary spermatocytes most likely relates to the regulation of the translation of proteins that are required during spermatogenesis, safeguarding the gamete genome against inordinate transposon mobilization during genome replication and in cross-interchange of chromatids in early stages of spermatogenesis.

PIWIL2 regulates the orderly development of gamete. In mouse, *PIWIL2* was detected in the cytoplasm of spermatogonia and spermatocytes by immunostaining (Lee et al. 2006). Specifically, piRNA-*PIWIL2* complexes are present in pi-bodies, also known as intermitochondrial cement (Siomi et al. 2011). The absence of *PIWIL2* and *PIWIL4* can lead to arrest at the zygotene-pachytene stage

of meiosis I, implying that they play crucial roles in primary spermatocyte stage. In adult turbot, *PIWIL2* is highly expressed in ovary and testis rather than in other tissues, its mRNA signal is present in spermatogonia and spermatocytes as well as in all stages of oocyte development, whereas almost no signal was detected in spermatids. Wang et al. (2017) observed no changes in the expression of *PIWIL2* during testicular development in turbot from 8 to 18 months of age. In human adults, *PIWIL2* was expressed in all types of germ cells of the male testis but its expression was stronger in round spermatids and female oocytes (Gomes Fernandes et al. 2018), and it is closely associated with testicular development and oogenesis (Fang et al. 2019). We observed that *PIWIL2* was highly expressed in ovine gonadal tissues and was mainly localized in spermatogonia, primary spermatocyte and oocyte, suggesting that *PIWIL2* might play an important role in ensuring that the whole genome lacks defects during genome replication and cross-interchange of chromatids in the early stages of spermatogenesis and oogenesis in sheep, which needs further study in the next step.

PIWIL4, as a molecular chaperone of *PIWIL2*, participates in gametogenesis. *PIWIL4* can interact with *PIWIL2* in the germ line of mouse, resulting in DNA methylation at piRNA target loci. Therefore, the expression level of *PIWIL4* in spermatogonia corresponds to the time period during cell-cycle arrest and de novo DNA methylation (Bortvin 2013; Hadziselimovic et al. 2015). Although mouse *PIWIL4* is homologous to the human one, its expression pattern differs between the two species. In mouse, *PIWIL4* occurs predominantly in normal germ cells and its expression declines soon after birth (Aravin et al. 2008). It is not expressed in oocytes during the developmental stage (Kabayama et al. 2017). In humans, most of its expression occurs in gonadal germ cells in adult males (Gomes Fernandes et al. 2018), and it is ubiquitously expressed in cells of multiple tissues including oocytes at different developmental stages (Sugimoto et al. 2007; Gomes Fernandes et al. 2018). Like in humans, *PIWIL4* is expressed in yak testis but with little variation in expression at different stages of sexual maturity (Gong et al. 2018). In rhesus monkey, the expression of *PIWIL4* in testis is the highest among all tissues, and *PIWIL4* is expressed in perinuclear cells of seminiferous tubules and in various types of germ cells in infant and adult

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monkeys, respectively (Zhao et al. 2011). Based on our results, it will be necessary to determine the correlation between the expression of PIWIL2 and PIWIL4 with the levels of DNA methylation at piRNA target loci throughout the development periods of germ cells, to understand the role it plays during spermatogenesis and oogenesis in sheep.

AGO3 is mainly involved in the ping-pong amplification of piRNA pathway in spermatogonia and germline stem cells of *Drosophila* (Brennecke et al. 2007; Nishida et al. 2007; Kirino et al. 2009), and its expression is negatively associated with transposons (as *Mos1*) in spermatogonia and spermatocytes (Saint-Leandre et al. 2017). Nucleoporin 358 enables PIWI (AGO3 and AUB) to enter the nucleus, following which PIWI binds piRNAs to silence transposons and regulate gene expression in ovary (Parikh et al. 2018), indicating that AGO3-piRNAs can protect the animal germline genome by silencing transposons. It has been observed that AGO3 was generally expressed in rat testis from preadolescence to adolescence (Kang et al. 2014) and yak testis at different ages (Gong et al. 2018). We detected AGO3 was highly expressed in reproductive tissues (ovary, testis) and non-reproductive tissues like cerebellum and heart of sheep, which suggested a large expression of transposable elements in those tissues. This coincides with the fact that cerebellum and heart are mainly composed of almost terminally differentiated cells. In this study, AGO3 protein was identified mainly in primary spermatocytes and oocytes from mature follicles, suggesting AGO3 may correlate with the formation of AGO3-piRNAs to protect the animal tissue genome universally by silencing transposons (Wang et al. 2012; Krawczynski et al. 2015; Parikh et al. 2018).

Additionally, we surprisingly found that the PIWI proteins also occurred in leukocytes of ovarian blood, which implies they might have a potential immune function. Both piRNA and PIWI proteins were detected in whole blood cells in humans (Freedman et al. 2016; Tosar et al. 2018; Huang et al. 2015; Fang et al. 2019). *PIWIL1* and *AGO3* can enhance animal's immune defences against tumorigenesis (Xie et al. 2018) and flu (Van Stry et al. 2012), respectively. The antiviral responses of PIWI proteins in the virus-specific piRNA (vpiRNA) pathway have been assessed. Alazem et al. (2017) found that the AGOs are associated with immune defence functions in *Arabidopsis*. Knockdown

of *AGO3*, *PIWIL5* and *PIWIL6* reduced the antiviral ability of Semliki Forest virus (SFV)-specific piRNA, Zika virus (ZIKV)-specific or alphavirus, and Chikungunya virus (CHIKV)-specific piRNA (Miesen et al. 2015; Varjak et al. 2017a, b; Varjak et al. 2018). *PIWIL4* has also been reported to be a key mediator for antiviral response and it can bind piRNA or siRNA to resist SFV or ZIKV, although it is not required for the production of vpiRNAs (Varjak et al. 2017a, b). For the siRNA pathway, knockdown of the key proteins such as PIWIL4 led to an increase in virus replication (Varjak et al. 2018). Our results and those references herein provide a better understanding of the immune defence mechanisms of PIWI proteins in prevention of pathogenesis and antiviral response.

CONCLUSION

The expression levels of *PIWIL1*, *PIWIL2*, *PIWIL4* in testis and ovary were significantly higher than in most other tissues in sheep. PIWI proteins were mainly localized in primary spermatocytes of testes and oocytes from mature follicles of ovary in sheep. Additionally, PIWI proteins were also surprisingly found to be distributed in leukocytes in ovarian blood. Our findings provide useful insights that can further help to understand the function of PIWI proteins during spermatogenesis, oogenesis and immune defence in sheep.

Conflict of interest

The authors declare no conflict of interest.

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