KH-type splicing regulatory protein is a new component of chromatoid body

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Abstract

The chromatoid body (CB) is a specific cloud-like structure in the cytoplasm of haploid spermatids. Recent findings indicate that CB is identified as a male germ cell-specific RNA storage and processing center, but its function has remained elusive for decades. In somatic cells, KH-type splicing regulatory protein (KSRP) is involved in regulating gene expression and maturation of select microRNAs (miRNAs). However, the function of KSRP in spermatogenesis remains unclear. In this study, we showed that KSRP partly localizes in CB, as a component of CB. KSRP interacts with proteins (mouse VASA homolog (MVH), polyadenylate-binding protein 1 (PABP1) and polyadenylate-binding protein 2 (PABP2)), mRNAs (*Tnp2* and *Odf1*) and microRNAs (microRNA-182) in mouse CB. Moreover, KSRP may regulate the integrity of CB via DDX5-miRNA-182 pathway. In addition, we found abnormal expressions of CB component in testes of *Ksrp*-knockout mice and of patients with hypospermatogenesis. Thus, our results provide mechanistic insight into the role of KSRP in spermatogenesis.

Reproduction (2017) **154** 723–733

Introduction

After meiotic divisions of spermatogenesis, transcription is inactive due to highly compacted chromatin in post-meiotic round spermatids. The regulation of gene expression in this stage is mainly under post-transcriptional control (Kimmins & Sassone-Corsi 2005). This phase of spermatogenesis can form germ cell-specific structure—the chromatoid body (CB). In mammals, the CB appears in the cytoplasm from pachytene spermatocytes, forms a fibrous perinuclear granule in mature round spermatids and gradually disappears in late spermatids (Parvinen 2005, Kotaja & Sassone-Corsi 2007, Yokota 2008). The CB is composed of an abundance of RNA-binding proteins, mRNA, microRNA (miRNA) and piRNA. Therefore, the CB is identified as a male germ cell-specific RNA storage and processing center (Kotaja & Sassone-Corsi 2007, Yokota 2008, Meikar *et al.* 2014). Recently, introns were identified in the chromatoid body (Meikar *et al.* 2014, Cullinane *et al.* 2015). Although the CB has been studied

for several decades, its function in spermatogenesis and male infertility is little known. The sterility phenotype of various knockout mice lacking CB constituent proteins suggests the CB may play an important role in spermatogenesis (Tanaka *et al.* 2000, 2011, Deng & Lin 2002, Paronetto *et al.* 2009, Yabuta *et al.* 2011).

The KH-type splicing regulatory protein (KSRP, also known as FBP2) is one member of the far upstream element (FUSE)-binding protein (FBP) family (Davis-Smyth *et al.* 1996, Gherzi *et al.* 2010). KSRP is distributed in both nuclear and cytoplasmic compartments of the somatic cells (Trabucchi *et al.* 2009, Gherzi *et al.* 2010, Briata *et al.* 2013). KSRP has three distinct regions: a mainly structured central region, which includes four KH domains responsible for nucleic acid binding and two N- and C-terminal low sequence complexity regions that are involved in post-translational modifications and protein-interactions (Gherzi *et al.* 2010, Briata *et al.* 2013). KSRP as a multifunctional RNA-binding protein can regulate AU-rich-element (ARE)-containing mRNAs decay (Gherzi *et al.* 2004, Garcia-Mayoral

et al. 2007) and promote the maturation of select miRNAs by interacting with both Drosha and Dicer (Ruggiero *et al.* 2009, Trabucchi *et al.* 2009). During miRNA biogenesis, KSRP via an AGGGU sequence at miRNA terminal loop stimulates primary miRNAs (pri-miRNAs) and precursor miRNAs (pre-miRNAs) processing (Trabucchi *et al.* 2009, Nicastro *et al.* 2012). Studies in somatic cells revealed that KSRP is essential for regulating cell proliferation and differentiation, innate immune response and DNA damage response (Lin *et al.* 2011, Zhang *et al.* 2011, Repetto *et al.* 2012). However, the function of KSRP in spermatogenesis is poorly understood.

In this study, the role of KSRP in spermatogenesis is investigated. We demonstrate that KSRP partially localized in CB as a component. KSRP could interact with proteins, mRNAs and miRNAs in CB. Moreover, KSRP may regulate the integrity of CB via DDX5-miRNA-182 pathway. We also found abnormal expressions of CB components in testes of *Ksrp*^{-/-} mice and of patients with hypospermatogenesis. Our study provides insights into an important role of KSRP in spermatogenesis.

