Cellular ontogeny of RBMY during human spermatogenesis and its role in sperm motility

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The Y-chromosome-encoded gene *RBMY* (RNA-binding motif on Y) is a male germline RNA-binding protein and is postulated to be a RNA-splicing regulator. In order to understand the roles of RBMY in different stages of male gamete maturation, the present study aimed at determining its cellular expression during spermatogenesis, spermeogenesis and in mature spermatozoa. In the spermatogonia (cKIT-positive cells), RBMY immunolocalized as two distinct foci, one in the nucleolus and the other in the subnuclear region; in the spermatocytes (cKIT-negative cells), the nucleus had punctuate staining with a subnuclear foci; in the pachytene cells, the protein was localized as a punctuate pattern in the nucleus spread along the elongating chromosomes. In the round and the elongating spermatids, the protein expression was polarized and restricted to the cytoplasm and in the developing mid-piece. In testicular and ejaculated sperm, RBMY was localized to the mid-piece region and weakly in the tail. Incubation of spermatozoa with the RBMY antibody reduced its motility. The spatial differences in expression of RBMY in the germ cells and the presences of this protein in post-meiotic cells and in transcriptionally inert spermatozoa suggest its involvement in multiple functions beyond RNA splicing. One such possible function of RBMY could be its involvement in sperm motility.

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1. Introduction

Genetic analysis of men with infertility and subfertility has led to identification of genes that are crucial for spermatogenesis. Amongst these, is the Y-chromosome-encoded gene *RBMY* (RNA-binding motif on Y), which is present in multiple copies and distributed throughout the Y-chromosome. Complete or partial deletions of the Y-chromosome regions encompassing the *RBMY* genes are observed in infertile men (Foresta *et al.* 2001); partial deletion of the *Rbmy* gene family in the mouse is associated with sperm abnormalities (Mahadevaiah *et al.* 1998), underscoring a critical requirement of RBMY in male fertility.

The sequence and cellular distribution of RBMY protein are consistent with their function in nuclear RNA processing. The open reading frame of *RBMY* cDNA contains an RNA-binding domain and the carboxy-terminal domain has four repetitions of a Ser-Arg-Gly-Tyr tetrapeptide motif called SRGY box which is a characteristic of many RNA-binding proteins (Ma *et al.* 1993). Also, RBMY shares sequence homology with hnRNP G, suggesting that RBMY functions as an RNA processing factor (Ma *et al.* 1993; Delbridge *et al.* 1999; Chai *et al.* 1998; Mazeyrat *et al.* 1999). Indeed, RBMY co-localizes and interacts with pre-mRNA splicing factors (Elliot *et al.* 1998) and is thought to inhibit RNA-splicing activities by sequestering splicing factors from nascent RNA (Elliot *et al.* 1997; Venables *et al.* 2000; Dreumont *et al.* 2010). The murine and the human RBMY-RNA interactome has been recently reported (Zeng *et al.* 2008, 2011); the NMR structure of RBMY complexed with RNA has also been solved (Skrisovska *et al.* 2007). These results are strong evidences to support the function of RBMY in splicing and its involvement in regulation of transcription and translational control in the male germline.

In the testicular germ cells, the process of active transcription and translation occurs in the pre-meiotic and meiotic

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stages, the germ cells are transcriptionally and translationally quiescent post meiosis (Yu *et al.* 2003; Almstrup *et al.* 2004). Since RBMY is a RNA splicing controlling factor, it is conceivable that it may play a crucial role in pre-meiotic cells and alterations of RBMY might affect spermatogenesis in the early stages. However, human males who harbour deletions of the AZFb locus (encompassing the RBMY genes) generally suffer from arrest of spermatogenesis at post-meiotic stages (Foresta *et al.* 2001; Abid *et al.* 2008a; Hadjkacem-Loukil *et al.* 2011); mice knockouted for *Rbmy* also have normal spermatogenesis but develop structurally defective spermatozoa (Mahadevaiah *et al.* 1998). These observations suggest that beyond RNA editing and splicing in the pre-meiotic and meiotic germ cells, RBMY might have additional functions in the post-meiotic gametes. However, little is known about the existence of RBMY in the male germline post meiosis, and its presence and roles of any in spermatozoa are unknown.

