Tudor Domain Containing Protein TDRD12 Expresses at the Acrosome of Spermatids in Mouse Testis

Min Kim[a], Byeong Seong Ki[a], Kwonho Hong[1], Se-pill Park[2], Jung-Jae Ko*, and Youngsok Choi*

Department of Biomedical Science, CHA University, 335 Pangyo-ro, Seongnam-si, Gyeonggi-do 13488, Korea

ABSTRACT: *Tdrd12* is one of tudor domain containing (*Tdrd*) family members. However, the expression pattern of *Tdrd12* has not been well studied. To compare the expression levels of *Tdrd12* in various tissues, real time-polymerase chain reaction was performed using total RNAs from liver, small intestine, heart, brain, kidney, lung, spleen, stomach, uterus, ovary, and testis. *Tdrd12* mRNA was highly expressed in testis. Antibody against mouse TDRD12 were generated using amino acid residues SQRPNEKPLRLTEKKDC of TDRD12 to investigate TDRD12 localization in testis. Immunostaining assay shows that TDRD12 is mainly localized at the spermatid in the seminiferous tubules of adult testes. During postnatal development, TDRD12 is differentially expressed. TDRD12 was detected in early spermatocytes at 2 weeks and TDRD12 was localized at acrosome of the round spermatids. TDRD12 expression was not co-localized with TDRD1 which is an important component of piRNA pathway in germ cells. Our results indicate that TDRD12 may play an important role in spermatids and function as a regulator of spermatogenesis in dependent of TDRD1. (**Key Words:** Tdrd12, Tudor, Spermatogenesis, Acrosome, Spermatid, Testis)

INTRODUCTION

Spermatogenesis is a complicated process in the testis. Once arrived at primitive embryonic testis, primordial germ cells are differentiated to spermatogonia. The sperm is differentially generated from spermatogonia via a series of meiotic process. At puberty, some of spermatogonia become primary spermatocytes entering meiosis and produce two secondary spermatocytes. Secondary spermatocytes turn to four haploid round spermatids by completion of meiosis. The round spermatids are transformed into sperms under spermiogenesis (Matzuk and Lamb, 2008). The round spermatid has a unique cellular structure, a chromatoid-body in the cytoplasm (Meikar et al., 2011). The chromatoid-body contains a variety of RNAs and RNA-binding proteins that are involved in RNA protecting, decay, and silencing (Kotaja and Sassone-Corsi, 2007). Among RNA-processing proteins, tudor domain containing (TDRD) family proteins are important for germ-cell specific small RNA, piRNA biogenesis (Bak et al., 2011).

In mammals, 12 members of TDRD family proteins were identified since *Drosophila* tudor protein was discovered (Ying and Chen, 2012). Many members of TDRD family proteins are involved in germ cell development. For example, TDRD1 known as mouse tudor repeat-1 was originally found in spermatogonia and functions as an essential regulator for male germ-cell development (Wang et al., 2001; Chuma et al., 2003; Chuma et al., 2006). TDRD1 is highly expressed in fetal prospermatogonia and postnatal primary spermatocytes (Chuma et al., 2003). The localization of TDRD1 is exclusively restricted to the chromatoid bodies of late stage spermatocytes and round spermatids. *Tdrd1* deficient mice