Materials and methods

Mice and human testicular samples

Ksrp-knockout and wild-type mouse testes were obtained from the Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham (Lin *et al.* 2011). *Miw1*^{+/-} and *Miw1*^{-/-} mouse testes were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Male ICR mice were obtained from the Animal Center, School of Medicine, Shanghai Jiaotong University (SJU, Shanghai, China). All the experiments on live vertebrates were performed in accordance with the relevant guidelines and regulations. This study received ethical approval from the institutional review boards of the International Peace Maternity & Child Health Hospital, School of Medicine, SJU (The number of ethical protocols for animal experiments is (GKLW) 2015-80).

Human testicular samples, biopsy specimens from patients with hypospermatogenesis (ages 24–34 years) and normal controls (obstructive azoospermia with normal spermatogenesis, ages 24–43 years), were obtained from the First Affiliated Hospital of Anhui Medical University (Hefei, China). All patients had at least two semen analyses showing azoospermia. All patients gave informed consent and the research received ethical approval from the institutional review boards of the International Peace Maternity & Child Health Hospital, School of Medicine, SJU and the Anhui Medical University (The number of ethical protocols for human data is (GKLW) 2015-80). Written, informed consent, which conformed to the tenets of the Declaration of Helsinki, was obtained from each participant prior to the study.

DNA constructs

The pcDNA-flag-KSRP expression vector was kindly provided by Dr Ching-Yi Chen (Department of Biochemistry & Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA). The GFP-SAM68 expression vector was provided by Dr Stéphane Richard (Lady Davis Institute for Medical Research, Jewish General Hospital, and Departments of Medicine and Oncology, McGill University, Canada). The full-length cDNA of mouse *Mvh*, *Pabp1*, *Pabp2* and *Ddx5* were obtained by RT-PCR from total RNA of mouse testis using primers containing specific restriction sites. For the construction of expression vectors, *Mvh* cDNA was cloned into p3XFLAG-myc-CMV-24 (Sigma) vectors at Sal I and Not I sites. *Pabp1* and *Pabp2* cDNAs were cloned into pEGFP-C1 (Clontech Laboratories) vectors at EcoR I and Sal I sites. *Ddx5* cDNA was cloned into p3XFLAG-myc-CMV-24 or pEGFP-C1 vectors at Sal I and BamH I sites. A series of truncated forms of *Pabp1* and *Pabp2* were amplified by PCR and sub-cloned into pEGFP-C1 vectors. Primer sequences are listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

siRNA duplexes homologous in sequence with the miRNA-182 mimic and a scrambled NC were synthesized and purified by the Shanghai Gene-Pharma Co. (Shanghai, China).

Cell culture and transfection

Cell culture and transfection were performed as previously described (Liu *et al.* 2014).

Isolation of germ cells and RNA interference

Spermatocytes (Sper) and round spermatids (Sp) were isolated from 18-dpp mice and 25-dpp mice, respectively, using the unit gravity sedimentation procedure as described previously (Bellve *et al.* 1977, Gan *et al.* 2011). Briefly, de-capsulated testes were digested with 2 mg/mL collagenase (Type IV, Sigma) (15 min, 37°C) and 0.25% trypsin containing 75 U/mL DNase I (Sigma) (15 min, 37°C). The suspension was filtered through a stainless steel filter (70 mesh) and centrifuged. The supernatant was removed, and the cells were washed twice in ice-cold PBS. Cells were then re-suspended in DMEM/F12 (1:1; Life Technologies). Cells were infected by *Ksrp* shRNA and control shRNA virus, plasmids of which were obtained from Dr Mian Wu (University of Science and Technology of China) and cultured for 24 h at 34°C in a humidified atmosphere of 5% CO₂ and 95% air. Then cells were harvested and lysed in TRIzol reagent (Invitrogen) for extracting total RNA.

The shRNA lentivirus was generated as previously described (Mei *et al.* 2011). To generate *Ksrp* shRNA and control shRNA lentivirus, HEK293T cells (grown on a 6-cm dish) were transfected with 2 µg of PLKO.1 *Ksrp* shRNA (the sequences of the KSRP mRNA: CCGGCCCTGAGAAGATTGC TCACATCTCGAG ATGTGAGCAATCTT CTCAGGGTTTTTG) or control vector, 2 µg of pREV, 2 µg of pGag/Pol/PRE and 1 µg of pVSVG. After 24-h transfection, cells were cultured with DMEM medium containing 20% FBS for an additional 24 h. The culture medium containing lentivirus particles was centrifuged

at 1000g for 5 min, and lentiviruses in the supernatant were used for infection.

Isolation of chromatoid body

CBs were isolated as previously described (Meikar *et al.* 2010). For protein assay, washed beads were boiled for 10 min in sodium dodecyl sulfate loading buffer. For RNA assay, CB RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, primer sequences of *Tnp2* are as previously described (Meikar *et al.* 2010). For immunofluorescence, CBs were eluted from beads with 100 mM Na-phosphate buffer (pH 12.0).