To gain an insight into the potential roles of RBMY in male gametes, we aimed towards determining the detailed ontogeny of RBMY protein during germ cell maturation and in ejaculated spermatozoa. Since RBY was detected in the spermatozoa, the study was extended to determine its involvement in sperm motility.

2. Materials and methods

2.1 *Sample collection and processing*

Human testicular tissues were collected after informed consent and were a part of previous studies (Modi *et al.* 2005; Shah *et al.* 2005a, b; Abid *et al.* 2008b). Semen samples were collected by masturbation from anonymous donors, and those samples meeting the criteria of normozoospermia were included in the study. All experiments were repeated twice using three independent samples.

2.2 *Cellular localization of RBMY*

2.2.1 *Germ cell isolation:* Testicular tissue was minced and initially digested in Collagenase IV (1 mg/mL, Sigma USA, St. Louis, USA) and subsequently in 0.25% Trypsin/ EDTA at 37°C. The cells were filtered through 100, 70 and 40 µm nylon filters and the flow through containing the germ cells was washed thrice in PBS and used for immunofluorescence.

2.2.2 *Indirect immunofluorescence:* The protocol for immunofluorescence has been detailed previously (Shah *et al.* 2005a, b). Washed spermatozoa or isolated germ cells were smeared, air-dried and fixed in chilled acetone. The slides were incubated in 5% bovine serum albumin (BSA) and the cells were probed overnight at 4°C overnight with a goat polyclonal antibody (Santacruz Biotechnology Inc, sc - 14572, USA) that recognizes the N-terminus of human RBMY (N-RBMY) at a dilution of 1:50. For negative control, normal goat serum was used in place of the primary antibody. The following day, the slides were washed extensively in PBS containing 0.1% Tween 20 (PBS-T) and incubated with 1:1000 diluted FITC-labelled antigoat IgG (Jackson laboratories, USA). The slides were washed with PBS-T, counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma USA) or propidium iodide and mounted in antifade medium. The sections were viewed under a confocal microscope (LSM 510 Meta, Carl Ziess USA) (Modi et al. 2003). Spermatozoa were also stained with I-RBMY antibody that was raised in rabbit against an internal fragment (amino acids 373-412) of human RBMY (Santacruz Biotechnology Inc, USA, sc - 28727). This antibody was used as a dilution of 1:50 and immunoflourescence was performed exactly as above.

The different germ cell types were identified under DIC based on morphology (Holstein *et al.* 2003). Large cells with high nucleo-cytoplasmic ratio and fine chromatin were considered spermatogonia. Spermatocytes were defined as cells with interphase nucleus and visible cytoplasm. Meiotic cells were identified based on their size and presence of chromosomes. Round spermatids were small interphase cells with condensed nucleus. Elongating spermatids were identified based on shape, placement of cytoplasm, condensed nucleus and the tail.

2.2.3 *Double immunofluorescence for RBMY and cKIT in germ cells:* To differentiate between spermatogonia and spermatocytes, the cells were immunostained as above for RBMY along with cKIT. For detection of cKIT, a monoclonal antibody against cKIT (Cell Signaling, Danvers, USA) was mixed along with the RBMY antibody (as above). Post washing, the slides were incubated with FITC-labelled antigoat IgG for RBMY and Alexa fluor 594-labelled antimouse IgG to detect cKIT. Post stringency washes in PBS-T, the slides were counterstained with DAPI, mounted and observed as above. The negative controls included slides incubated with the secondary antibodies alone.

