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Characterization of a piRNA binding protein Miwi in mouse oocytes

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ABSTRACT

Argonaute proteins and Piwi proteins bind with microRNA (mRNA) and Piwi-interacting RNA (piRNA), respectively, to form functional complexes. Piwi proteins are mostly restricted to germ cells and stem cells, and the Piwi-piRNA pathway is required for normal spermatogenesis. Although piRNAs were also recently identified in mammalian oocytes, expression of Piwi proteins in the ovary has not been well characterized. Previous studies did not detect mRNA of Miwi, a murine homologue of Piwi proteins, in total RNA of mouse ovary tissue. We demonstrated herein the presence of Miwi in murine oocytes. Reverse transcription polymerase chain reaction (RT-PCR), Western blot, and immunofluorescence based on quantum dots immune labeling technique were used to investigate the expression profile of Miwi in oocytes of adult and neonatal females at 0, 1, 2, 3, and 4 weeks postpartum. Although RT-PCR was negative in total RNA of the adult ovary, both RT-PCR and Western blot detected Miwi in oocytes of adult mice, and ovaries of neonatal females. Miwi transcript and protein peaked at 1 and 2 weeks postpartum, respectively. Miwi mRNA was detectable in newborn mouse ovaries, implying its transcription was initiated at least in the primordial follicle. Its protein was strong in late primary and secondary follicles, but appeared to decrease as maturation proceeded. The exclusion of anti-Miwi immunofluorescence from some cytoplasmic granules was observed. Given that diverse biologic and molecular functions have been revealed for the Piwi-piRNA pathway in germline cells of many species, Miwi might be an important functional protein in murine folliculogenesis.

1. Introduction

Small RNAs, including microRNAs (mRNAs) and Piwi-interacting RNAs (piRNAs), are believed to have essential roles in male and female gametogenesis. Certain proteins bind with these small RNAs and form functional complexes. Among them, the Argonaute superfamily are highly conserved in diverse organisms and form the core of functional complexes of mRNAs and piRNAs. This family includes two branches—Argonaute proteins and Piwi proteins. The former clade is widely expressed in all cells and mainly involved in regulation of gene expression through binding with mRNAs, and the latter clade is mostly restricted to germ and stem cells [1–3].

Proteins of the Piwi clade have highly conserved roles in regeneration and development of germline cells by binding with piRNAs [1–10]. In *Drosophila* and Zebrafish, Piwi mutants are defective in gametogenesis [4–7]. In the mouse, three homologues of Piwi were identified: Miwi, Mili, and Miwi2. Their special spatial and temporal patterns in mouse testis and essential roles in spermatogenesis have been reported [8–10]. Miwi is located in pachytene spermatocytes and round spermatids [8]. The mutant of Miwi leads to an arrest of spermatogenesis at 24 days postpartum [8]. The expression of Mili in male germ cells is

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from primordial germ cells until the pachytene stage of meiosis [9], and Miwi2 is from 18 days postcoitum in gonocytes to 3 days postpartum in spermatogonial stem cells [3,10]. Mutants of Mili or Miwi2 resulted in arrested spermatogenesis at the stage of meiosis, and in a progressive loss of germ cells in adult males [9,10].

The expression and crucial roles of murine Piwi proteins have been well documented in the male germline, whereas except for Mili, less is known in the female germline. Mammalian folliculogenesis, which contains primordial folliculogenesis, establishment of a primordial follicle pool, and postnatal follicle development, is a complicated physiologic process regulated by many endocrine, paracrine, and autocrine factors [11,12]. Considering the similarity between gametogenesis of males and females, Piwi proteins might have roles in folliculogenesis and oogenesis. Also, some piRNAs were recently identified in the mouse ovary [13,14]; therefore, Piwi proteins as partners might also be present in the mouse ovary and have a role limited to ovarian function. Indeed, the expression of Mili has been detected in female germ cells from the arrested to the growing oocyte stages [15].