Immunofluorescence (IF)

Squash preparations were performed as described previously (Kotaja *et al.* 2004). Germ cells were spread out of seminiferous tubules to mono-layers, snap-frozen in liquid nitrogen and fixed in 4% PFA. After washing three times in PBS, germ cells were treated with 0.1% Triton X-100 (Sigma) in PBS for 5 min and then incubated for 1 h in 0.5% BSA in PBS. Cells were incubated with primary antibodies: rabbit anti-KSRP (Bethyl Laboratories, Montgomery, AL), rabbit anti-DDX5 (Bethyl Laboratories) in dilution 1:200 overnight at 4°C.

For paraffin-embedded testes slides, IF analysis was performed as described by Liang and coworkers (Liang *et al.* 2011). The primary antibodies of mouse anti-MVH (Abcam) and rabbit anti-KSRP (Bethyl Laboratories) were used in dilution 1:200. Alexa Fluor 488/555 conjugated secondary antibodies (Life Technologies) were used in dilution 1:200. Nuclei were stained with Hoechst 33342 (Sigma). Fluorescent signals were observed using an epifluorescence microscope (Eclipse 80i, Nikon).

Western blotting

Western blotting experiments were performed as previously described (Yin *et al.* 2013). The nuclear and cytoplasmic extracts were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. The following antibodies were used for Western blotting analysis: rabbit anti-KSRP and rabbit anti-DDX5 (Bethyl Laboratories); rabbit anti-MVH and rabbit anti- β -actin (Abcam); mouse anti-GFP (Clontech Laboratories); rabbit anti-FLAG (Sigma); rabbit anti-MIWI (Cell Signaling Technology); mouse anti-GAPDH and mouse anti-Lamin A/C (Santa Cruz Biotechnology). Protein levels were normalized to β -actin or GAPDH.

Immunoprecipitation (IP) assay

IP assay was performed as previously described (Yin *et al.* 2013) with some modifications.

To explore the KSRP and DDX5 interaction proteins in testis, adult mouse testes were lysed in TNE buffer (containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NonidetP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM activated sodium orthovanadate, 10 μ g/mL proteinase

inhibitor cocktail (Roche Diagnostics)). The lysates were centrifuged, and 10% volume of supernatants was loaded as an input fraction. The remaining supernatants were pre-cleared with Protein G agarose (GE Healthcare) for 2 h at 4°C. After rabbit anti-KSRP (Bethyl Laboratories) or rabbit anti-DDX5 (Bethyl Laboratories) or control rabbit IgG incubated with Protein G agarose (GE Healthcare) for 4–6 h at 4°C, the pre-cleared lysates were then incubated with antibody-coated recombinant Protein G agarose overnight at 4°C. Then, the beads were washed three times with TNE buffer and boiled in sodium dodecyl sulfate loading buffer.

To detect the interaction between KSRP and PABP1/2, DDX5 and PABP1/2, HEK 293T cells were transiently transfected with the indicated plasmids for 36 h. Cell lysates were prepared as described above. The remaining supernatants were incubated with rabbit anti-GFP (Abcam) or mouse anti-FLAG (Sigma) antibody overnight at 4°C. Protein samples were resolved by SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining, silver staining or immunoblotting. Separated protein bands in SDS-PAGE were excised from the gel and processed for LC-MS analysis.

RNA-IP, RNA isolation and real-time quantitative PCR

RNA-IP was carried out as described previously (Sundaram *et al.* 2013). Briefly, mouse testes were lysed in an ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NonidetP-40 and RNaseOUT (Invitrogen), 1 mM PMSF, 5 mM sodium orthovanadate and 10 μ g/mL proteinase inhibitor cocktail (Roche Diagnostics). Testes extracts were centrifuged at 14,000g for 15 min, and 10% volume of supernatant was loaded as an input fraction. Remaining supernatants were pre-cleared with Protein G agarose (GE Healthcare) for 2 h at 4°C. After 2 μ g rabbit anti-KSRP (Bethyl Laboratories) or control rabbit IgG (negative serum) incubated with recombinant Protein G agarose for 4–6 h at 4°C, beads were washed three times with ice-cold lysis buffer. The pre-cleared lysates were then incubated with antibody-coated recombinant Protein G agarose overnight at 4°C.

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in an Applied Biosystems StepOne real-time PCR system (Applied Biosystems). For mRNAs real-time PCR assays, cDNA was synthesized from total RNA using a PrimeScript RT reagent kit (Takara Bio), and the expression of mRNAs was measured using a SYBR Premix Ex Taq II kit (Takara Bio), as described previously (Yao *et al.* 2010). RNA-IP results were normalized to input RNA levels and plotted as fold enrichment compared to the IgG control RIP. The mRNA levels were normalized to GAPDH mRNA. Expression levels of miRNAs were measured using TaqMan microRNA assays (Applied Biosystems) according to the manufacturer's instructions, and data were normalized to U6 snRNA (Applied Biosystems). Primer sequences are listed in [Supplementary Table 2](#).

miRNA microarray analysis

See [Supplementary data](#) for details.