2.3 *Motility analysis*

Spermatozoa were washed once with DMEM medium (GIBCO, Grand Island, USA) and incubated with 1:50 dilution of N-RBMY or I-RBMY antibody that was raised against an internal fragment (amino acids 373-412) of human RBMY (Santacruz Biotechnology Inc, USA, sc -28727) for 30 min at 37°C. As controls, cells were incubated in 1:50 dilution of normal goat or rabbit serum. At the end of 30 min, the cells were loaded on to glass slides and motility was assessed at 37°C in a Computer Assisted Sperm Analyzer (CASA, Hamilton Thorn IVOS V12.3) at the rate

of 60 frames/s using default settings. CASA was performed for three independent samples and in duplicates. Mean ± SD was computed and statistical test was done by two-way ANOVA using Dunnet multiple comparison test.

3. Results

3.1 *Expression of RBMY in the germ cells during spermatogenesis*

Figure 1 demonstrates the expression of RBMY protein in the different germ cell types isolated from the human testis. As evident, in cells with high nucleo-cytoplasmic ratio (presumptively the spermatogonia), RBMY was localized as two distinct foci, one in the subnuclear region with some cytoplasmic staining and the other in the nucleus which almost always was within the nucleolus (figure 1A). In similar cell types but with more amounts of cytoplasm (presumptively the spermatocytes), a single green signal representing RBMY was detected only in the subnuclear region with some cytoplasmic staining (figure 1B). In these cells no staining was evident in the nucleolus; instead, a weak staining for RBMY was spread over the chromatin in a punctuate pattern (figure 1B, green channel).

Since spermatogonia and spermatocytes are difficult to differentiate based on morphology alone, double immunofluorescence experiments were performed. In this case, the cells were stained for RBMY along with cKIT as a marker for spermatogonia. As evident from figure 2A, the cKIT-positive cells had the nucleolar and the subnuclear RBMY expression, whereas the cells that had only the subnuclear foci of RBMY were negative for cKIT (figure 2B). Quantitatively, almost 90% of cKIT-positive spermatogonial cells had RBMY localized in the nucleolus. These results confirmed that RBMY is localized in the nucleolar and subnuclear region in the spermatogonia; in the spermatocytes the RBMY was not detected in the nucleolus but was detected at multiple sites spread along the nucleus with the subnuclear foci retained.

The pachytene cells were identified by the condensed chromatin organized into chromosomes. In these cells (figure 1C) RBMY was detected abundantly interspersed all over the nucleus. The focal subnuclear staining detected in the spermatogonia and spermatocytes was not observed in the pachytene cells.

Figure 1D shows the expression of RBMY in the post-meiotic round spermatids. As evident, RBMY expression was restricted to the cytoplasm – no nuclear signals were