were sterile due to prevention of the meiotic process in the spermatocyte (Chuma et al., 2006). *Tdrd2/Tdrkh* was identified as a component in the Miwi complex (Chen et al., 2009). TDRD2 is critical for piRNA biogenesis in the germline with Miwi protein. *Tdrd2* knockout mice are sterile resulting from the defect of spermatogenesis (Saxe et al., 2013). TDRD4/ring finger protein 17 (RNF17) and TDRD5 are mainly expressed in chromatoid bodies and involved in RNA processing for spermatogenesis (Smith et al., 2004; Pan et al., 2005). *Tdrd5* deficiency in mice leads to spermatogenetic arrest at the round spermatid stage through unregulated retrotransposon silencing (Yabuta et al., 2011). TDRD6 is a regulator for miRNA function and plays an important role in chromatoid body organization and spermiogenesis (Vasileva et al., 2009). TDRD7 is ubiquitously expressed (Lachke et al., 2011; Tanaka et al., 2011). *Tdrd7* disruption causes male sterility, cataract and glaucoma (Lachke et al., 2011; Tanaka et al., 2011). In the testis, TDRD7 is involved in suppression of long interspersed nuclear elements-1 (LINE-1) retrotransposons (Tanaka et al., 2011). TDRD8/Serine/Threonine kinase 31 (STK31) is expressed in mid-to-late spermatocyte cytoplasm and interacts with Piwi-like RNA-mediated gene silencing 1 (PIWI1) protein (Bao et al., 2012). TDRD9 also forms a protein complex with MIWI2 (Shoji et al., 2009). TDRD9 contains an ATPase/DExH-box ATPase (DExH)-type helicase and a Tudor domain and functions in silencing LINE-1 retrotransposon during spermatogenesis. Deficiency of *Tdrd9* in male mice is associated with sterility by failure of chromosome synapsis (Shoji et al., 2009). TDRD11/stapihylococcal nuclease domain containing 1 (SND1) is involved in multiple cellular process such as double-stranded RNA editing, pre-mRNA splicing, microRNA-mediating gene silencing and piRNA biogenesis in germlines (Callebaut and Mornon, 1997; Leverson et al., 1998; Yang et al., 2002; Caudy et al., 2003; Li et al., 2008; Paukku et al., 2008; Gao et al., 2012; Garcia-Lopez et al., 2013).

TDRD12 has been recently characterized as a TDRD family protein which has two tudor domains and a DEAD box. *Drosophila* TDRD12 ortholog is known to interact with the essential piRNA pathway factor Vreteno and regulates piRNA biogenesis in ovarian germline cells (Handler et al., 2011). TDRD12 in mice was also identified as a component of Piwi protein Piwi-like RNA-mediated gene silencing 2 (PIWI2) ribonucleoprotein complex including small RNAs, MILI and TDRD1, although TDRD12 does not directly interact with MILI and TDRD1. *Tdrd12* deficiency in male mice induces atrophied testes (Pandey et al., 2013). The defects in *Tdrd12* null mice resulted from loss of MIWI2-bound piRNA that is important for overall piRNA biogenesis. In this study, we

generated anti-mouse TDRD12 antibody to investigate the expression pattern of TDRD12 in the mouse testis and demonstrated the differentially expressing TDRD12 in testis during postnatal development.

MATERIALS AND METHODS

Animals

All mice experiments were performed on 2 to 8week-old ICR mice provided by Orient Bio Company (Seongnam, Korea). Mice were housed under temperature and light controlled conditions with the lights on for 12 hours daily and given with a free access to food and water. Care of mice and experimental procedures were conducted with the Guide for the Care and Use of Laboratory Animals (No. IACUC140032). All experiments were approved by the Institutional Animal Care and Use Committee of CHA University. Mouse testis was frozen and stored at –80°C to isolate RNA and protein extraction or fixed in 4% paraformaldehyde solution to make paraffin block.

Real time-polymerase chain reaction analysis

Total RNA from testis was isolated using Trizol, as per the manufacturers' instruction (Invitrogen, Carlsbad, CA, USA). Two micrograms of total RNA were converted to cDNA using TOPscript cDNA Synthesis kit (Enzynomics, Daejeon, Korea), followed by PCR amplification. The primers used for real time-polymerase chain (RT-PCR) were as follows: *Tdrd1* (5′-AGCAGAGACTGCTGTTGCAC-3′ and 5′-CACAGCGGAGAGGCTAAAAG-3′), *Tdrd2* (5′-AAGGACATGGCCACAGAAAC-3′ and 5′-CGTAGGA GCACAGGGAAGAG-3′), *Tdrd3* (5′-ACGCAGCAGTTT TACCAAC-3′ and 5′-CCCCATTGCCTTGTGTAAAT-3′), *Tdrd4* (5′-CCAAGCCATAAAGGTGCTCT-3′ and 5′-TTATCTGCAAGCCCCATTTC-3′), *Tdrd5* (5′-GGCACA AAAGGACTGGTGTT-3′ and 5′-CAGGTTCAGACTGGC TCCTC-3′), *Tdrd6* (5′-TCCAAACAGTGGGAAAGGAC-3′ and 5′-GACAACTTCCAGGGGTCAAA-3′), *Tdrd7* (5′-AGTGCTGCTTGGCTGATCTT-3′ and 5′-GTTTCCACA GGTCTGCGTTT-3′), *Tdrd8* (5′-AGCAGCCAGAAGGTT TTTGA-3′ and 5′-GCAAGGTGGCAATGAACTTT-3′), *Tdrd9* (5′-CACCAGTGATGGAGCTGAG-3′ and 5′-GTGC ATTCTCCTGCAACTGA-3′), *Tdrd11* (5′-GAATACCTGA ATGCCCAGG-3′ and 5′-TTCAATGCTAGCCACGACAG-3′), Tdrd12 (5′-CACCAGTTGCAGAAGGTGA-3′ and 5′-CGCAAAGCCCTGTATTTCAT-3′), and *Gapdh* (5′-AGGT CGGTGTGAACGGATTT-3′ and 5′-GTAGACCATGTAG TTGAGGTCA-3′).