In vitro transcription of biotinylated *Tnp2*

See [Supplementary data](#) for details.

Biotin pull-down assay

See [Supplementary data](#) for details.

Statistical analysis

All experiments were repeated at least three times. Data were showed as mean $\pm$ S.E.M. Statistical analysis was applied by Student's *t*-test, using SAS Software. *P* values <0.05 were considered to be statistically significant.

Results

KSRP localizes in CB

To understand the role of KSRP in testis, the protein levels and cellular localization of KSRP were determined (Fig. 1). Western blotting analysis showed that KSRP was expressed through spermatogenesis and present in both nuclei and cytoplasm (Fig. 1A and B). Cellular localization of KSRP showed that KSRP is mainly expressed in spermatocytes and round spermatids (Fig. 1C and D). We also observed that KSRP partially co-localized with mouse VASA homolog (MVH) in some round spermatids (Fig. 1E). CB appears in pachytene spermatocytes and round spermatids, of which MVH is considered to be a marker (Parvinen 2005, Kotaja & Sassone-Corsi 2007, Yokota 2008). To further verify whether KSRP had a relationship with CB, we isolated mouse CB, and validated that CB were enriched with high purity by immunostaining, Western blot and RT-PCR assay (Supplementary Fig. 1A, B, C, D and E). In addition, we characterized global miRNA profiling of CB (Supplementary Fig. 1F). MiR-34c* and miR-182 levels in CB were further quantified by real-time PCR to verify the accuracy of microarray (Supplementary Fig. 1G). The CB pulled-down by MVH antibody was then stained with KSRP antibody. As shown in Fig. 1F, the KSRP signal was only detectable in MVH-pull-down samples but not in IgG controls. These data suggest that KSRP is a CB component.

KSRP interacts with CB constituent proteins in the cytoplasm

As KSRP showing co-localization with MVH in CB, we first examined whether KSRP interacted with MVH in CB as well. Co-IP assays verified that KSRP indeed interacted with MVH in adult mouse testes (Fig. 2A) and isolated CB (Fig. 2B). Furthermore, co-IP with MVH antibody in testicular nuclear and cytoplasmic fractions showed that the interaction between KSRP and MVH occurred in the cytoplasm (Fig. 2C). Due to the RNA-binding feature of KSRP (Gherzi *et al.* 2004, Garcia-Mayoral *et al.* 2007),

Figure 1 KSRP is expressed in the mouse testis and localized in CB. (A) Western blotting analysis of KSRP protein expression in the mouse testes on days postpartum (dpp) 0, 5, 8, 14, 21 days (d), 1 month and 2 months (m). Actin was used as a protein loading control. (B) Western blotting analysis of KSRP distribution in testicular fractions from adult mouse testes. GAPDH and Lamin A/C were used as cytoplasmic and nuclear markers respectively. (C) Western blotting analysis of KSRP protein expression in testicular spermatocytes (Sper), which are isolated from 18-day-old mice and round spermatids (Sp), which are isolated from 25-day-old mice. Actin was used as a protein loading control. (D) IF staining for KSRP protein. Squash preparations from adult mouse testes were stained with KSRP antibody (green). Nuclei (blue) were stained with Hoechst. Scale bars: 10 μ m. (E) KSRP (red) and MVH (green) are co-localized at CB (white arrows) in adult mouse round spermatids. Nuclei (blue) were stained with Hoechst. Scale bars: 10 μ m. (F) CBs were eluted from beads with 100 mM Na-phosphate buffer (pH 12.0). MVH: anti-MVH antibody, as CB marker. IgG: negative rabbit serum, as negative control. IF staining with KSRP antibody (red), and CBs are shown as anti-KSRP positive granules (white arrows). Scale bars: 10 μ m.

and the accumulation of RNA in CB (Kotaja & Sassone-Corsi 2007, Yokota 2008, Meikar *et al.* 2014), we speculated that the interaction between KSRP and MVH may be mediated by RNA. Therefore, we treated the testes lysates with RNaseA and then performed co-IP with