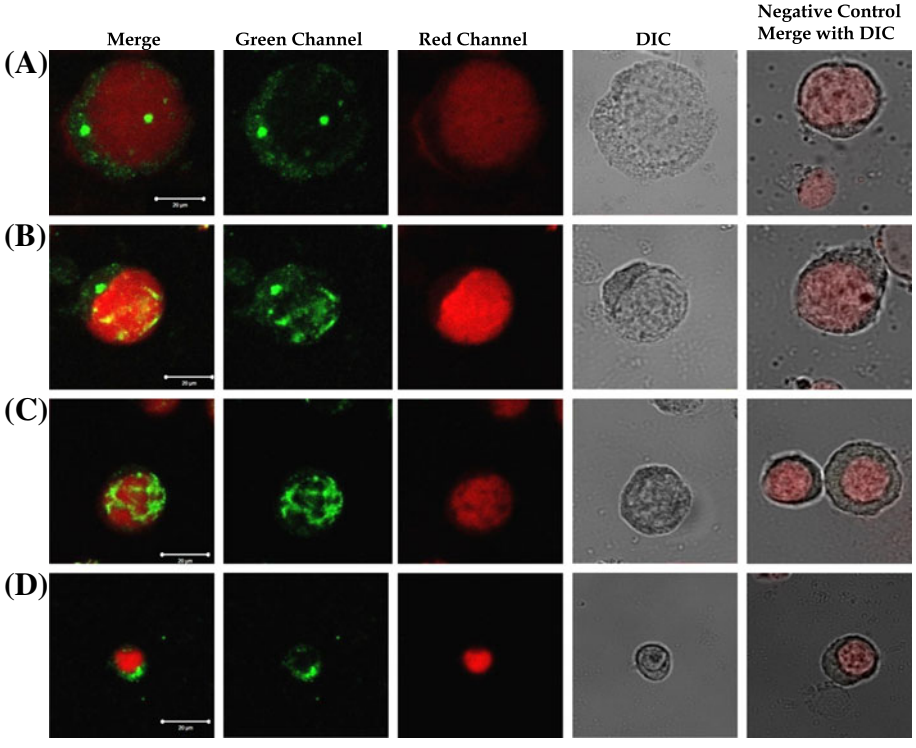

Figure 1. Localization of RBMY in human testicular germ cells: (**A**) spermatogonia, (**B**) spermatocyte, (**C**) pachytene cells and (**D**) round spermatid. The first panel is the merged image of the green channel for RBMY and red channel for nuclear counter stain. The cell morphology is evident in the DIC panel. Negative controls incubated with normal goat serum are shown in the last panel.

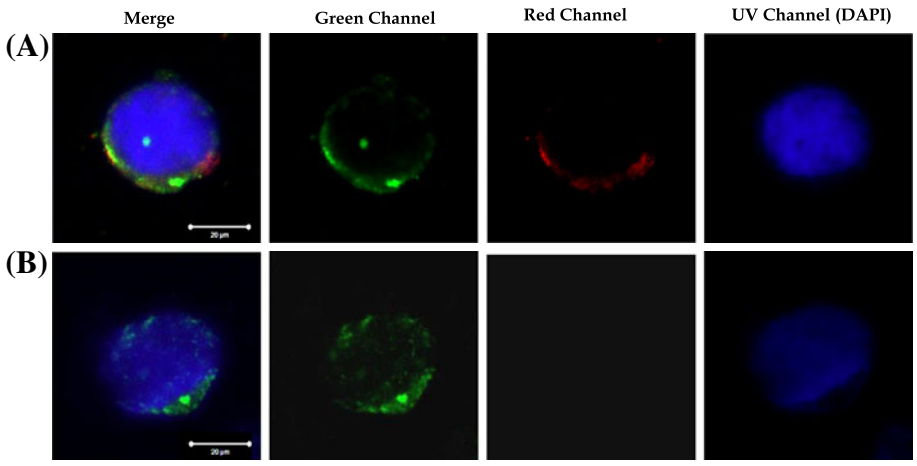

Figure 2. Co-localization of RBMY and cKIT in human testicular germ cells. The first image in each panel is the merged image of the green channel corresponding to RBMY, the red channel corresponding to cKIT and UV channel for the DAPI. Panel (**A**) shows the cKIT-positive (red staining) spermatogonia with nucleolar and subnuclear RBMY foci (green staining). Panel (**B**) is the cKIT-negative spermatocyte showing the RBMY protein (green staining) speckled along the nucleus and distinct foci in the cytoplasm.

detected. The negative control did not show any staining (figure 1, extreme right panel).

3.2 *Expression of RBMY in the developing testicular and ejaculated spermatozoa*

Figure 3 demonstrates the changes in expression profile during spermiogenesis. In elongating spermatids, RBMY is restricted to the cytoplasm and polarized to the base along the cytoplasm (figure 3A). In cells with the elongating tail, the expression was found getting restricted the base of the spermatid (figure 3B). In the terminal stages of spermiogenesis where the cytoplasm was found to be extruding, RBMY was present in the cytoplasmic droplet (figure 3C). In the testicular sperm, where the cytoplasm has been completely extruded, the expression of RBMY was restricted to the mid-piece with some weak staining often seen in the tail (figure 3D). The corresponding negative controls did not show any green fluorescence, indicating the specificity of the staining (figure 3, extreme right panel).