Antibody generation and Western blot analysis

Antibody against mouse TDRD12 was generated by immunizing rabbits with the keyhole-limpet hemocyanin-conjugated synthetic peptide SQRPNEKPLPLTEKKDC

that corresponded to amino acid residues 318-334 (Young-in Frontier, Seoul, Korea). The antibody was affinity-purified and the specificity of the antibody was validated by Western blotting and immunofluorescence staining. Protein extract from testis was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4% to 20% gradient gel), transferred to nitrocellulose membrane and blocked with 5% non-fat milk in tri-buffered saline (TBS) containing 0.1% Tween 20 (TBST). The membrane was incubated with anti-TDRD12 and anti-α-tubulin antibodies diluted in TBST-5% milk at 4°C for overnight, followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. The membrane was developed using ECL Western Blotting substrate kit (GenDEPOT, Barker, TX, USA).

Immunofluorescence

The deparaffinized tissue section was incubated with blocking buffer (4% bovine serum albumin and 5% normal IgG in phosphate-buffered saline (PBS) for 1hour at room temperature and incubated with primary antibody in the blocking buffer for overnight at 4°C. The antibodies used in this study were anti-TDRD12 (1:200), Lectin-peanut agglutinin (PNA) (1:2,000, Molecular Probes, Eugene, OR, USA), TDRD1 (1:200, R&D systems, Minneapolis, MN, USA), and DEAD-box helicase 4 (DDX4) (1:500, Abcam, Cambridge, UK) antibodies. After washing with PBS, the sections were incubated with the secondary antibodies (1:1,000) in blocking buffer for 2 hours at room temperature. The testis sections were washed with PBS and mounted. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear counter stating of testis section. Slides were viewed and imaged with a confocal microscope, LSM 510 Meta (Carl Zeiss Co., Hamburg, Germany) equipped with three lasers.