Figure 2 KSRP interacts with MVH and SAM68, but not with MIWI. (A) Co-IPs were performed using antibody against MVH (i) or KSRP (ii), followed by Western blotting using antibodies against MVH and KSRP in adult mouse testes. IgG: negative rabbit serum, as negative control. (B) The expression of KSRP in CB was detected by Western blotting. MVH and MIWI were used as CB markers. MVH: anti-MVH antibody, as CB marker. IgG: negative rabbit serum, as negative control. (C) Co-IP using antibody against MVH, followed by Western blotting using antibody against KSRP in cytoplasmic and nuclear fractions from adult mouse testes. IgG: negative rabbit serum, as negative control. GAPDH and Lamin A/C were used as cytoplasmic and nuclear markers, respectively. (D) Co-IP using antibody against MVH after treatment with RNaseA in adult mouse testicular extracts, followed by Western blotting using antibodies against KSRP and MVH. IgG: negative rabbit serum, as negative control. (E) HEK293T cells were lysed in TNE buffer for co-IP assay after co-transfection with GFP-SAM68 plasmids and FLAG (or pcDNA-flag-KSRP) plasmids for 36 h. Co-IP using anti-FLAG antibody, followed by Western blotting using antibodies against FLAG and GFP. (F) Co-IP using anti-MIWI antibody in adult mouse testes, followed by Western blotting using antibodies against MIWI and KSRP. IgG: negative rabbit serum, as negative control.

MVH antibody. The results showed that the interaction was RNA independent (Fig. 2D). To further investigate whether KSRP interacts with other components of CB, we performed *in vitro* and *in vivo* co-IP assays. The data showed that KSRP interacts with SAM68 *in vitro* (Fig. 2E), but not with MIWI *in vivo* (Fig. 2F).

KSRP interacts with PABP1, PABP2 and RNA in CB

To investigate the function of KSRP in the testis and CB, IP assays were performed using KSRP antibody in adult mouse testes to identify associated proteins (Supplementary Fig. 2A). Several KSRP-specific bands were observed that were absent in the IgG control immunoprecipitation, and two bands were identified as polyadenylate-binding protein 1 (PABP1) and polyadenylate-binding protein 2 (PABP2) using mass spectrometric analysis. Previous studies have shown that PABP1 and PABP2 are present in CB and regulate the translation in haploid spermatogenic cells (Kimura *et al.* 2009, Meikar *et al.* 2014). The interaction between KSRP

and PABP1/PABP2 was confirmed by co-IP in HEK293T cells (Supplementary Fig. 2B and C). PABP proteins have four highly conserved RNA-recognition motifs (RRM1–4) that mediate both RNA and protein interactions, and a C-terminal domain (termed the PABC domain), which only mediates protein interactions (Berlanga *et al.* 2006, Brook *et al.* 2009, Burgess & Gray 2010). To identify the interaction domains of PABP1/PABP2, we generated GFP-PABP1/GFP-PABP2 full length (FL), GFP-PABP1/GFP-PABP2 RRM1–4 motifs and GFP-PABP1/GFP-PABP2 C domain recombinant plasmids, and then performed co-IP assays with transfected HEK293T cells. The results showed that the interaction between KSRP and PABP1/PABP2 is mediated by RRM1–4 motifs but not C domain (Supplementary Fig. 2D and E), which indicates RNA might also be involved in the interaction. To confirm that, HEK293T cell lysates were treated with RNaseA before co-IP was performed. As shown in Supplementary Fig. 2F, the interaction between KSRP and PABP1/PABP2 was reduced after RNaseA treatment, which indicates that RNA could enhance

Figure 3 KSRP interacts with mRNA and miRNA of CB. (A) The specific interaction of KSRP and CB mRNA (*Tnp2*, *Prm2* and *Odf1*) in adult mouse testes was confirmed by RNA-IP (using anti-KSRP antibody) and real-time PCR. *Gapdh* was used as negative control, and β -Catenin was used as positive control. Data were presented for at least three independent experiments. (B) RNA pull-down using biotin-labeled *Tnp2* mRNA sense (+) and antisense (–) strand probe, followed by Western blotting using antibody against KSRP in adult mouse testes. (C) Stem-loop precursor sequence of *Mus musculus* miR-182. (D) The specific interaction of KSRP and CB miRNA (miR-182) in adult mouse testes was confirmed by RNA-IP (using anti-KSRP antibody) and real-time PCR. IgG: negative rabbit serum, as negative control. Data are presented as means $\pm$ S.E.M. ($n=3$).

their interaction. These results increase the possibility that some RNA in CB may be directly associated with KSRP and regulate its function.

To identify the KSRP-associated RNA in CB, RNA-IP with KSRP antibody was performed in mouse testes. The results showed that the enrichment of CB mRNA (*Tnp2* and *Odf1*) from KSRP RNA-IP samples was comparable with the positive control— β -Catenin level, which could bind with KSRP as previous studies (Gherzi *et al.* 2006) (Fig. 3A). These data demonstrate that KSRP binds to multiple CB RNA components. RNA pull-down assay with biotin-labeled *Tnp2* RNA provided further evidence that KSRP binds to *Tnp2* mRNA directly (Fig. 3B).