Figure 4A demonstrates the expression of RBMY in the ejaculated spermatozoa using two antibodies raised against different epitopes of RBMY (N-RBMY and I-RBMY). Using both the antibodies, intense fluorescence for RBMY was detected in the mid-piece of all spermatozoa with weak but specific staining in the tail region (figure 4A). No signals were detected in the negative controls (figure 4A).

3.3 *Effect of RBMY antibodies on sperm motility*

To determine any involvement of RBMY in sperm motility, washed spermatozoa were incubated with either normal rabbit/goat serum or sera containing the N-RBMY or the I-RBMY antibodies and the motility was determined by CASA. Since the motility parameters of the cells incubated with rabbit or goat serum at the specified dilutions did not differ significantly, the data of both these control groups were pooled and designated as control. As compared to controls, the numbers of motile and progressively motile spermatozoa were significantly reduced, the number of non-motile spermatozoa increased significantly in presence of N-RBMY but not the I-RBMY antibody (figure 4B). When the spermatozoa were classified based on their type of motility, as compared to controls, the number of rapid and medium motile spermatozoa significantly reduced, and a significant increase in number of slow and static spermatozoa was observed in presence of N-RBMY antibody (figure 4C). The indices did not differ significantly in case of cells incubated with I-RBMY antibody as compared to controls (figure 4C).

4. Discussion

The results of the present study demonstrate that RBMY is expressed in all the germ cell types of the testis and also in the ejaculated spermatozoa. In the pre-meiotic and meiotic germ cells, RBMY has distinct spatial pattern of staining depending on the stage of differentiation; in the post-meiotic cells, RBMY is retained and expressed in the mid-piece and the tail of the testicular and ejaculated spermatozoa. Functional studies have revealed that RBMY may play a role in sperm motility.

The Y-chromosome has been thought to have evolved from an ancient autosomal homologous chromosome and in the course has lost most of its genes except those involved

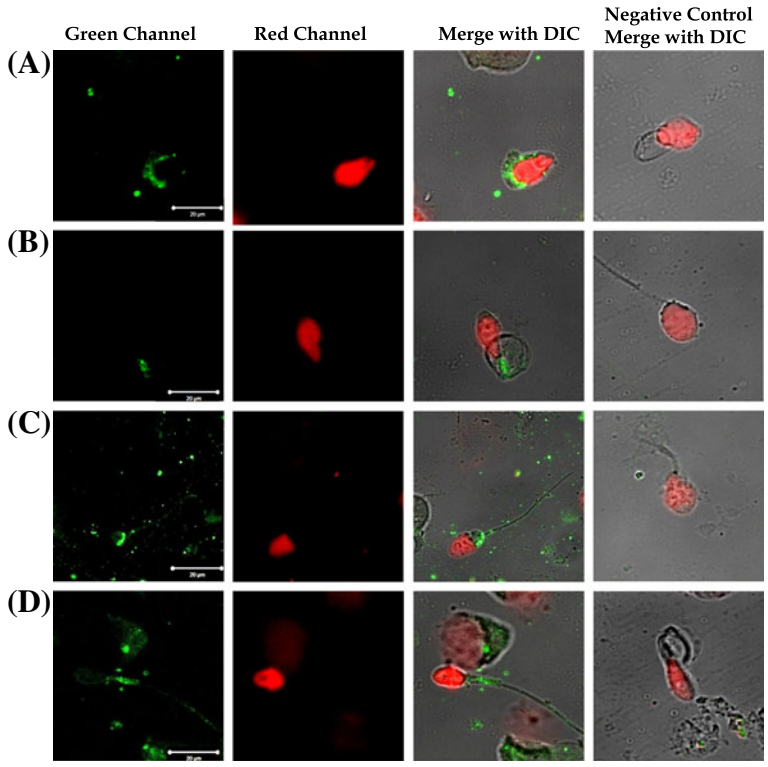

Figure 3. Localization of RBMY during spermiogenesis. (**A**–**C**) Spermatids at different stages of elongation. (**D**) Testicular sperm. The green channel is RBMY; the red channel is the nuclear counter stain propidium. The third panel is a merge of red and green channels overlapped with phase contrast (DIC). Negative controls are shown in the last panel.