RESULTS

Expression of *Tdrd* family members in the tissues

To examine the expression of *Tdrd* family members in tissues, RT-PCR analysis was performed using total RNAs from various tissues; liver, intestine, heart, brain, kidney, lung, brain, uterus, ovary, and testis (Figure 1). The expression patterns of twelve *Tdrd* genes are shown in Figure 1A. *Tdrd3*, *Tdrd7*, and *Tdrd11* were expressed in all tissues tested. The expression of *Tdrd2*, *Tdrd5*, and *Tdrd12* was detected in several tissues. On the other hand, *Tdrd1*, *Tdrd4*, *Tdrd6*, *Tdrd8*, and *Tdrd9* were exclusively expressed in the testis and/or the ovary (Figure 1A). These are consistent with previous reports showing *Tdrd1*, *Tdrd2*, *Tdrd4*, *Tdrd5*, *Tdrd6*, *Tdrd8*, *Tdrd9*, and *Tdrd12* in germline cells. *Tdrd12* was highly detected in the testis, whereas other tissues including the ovary and heart showed very low expression (Figure 1A). Next, we looked into the
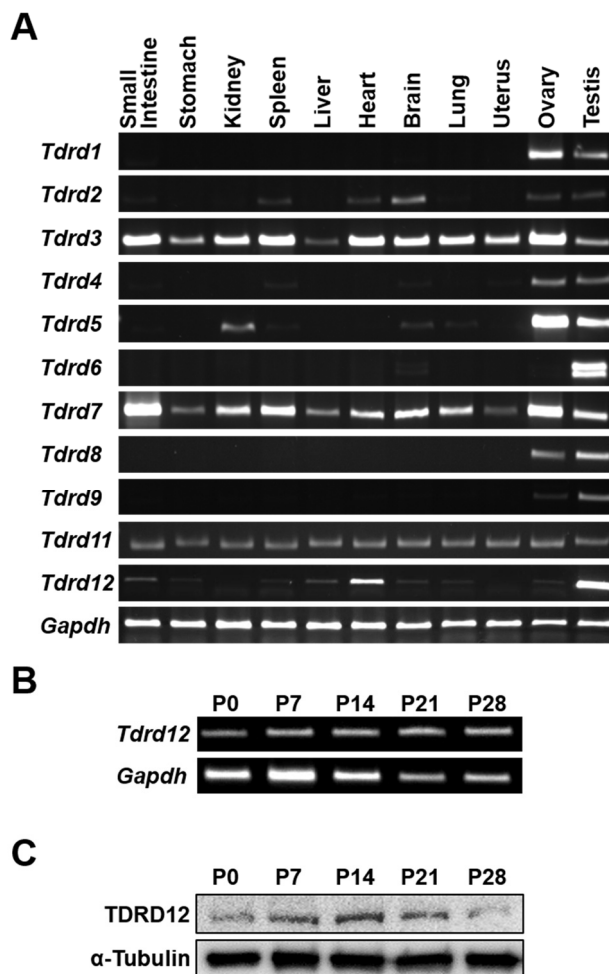

Figure 1. Gene expression of *Tdrd* family members in mouse tissues. (A) Expression of *Tdrd* family members were analyzed by RT-PCR. Total RNAs were extracted from mouse tissues; small intestine, stomach, kidney, spleen, liver, heart, brain, lung, uterus, ovary and testis. *Gapdh* was used as control. (B) Differential expression of *Tdrd12* mRNA in the testis during postnatal development. Total RNAs were prepared from testes of postnatal day 0 (P0), P7, P14, P21, and P28 ICR mice. *Gapdh* was used as control. (C) Differential expression of TDRD12 in the testis during postnatal development. Testicular extracts were prepared from testes of postnatal day 0 (P0), P7, P14, P21, and P28 ICR mice. The alpha-tubulin (α-tubulin) used as loading control. Tdrd, tudor domain containing; RT-PCR, reverse transcription-polymerase chain reaction; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; ICR, Institute for cancer research mice.

expression level of *Tdrd12* mRNA in the testis during postnatal development (Figure 1B). *Tdrd12* mRNAs were detected in the testis from postnatal day 0 (P0) and then remained high to P28.

To analyze TDRD12 protein level in testis, we generated specific antibody against mouse TDRD12 using a small peptide SQRPNEKPLPLTEKKDC (318th to 334th amino acids of mouse TDRD12). TDRD12 (~130 kDa) was detected in the testis during postnatal development at P0, P7,

P14, P21, and P28. The protein was highly detected at P14 and then decreased at P21 (Figure 1C).

Expression and localization of Tdrd12 protein in the testis

To investigate the localization of TDRD12 in mouse testis, immunofluorescence was performed using anti-TDRD12 antibody together with the stage specific markers. Anti-DDX4 antibody for germ cell, anti-TDRD1 antibody for spermatocyte, and anti-lectin-PNA antibody for spermatid marker were used for the stage specific markers in the testis. DDX4 was detected in the cytoplasm of spermatocyte and chromatoid body of round spermatid (Figure 2A). However, TDRD12 was not co-localized with DDX4. The staining pattern of TDRD12 was similar to a protein in acrosome structure in round spermatids (Figure 2A). To test whether TDRD12 is expressed in acrosome, we co-stained tissues with antibodies against TDRD12 and lectin-PNA (Kallajoki et al., 1986). The localization of TDRD12 was completely overlapped with lectin-PNA in acrosome of round spermatid (Figure 2B). Next, we examined the co-localization of TDRD12 with TDRD1 which is a well characterized TDRD family member. TDRD12 was not co-localized with TDRD1 in the seminiferous tubule of testis, which was detected only in the spermatocyte, not in spermatid (Figure 3). This result suggested that the function of TDRD12 might not be associated with TDRD1 which acts as a molecular scaffold for piRNA biogenesis factors.