All the above evidence suggested the possibility of existence and function of Piwi proteins in mouse female germline cells, similar to other species. However, whether Miwi is present in the mouse ovary has not been well characterized. Previous studies [16,17] did not detect Miwi mRNA in mouse ovarian RNA by reverse transcription polymerase chain reaction (RT-PCR), but it can not be excluded that the Miwi mRNA in the total ovarian RNA was too low to be detected, because oocytes only account for a small proportion of cells in the adult ovary. For instance, mouse Vasa homologue (Mvh) mRNA exists in mouse oocytes, but was undetectable by RT-PCR from total ovarian RNA of the adult mouse [18]. In this study, we detected the restricted expression of Miwi in mouse oocyte, determined its temporal expression during postnatal ovary development, and revealed the distribution features of Miwi in various stages of folliculogenesis.

2. Materials and methods

2.1. Mice

This study was approved by the institutional review board at our facility. Mouse (BALB/c strain) were used. Natural mating was performed to produce neonates for the study of Miwi expression in neonatal ovaries. Adult male mice were caged at night with adult female mice. Female mice were examined the next morning for vaginal plugs.

2.2. Preparation of ovaries, oocytes, and granulosa cells

Mice were humanely killed by cervical dislocation after intraperitoneal injection of 0.2 mL of 10% (wt/vol) chloral hydrate.

Oocytes and granulosa cells of adult mice were harvested by follicle puncture. Ovaries were excised and placed in 35 mm culture dishes within HTF-HEPES medium (InVitroCare Inc., Frederick, MD, USA) supplemented with 0.5% (wt/vol) human serum albumin. After follicle puncture using an injection needle (27.5 ga), oocytes (with granulosa cells) were collected with a Pasteur pipette (under a stereomicroscope) and transferred into an IVF-30 (Vitrolife, Göteborg, Sweden) droplet. Subsequently, granulosa cells were collected carefully by a Pasteur pipette and transferred into a tube. Oocytes and granulosa cells were collected for Western blot and RNA extraction (for RT-PCR).

Ovaries were individually collected from adult (aged 10 weeks) and neonatal females at 0, 1, 2, 3, and 4 weeks postpartum. For each age group, five or six mice were used to obtain the ovaries, and both ovaries were removed. Ovaries were either fixed in 4% (wt/vol) neutral buffered paraformaldehyde and processed for immunofluorescence, or immediately cryostored in liquid nitrogen for subsequent RNA isolation for RT-PCR analysis, and protein extraction for Western blot.

Testis of adult mice were used as positive controls in experiments of RT-PCR, Western blot, and immunofluorescence.

2.3. RNA isolation and RT-PCR

Total RNAs from liquid nitrogen frozen tissues (mouse ovaries and testes), and cells (oocytes and granulosa cells) were extracted with TRI Reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini kit (Qiagen, Hilden, German) respectively, according to the manufacturer instruction. The purity of total RNA was checked spectrophotometrically at 260 nm and 280 nm. A minimum optical density (OD260/280) ratio 1.80 was required for the following RT-PCR.

Nonquantitative RT-PCR was performed as described [19], to identify the presence of Miwi transcript in mouse oocytes. Briefly, DNase I treatment (Takara, Dalian, China) was carried out according to the manufacturer instructions to remove any contaminating DNA. Then RNA was reverse transcribed to complementary DNA using oligo(dT) primers and MMLV reverse transcriptase (Toyobo, Osaka, Japan). Mouse testicular RNA reverse-transcribed without MMLV reverse transcriptase was used as negative control. The complementary DNAs were further amplified by polymerase chain reaction (PCR). To eliminate the contamination by any possible remnant genomic DNA, primers were chosen in different exons: forward 5′-CCATCGCAGGATTCGTCG-3′ (in exon 17), reverse 5′-ATGTGGTC GGGCTTCAGG-3′ (in exon 21). The PCR amplification was performed on a Gradient thermal cycler (Eppendorf, Hamburg, Germany) in a final volume of 25 μL PCR mixture consisted of 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM deoxy-ribonucleoside triphosphates, 1.5 IU TaKaRa Taq DNA polymerase (Takara) and 0.4 μM of each primer. The thermal profile was as follows: A 5-minute denaturation at 94 °C; 35 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds; followed by a final extension at 72 °C for 5 minutes. The PCR products were analyzed on a 1.5% (wt/vol) agarose gel. The gel-extracted PCR products were inserted in the pCR2.1-TOPO vector (Invitrogen) and were subjected to DNA sequencing to confirm the correct amplification. Experiments were repeated at least three times using oocytes, granulosa cells, and ovaries obtained from different animals.