in male germ cell development. Not surprisingly, the genes left on the Y-chromosome such as *RBMY* have male-specific functions and are important for male fertility. Human males with deletions of the AZFb locus that harbours the *RMBY* genes have failure of spermatogenesis and are infertile (Foresta *et al.* 2001; Abid *et al.* 2008a). Considering the potential importance of the *RBMY* gene for maintenance of male fertility, we investigated the expression of RBMY protein in the process of spermatogenesis and gamete functions. During spermatogenesis, quantitative changes in rates of RNA synthesis and alterations in the number of transcripts in the different germ cell types have been reported. Since RBMY is involved in RNA splicing and interacts with transcripts of proteins involved in meiosis (Zeng *et al.* 2008, 2011), it is likely that its expression and/or its subcellular distribution may change during different stages of germ cell maturation. Extending the previous observations in the mouse and man (Lee *et al.* 2004), we demonstrate that RBMY is not only present in the spermatogoina and the spermatocytes but the subcellular distribution differs significantly. In the spermatogonia, RBMY immunolocalized as two distinct foci, one in the nucleus that always overlapped with the nucleolus and the other in the subnuclear region. In the spermatocytes, the nucleolar RBMY disappeared and the protein was distributed in a speckled pattern in the nucleus; however, the subnuclear RBMY seen in the spermatogonia was retained. The identity of the two cell types was confirmed by double immunofluorescence using cKIT positivity as a marker for spermatogonia. However, unlike in the mouse, human RBMY continued to be expressed during meiosis, where in the pachytene cells, RBMY was localized in a punctate pattern exclusively within the nucleus spread all around the condensing chromosomes, and the focal subnuclear RBMY was not observed. These results suggest that RBMY requirements in the three germ cell types differ spatially and might have different functions. Experimental evidences have suggested that the nucleoplasmic population of splicing factors may be involved in pre-mRNA splicing; the punctuate sites contain both pre-mRNA splicing factors and RNA polymerases (Gama-Carvalho *et al.* 1997; Misteli *et al.* 1997; Zeng *et al.* 1997; Dreumont *et al.* 2010). Thus, it is tempting to propose that in the spermatocytes and meiotic cells, RBMY may associate with transcriptionally active regions within the nucleus to enrich in nascent RNA and participate in the regulation of splicing events. The significance of the nucleolar and the subnuclear RBMY detected in the spermatogonia needs to be determined. It will be of potential interest to study the spatial localization of the