We also investigated the expression and localization TDRD12 in mouse testis during postnatal development at 2, 4, 6, and 8 weeks. At early stage of 2 weeks, TDRD12 was detected in the cytoplasm of primary spermatocyte (Figure 4). However, the expression of TDRD12 protein was localized in the acrosome of the spermatid at 4 week when the round spermatids are present in the seminiferous tubule.
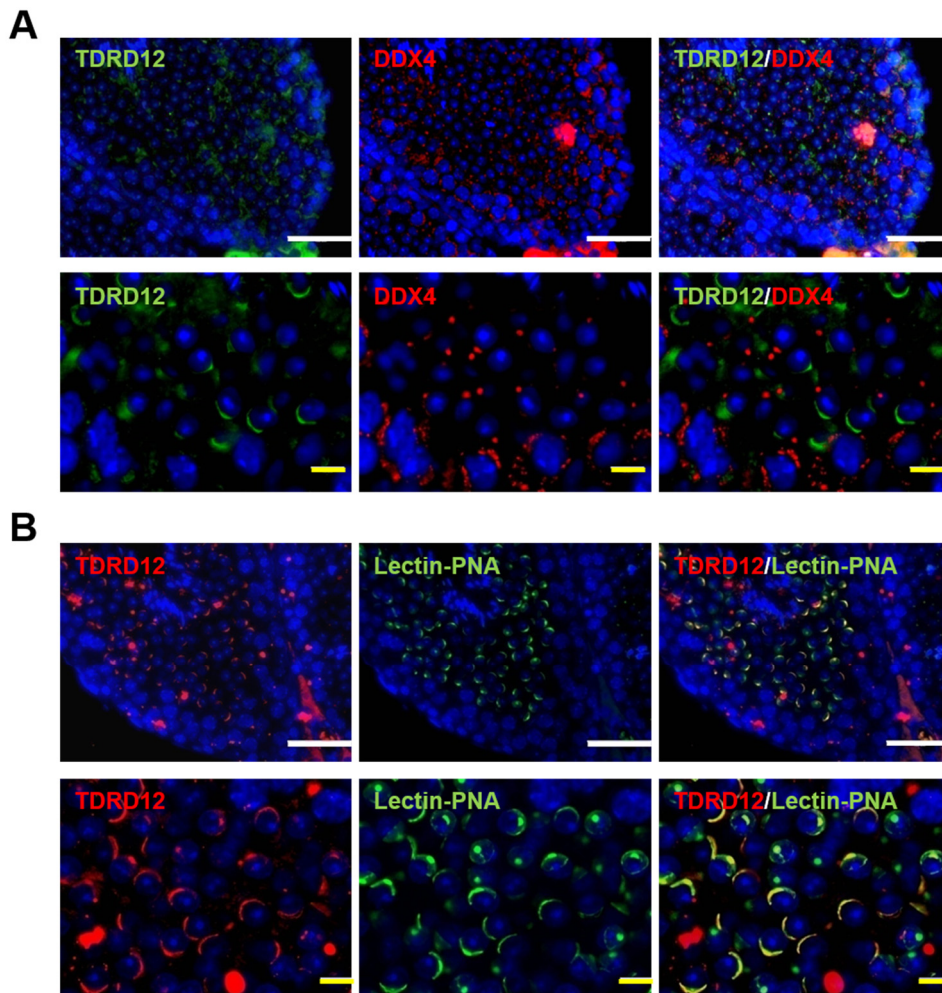