KSRP is reported to bind to selected miRNAs, of which precursors have an AGGGU sequence at miRNA terminal loop and regulate their maturation (Trabucchi *et al.* 2009, Nicastro *et al.* 2012). As shown in Fig. 3C, the precursor of miR-182 contains the KSRP recognition sequence—AGGGU. We then considered whether KSRP binds to miR-182. RNA-IP results verified that KSRP indeed binds to mature miR-182 (Fig. 3D). Taken together, we found that KSRP can bind to CB proteins PABP1 and PABP2, as well as CB mRNA and miRNA in germ cells.

KSRP may regulate the integrity of CB via DDX5-miRNA-182 pathway

CB firstly appears in spermatocytes and condenses into one single finely filamentous granule in round spermatids (Kotaja & Sassone-Corsi 2007, Yokota 2008). To identify the proteins specifically expressed in round spermatids and been possibly involved in the condensation process, we extracted proteins from isolated spermatocytes and round spermatids, compared protein constituents with SDS-PAGE and performed mass spectrometric analysis with round spermatids-specific bands (Supplementary Fig. 3A, arrow). The results showed that DDX5 expressed

higher in round spermatids than in spermatocytes (Supplementary Fig. 3A). DDX5 is present in both nuclei and cytoplasm in adult mouse testes (Supplementary Fig. 3B and C). Previous studies suggested that DDX5 is one of CB proteins (Meikar *et al.* 2014). To validate the

Figure 4 KSRP interacts with DDX5 via miR-182. (A) HEK293T cells were co-transfected with indicated plasmids (FLAG/pcDNA-flag-KSRP and GFP-DDX5) for 36 h. Co-IP using anti-FLAG antibody, followed by Western blotting using antibodies against FLAG and GFP. (B) HEK293T cells were co-transfected with indicated plasmids (FLAG/pcDNA-flag-KSRP and GFP-DDX5) and 100 nM miR-182 mimic/scrambled NC for 36 h. Co-IP using anti-FLAG antibody, followed by Western blotting using antibodies against FLAG and GFP. (C) Co-IP using antibody against KSRP in adult mouse testes, followed by Western blotting using antibodies against DDX5 and KSRP. IgG: negative rabbit serum, as negative control.

association between DDX5 and other CB components, *in vitro* co-IP was performed. The results showed that DDX5 indeed interacted with CB protein MVH, PABP1 and PABP2 (Supplementary Fig. 3D, E and F).

Since KSRP mediates the interaction between MVH, PABP1 and PABP2 in CB, we speculated that KSRP may bind to DDX5 as well. Surprisingly, *in vitro* co-IP results showed that KSRP could not interact with DDX5 directly (Fig. 4A). In somatic cells, it was reported that DDX5 can regulate cytoskeleton via miR-182 (Wang *et al.* 2012), which raises the likelihood that miR-182 may mediate the KSRP and DDX5 interaction. HEK293T cells were triple over-expressed with miR-182, KSRP and DDX5 recombinant plasmids, and the co-IP assays showed that miR-182 is essential for the interaction between KSRP and DDX5, comparing with controls (Fig. 4B). Furthermore, we confirmed that KSRP could bind DDX5 in adult mouse testis (Fig. 4C). Given that KSRP binds to miR-182 in spermatids (Fig. 3D), it is possible that miR-182 connects the KSRP and DDX5 interaction in CB.

Next, we measured the expression of DDX5 and miR-182 when CB was disrupted, such as in *Miwi*-knockout mice (Kotaja *et al.* 2006). Results showed that the protein level of DDX5 was significantly

decreased (Fig. 5A), but the expression of miR-182 had no significant change in *Miwi*^{-/-} mice (Fig. 5B). We also mocked the disruption of CB structure *in vitro* by treating the seminiferous tubule segments with nocodazole for 48 h (Fig. 5C). After treatment, the mRNA levels of CB components, such as *Ksrp* and *Ddx5*, were significantly decreased, and the expression of pri-miR-182 was significantly increased (Fig. 5D). However, the expression of miR-182 was not significantly changed (Fig. 5E). Taken together, we speculated that KSRP may regulate the integrity of CB via DDX5-miR-182.

