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

CHUNYAN LI1,2, QIUYUE LIU1, XIANGYU WANG1, WENPING HU1, DEPING HAN3, JORAM MWASHIGADI MWACHARO4, CAIHONG WEI1, MINGXING CHU1*, RAN DI1*

1Key Laboratory of Animal Genetics and Breeding and Reproduction of Ministry of Agriculture and Rural Affairs, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, P. R. China
2College of Animal Science and Technology, Anhui Agricultural University, Hefei, P. R. China
3College of Veterinary Medicine, China Agricultural University, Beijing, P. R. China
4Small Ruminant Genomics, International Centre for Agricultural Research in the Dry Areas (ICARDA), Addis Ababa, Ethiopia

*Corresponding authors: mxchu@263.net (M. Chu), dirangirl@163.com (R. Di)


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 (spermaduct) 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 RNAprep Pure Tissue Kit (Tiangen, 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/µL. The RNA was stored at −80 °C prior to analysis. The extracted RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the PrimeScript™ 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
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, PIWIL4 and AGO3 were significantly higher (P < 0.05) in testis than in other reproductive (epididymis and vas deferens) and somatic (cerebrum, cerebel-
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 further 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).
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-reproductive 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 germ line 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 germ line cell types and mature oocyte. The results lay a foundation to further verify the role of PIW1 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
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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