Figure 2. Localization of TDRD12 in acrosome of round spermatids. (A) Co-immunostaining of testis with antibodies against DDX4 and TDRD12. (B) Co-immunostaining of testis with antibodies against lectin-PNA and TDRD12. Tissue sections were prepared from 6-weeks-old testis. White scale bars indicate 100 μm and yellow bars indicate 10 μm. TDRD, tudor domain containing; DDX, DEAD-box helicase 4; PNA, peanut agglutinin.
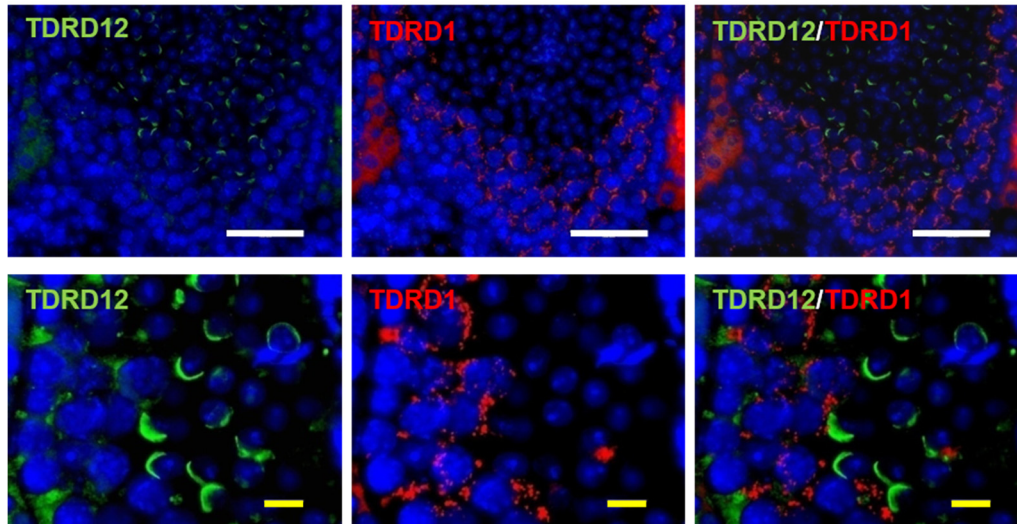
Figure 3. Co-localization of TDRD12 and TDRD1 in the mouse testis. Tissue sections were prepared from 6-weeks-old mouse. Anti-TDRD12 and anti-TDRD1 antibodies were used for immunofluorescence. White scale bars indicate 100 μm and yellow bars indicate 10 μm. TDRD, tudor domain containing.

The localization of TDRD 12 was limited in the spermatids at 6 and 8 week (Figure 4).

DISSCUSSION

In this study we demonstrated that the expression of TDRD12 is limited at the acrosome of spermatids in the mouse testis. As described in the introduction, Tdrd family possesses 12 members including TDRD12. Their expressions vary in the tissues. *Tdrd3*, *Tdrd7*, and *Tdrd11* ubiquitously express in various tissues. However, *Tdrd1*, *Tdrd5*, *Tdrd6*, *Tdrd8*, and *Tdrd9* are exclusively expressed in the gonad, ovary and testis. This implies that they play an important role in the gonad. Mutant mice models show that these members regulate the development and differentiation of male gonad. The deficiency of *Tdrd1* causes the defect in the spermatogenesis and germinal granule formation in mice resulting in infertility of male mice (Chuma et al.,
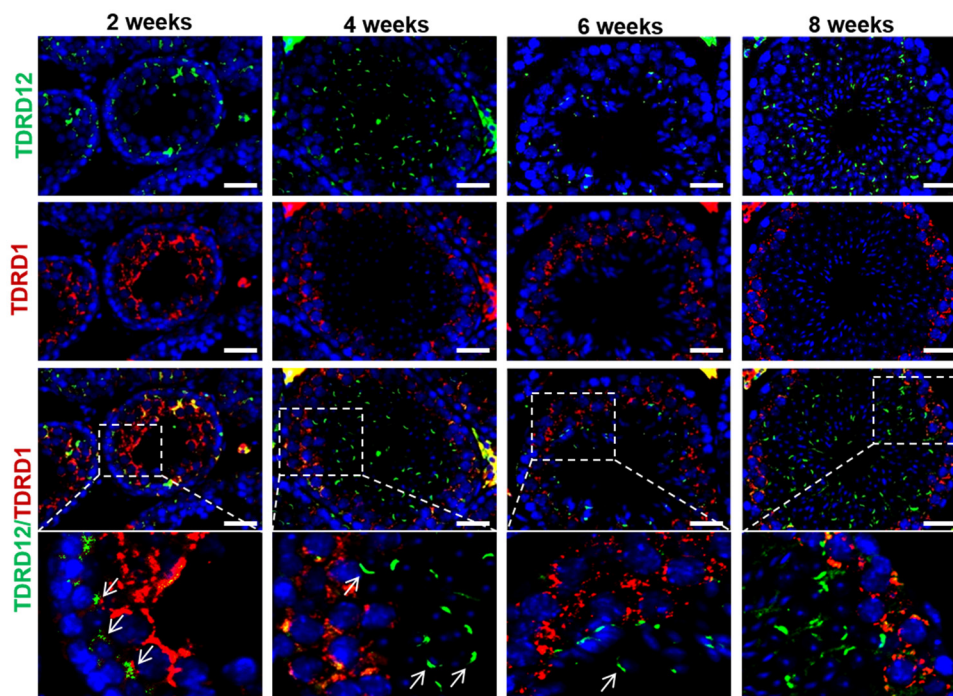