Abnormal expressions of KSRP and other CB components in testis of KSRP-knockout mice and of patients with hypospermatogenesis

One phenotype of *Ksrp*-knockout mice is partial sterility (Lin *et al.* 2011). The histology of epididymis and testis in *Ksrp*-knockout mice does not show any significant difference as in wild types. However, their fertility reduced while age raising (unpublished data). To better understand whether CB formation is impaired in the absence of *Ksrp*, the expression of MVH, DDX5 and miR-182 were first investigated in the knockout testis (Fig. 6A and B). In

Figure 5 KSRP regulates the integrity of CB via DDX5-miRNA-182 pathway. (A) The expression of CB proteins (MVH, MIWI, DDX5, and KSRP) in *Miwi*^{+/+} and *Miwi*^{-/-} mice testes was detected by Western blotting. GAPDH was used as a protein loading control. (B) The expression of miR-182 in *Miwi*^{+/+} and *Miwi*^{-/-} mice testes was examined by real-time PCR. Data are presented as means $\pm$ S.E.M. ($n = 3$). (C) Squash preparations from seminiferous tubule were stained with MVH antibody (green), after seminiferous tubule segments were incubated with nocodazole or DMSO for 48 h. CBs are shown as anti-MVH positive granules. Nuclei (blue) were stained with Hoechst. Scale bars: 10 μ m. (D and E) Real-time PCR analysis of CB components (D) and miR-182 (E) expression in seminiferous tubule, incubated with nocodazole or DMSO for 48 h. Data are presented as means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

Figure 6 The expression of CB components in *Ksrp* knockout mice testes and *Ksrp* knockdown-germ cells. (A) IF staining with MVH antibody (green) in *Ksrp* knockout (*KSRP* KO) mice and wild type (WT) mice testes, and CBs are shown as MVH positive granules (white arrows). Scale bars: 10 μm. (B) The expressions of *Ksrp*, *Mvh*, *Ddx5* and *miR-182* in the testes of *Ksrp*-knockout (*KSRP* KO) mice and wild type (WT) mice were examined by real-time PCR. Data are presented as means ± S.E.M. (n=4). *P<0.05, **P<0.01. (C) Real-time PCR assays of CB components and *pri-miR-182* expression in *Ksrp*-knockdown germ cells, infected by *Ksrp* shRNA or control shRNA viruses for 24 h. Data are presented as means ± S.E.M. (n=3). *P<0.05, **P<0.01.

Ksrp^{-/-} testis, CB, marked by MVH, was disappeared in some of the round spermatids (Fig. 6A). Moreover, real-time PCR results showed that the expression of *Ksrp*, *Mvh*, *Ddx5* mRNA and *miR-182* significantly declined in *Ksrp*^{-/-} mice compared with wildtype (Fig. 6B). In addition, similar decreased expressions of CB components mRNA (such as *Mvh*, *Miwi*, *Kif17b*, *Ddx5*, *Tnp2* and *Prm2*) and the increased expressions of *pri-miR-182* were detected in *in vitro*-cultured germ cells infected with *Ksrp* shRNA lentivirus (Fig. 6C).

The expression patterns of *KSRP*, *MVH*, *DDX5*, *pri-miR-182* and *miR-182* were also measured in testicular specimens of hypospermatogenic patients by real-time PCR (Fig. 7). The results showed that the expression of *Ksrp*, *Mvh* and *pri-miR-182* was remarkably increased in hypospermatogenic patients (Fig. 7A, B and C), but the expression of *Ddx5* and

miR-182 was significantly reduced (Fig. 7D and E). These results indicate that the abnormal expression of *KSRP* and other CB components may be correlated with human spermatogenic failure. Taken together, these data support the postulate that *KSRP* plays an important role in spermatogenesis.

Discussion

Recent studies have indicated that *KSRP* plays important functional roles in transcriptional regulation, post-transcriptional regulation and miRNA maturation in somatic cells (Gherzi *et al.* 2010, Danckwardt *et al.* 2011, Li *et al.* 2012). However, the function of *KSRP* in testis and spermatogenesis is little known. In this study, we found that *KSRP* is a component of CB. *KSRP* interacts with CB proteins (*MVH*, *PABP1* and *PABP2*), mRNA

Figure 7 The expression of CB components in the testes of hypospermatogenic patients. (A) Expression levels of *KSRP* in the testes of normal controls ($n=7$) and hypospermatogenic men ($n=8$) were measured by real-time PCR. (B) Expression levels of *MVH* in the testes of normal controls ($n=7$), and hypospermatogenic men ($n=11$) were measured by real-time PCR. (C) Expression levels of pri-miR-182 in the testes of normal controls ($n=7$) and hypospermatogenic men ($n=8$) were measured by real-time PCR. (D) Expression levels of *DDX5* in the testes of normal controls ($n=11$) and hypospermatogenic men ($n=11$) were measured by real-time PCR. (E) Expression levels of miR-182 in the testes of normal controls ($n=9$) and hypospermatogenic men ($n=12$) were measured by real-time PCR. Data are presented as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, compared with negative controls.