Figure 4. Differential expression of TDRD1 and TDRD12 in mouse testis during development. Tissue sections were prepared from 2, 4, 6, and 8 weeks-old testis. Anti-TDRD12 and anti-TDRD1 antibodies were used for immunofluorescence. Arrow represents acrosome. Scale bars indicate 50 μm. TDRD, tudor domain containing.

2006). *Tdrd5* is essential for transposon silencing and spermiogenesis in the mice (Yabuta et al., 2011). *Tdrd6* is also crucial for spermiogenesis by regulation of miRNA (Vasileva et al., 2009). The deficiency of *Tdrd7* causes male sterility, age-related cataract and glaucoma (Lachke et al., 2011; Tanaka et al., 2011). In the testis, *Tdrd7* is crucial for spermatid development by remodeling of chromatoid bodies (Tanaka et al., 2011). The disruption of *Tdrd9* leads to the activation and increase of Line-1 transposons in the germ cell resulting in male infertility (Shoji et al., 2009). The TDRD9-MIWI2 complex cooperates with another tudor-piwi complex, Tdrd1-MILI, for the regulation of piRNA biogenesis and pathway. TDRD7 is also involved in regulation of retrotransposons independently of piRNA biogenesis.

The regulation of piRNA biogenesis is very important in germ cells. The piRNAs are germ-cell specific small noncoding RNAs and act through Piwi-like proteins (Piwil) and Tudor-domain repeat proteins (Bak et al., 2011). Piwil proteins include three family members; Piwil1 (MIWI1), Piwil2 (MILI), Piwil4 (MIWI2) (Bak et al., 2011). Piwil proteins interact with piRNA to form Piwil-piRNA complex which is critical for preventing desperate activity of transposable elements (Bortvin, 2013; Luteijn and Ketting, 2013). TDRD1 is very important for piRNA pathway during spermatogenesis. TDRD1 interacts with Piwil2 in the intermitochondrial cement (known as pi-body) (Aravin et al., 2009; Reuter et al., 2009). In the pi-body, the complex interacts with mouse vasa homolog Mvh RNA helicase (DDX4). Whereas, TDRD9-MIWIcomplex is present in piP-bodies (van der Heijden et al., 2010). The piP-bodies contain also DDX4. DDX4 plays a role for RNA substrate exchange between pi-bodies and piP-bodies as predicted by the ping–pong mechanism (Reynolds et al., 2005).

TDRD12 was not co-localized with either DDX4 or TDRD1 during spermatogenesis. TDRD12 is exclusively localized at the acrosome of the spermatid. This pattern is totally different from other members that are present in pi-body or piP-body. The transcript of *Tdrd12* seems to be hardly detected in several tissues. However, the expression of *Tdrd12* was high in the testis and the heart compared to that in other tissues (Figure 1A). This implies that TDRD12 might play a role in the testis and the heart. Recent study revealed that *Tdrd12* disruption causes male sterility and is very important for secondary piRNA biogenesis in mice (Pandey et al., 2013). However, the localization and expression pattern of TDRD12 protein remains unknown in the testis. We showed that Tdrd12 was exclusively localized in the acrosome of spermatid. These results suggest that Tdrd12 might play an important role in spermatogenesis in the testis.

In this study, we showed that TDRD12 was exclusively localized in the acrosome of spermatid during spermatogenesis in the testis. It needs further investigation for a better understanding a role of TDRD12 in the acrosome of spermatid, which will provide a new function of tudor containing protein during germ cell development.

AUTHOR CONTRIBUTIONS

Experiment and writing (MK, BSK), Experiment (KH, SP), and Experimental Design and writing (JJK, YC).

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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