(*Tnp2* and *Odf1*) and miRNA (miR-182). KSRP also regulates the integrity of CB via DDX5-miR-182 pathway. Moreover, the loss of *Ksrp* in mouse testis or a reduced expression of *Ksrp* in mouse germ cells are associated with CB disappearance and reduced expressions of other CB components. Dysregulated expressions of *KSRP* and other CB components were also found in the testes of hypospermatogenic patients. Therefore, our data suggest that KSRP may play an important role in spermatogenesis.

A number of poly (A)-binding proteins (PABP) are reported to be found in CB, and that some CB proteins, such as MIWI, have been found to interact with a specific set of protein-coding mRNAs (Deng & Lin 2002, Meikar et al. 2010, 2011). Thus, Meikar and coworkers proposed a hypothesis that the CB is involved in storing and regulating mRNA transcripts whose translation is repressed until the proteins are required in elongating spermatids (Meikar et al. 2011). Here, we found that

KSRP interacts with PABP1, PABP2 and CB mRNA (*Tnp2* and *Odf1*). Furthermore, we proved that RNA could enhance the interaction of KSRP and PABP1/PABP2. Our data suggest that KSRP might participate in the regulation of mRNAs translation in haploid germ cells, which also support the hypothesis of Meikar and coworkers.

MicroRNA plays important roles in translational repression of mRNA in spermatogenesis (Braun 1998, Bartel 2009). The primary miRNA (pri-miRNA) are cleaved to precursor-miRNA (pre-miRNA) in the nuclei by Drosha. Then, pre-miRNA are exported to the cytoplasm and are cleaved to mature miRNA by Dicer (Krol et al. 2010). miRNA pathways have been found in CB, and many miRNA have been demonstrated to be accumulated in CB (Kotaja & Sassone-Corsi 2007, Meikar et al. 2014). In this study, we have identified a KSRP binding miRNA—miR-182. Decreased expression of mature miR-182 was found in *Ksrp*^{-/-} mice testes. Moreover, we discovered that the expression of pri-miR-182 was increased in *Ksrp*-knockdown germ cells. Since previous studies showed that KSRP serves as a component of both Drosha and Dicer complexes and regulates the biogenesis of a subset of miRNA (Trabucchi et al. 2009), we speculate that KSRP may be involved in the processing of miRNA, and eventually regulate the translation of mRNA in CB.

It is known that CB gets matured and compressed into one single condensed granule in round spermatids (Kotaja & Sassone-Corsi 2007, Yokota 2008). Highly expression of DDX5 was found in round spermatids, which suggests that DDX5 may be involved in regulating the maturation of CB. DDX5, as one of CB proteins (Meikar et al. 2014), is involved in RNA metabolism, transcriptional regulation and cytoskeleton formation via miR-182 (Wang et al. 2012). Our data showed that miR-182 mediates the interaction between KSRP and DDX5, which reveals that the components of CB form an interaction network. We also observed that, in the CB damaging situation, such as in *Miw1*^{-/-} testis and nocodazole-treated seminiferous tubule segments, the expression of *Ksrp*, *Ddx5* and *pri-miR-182* are significantly changed. However, the expression of mature miR-182 has no significant change. We speculate that there might be other pathways to regulate the biogenesis of miR-182 *in vivo*. Thus, we think that KSRP may regulate the integrity of CB via DDX5-miR-182. Further work is needed to find out other pathways of regulating miR-182 in testis.

In addition, the abnormal expressions of CB components (*Mvh*, *Ksrp*, *Ddx5* and miR-182) and pri-miR-182 were observed in testes of hypospermatogenic patients. The altered expression level of *Mvh* in patients indicates loss of function or impaired assembling of CB in their germ cells. Remarkably, the increased expression of *Ksrp* and pri-miR-182, while the declining expression of *Ddx5* and miR-182. The decreased expression of *Ddx5* implies that *Ddx5* may have different regulation

pathways in spermatogenesis. These results suggest that KSRP and CB may be correlated with male infertility.

In summary, we proposed the potential functions of KSRP in chromatoid body and spermatogenesis. The mechanistic studies providing a model of protein–mRNA–miRNA interactions in CB could provide us a better understanding of the function of CB in spermatogenesis, and a new viewpoint to cure male infertility.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0169>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Studies were supported by the following grants (to F S): the National Natural Science Foundation of China (81430027, 81671510); the Nation Program of China (2014CB943104).

Acknowledgements

The authors thank Dr Stéphane Richard (Lady Davis Institute for Medical Research, Jewish General Hospital, and Departments of Medicine and Oncology, McGill University, Canada) for the GFP-SAM68 expression vector; Dr Ching-Yi Chen (Department of Biochemistry & Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA) for the pcDNA-flag-KSRP expression vector; Dr Mian Wu (University of Science and Technology of China) for the HEK293T cell line and KSRP shRNA and control shRNA virus.

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Received 9 May 2017

First decision 31 May 2017

Revised manuscript received 28 August 2017

Accepted 4 September 2017