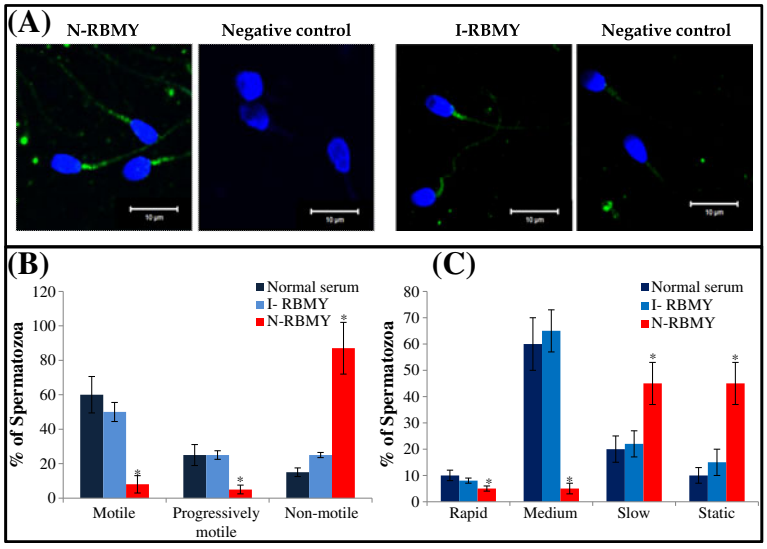

Figure 4. Expression of RBMY in ejaculated spermatozoa and effects of RBMY antibody on sperm motility. **(A)** Localization of RBMY protein (green staining) in ejaculated spermatozoa using the N-RBMY and the I-RBMY antibodies. The blue fluorescence corresponds to the nuclear counter stain for DAPI. The negative controls are probed with goat and rabbit serum and the secondary antibody. Effect of RBMY antibody on motility parameters (**B**) and types of motility (**C**) of ejaculated spermatozoa. Washed sperm were incubated with RBMY antibody directed against an internal epitope (I-RBMY) or the antibody directed against the N-terminal epitiope (N-RBMY). Cells incubated with normal goat or rabbit serum (normal serum) served as control. The various motility parameters were assessed by CASA. Values on Y-axis are mean ± SE of percentage of spermatozoa in three independent experiments. * indicates mean value significantly different as compared to control cells.

RBMY interacting partners and other splicing factors in different germ cells to have greater insights into the functional role of RBMY at different stages of spermatogenesis.

During spermatogenesis, RNA synthesis and transcript numbers have been observed to be maximal at the spermatogonia and spermatocyte stage and are reduced in round spermatids and then ceases while haploid round spermatids differentiate into elongated spermatids (Yu *et al.* 2003; Almstrup *et al.* 2004). Considering the major role of RBMY in RNA editing and splicing, it is envisaged that the expression of RBMY would be reduced or absent in the post-meiotic germ cells. Indeed, in the mouse *Rbmy*, mRNA is not detected in the meiotic and post-meiotic cells (Lee *et al.* 2004). However, human RBMY continue to express in post-meiotic cells, where protein is polarized to the base of the elongating spermatids and condensed in the cytoplasmic droplet. Subsequently, in the mature testicular and ejaculated sperm, RBMY is retained in the mid-piece region and in the tail. The apparent variation in the mouse and the human RBMY expression profiles suggest fundamental differences in the mechanisms by which RBMY may regulate spermatogenesis. Alternately, it is possible that RBMY protein requirements in the germ cells differ between the mouse and man. Indeed, the testicular phenotypes of mouse knockout for *Rbmy* and the humans deleted for AZFb encompassing the *RBMY* gene significantly differ. While humans having the partial or complete deletions of AZFb generally suffer from severe testiculopathy generally involving meiotic arrest (Foresta *et al.* 2001; Abid *et al.* 2008a, Hadjkacem-Loukil *et al.* 2011), the murine counterparts develop spermatozoa, albeit abnormal in structure (Mahadevaiah *et al.* 1998). It will be of potential interest to determine the presence of RBMY in testicular germ cells (mainly the post-meiotic cells) of other species to determine whether there are any species-specific requirements of RBMY.

The fact that the elongating spermatids and sperm are transcriptionally inert and the presence of RBMY in the cytoplasm but not in the nucleus suggest that it may not function as a splicing factor. Biochemical and structural studies have shown that the RRM motif of RNA-binding proteins are not only involved in RNA recognition but also in protein–protein interaction (reviewed in Maris *et al.* 2005; Chang and Ramos 2005). Whether the cytoplasmic RBMY in the spermatids and in the mid-piece and tail of spermatozoa functions via protein–protein interaction needs to be investigated. However, it is of interest to note that the ejaculated human spermatozoa retain miniscule amounts of RNA which is *in situ* localized to the mid-piece region (Modi *et al.* 2005; Shah *et al.* 2005b) where RBMY protein is enriched. Thus, it is possible that in the mid-piece, the RNA may be retained by binding to RBMY. RNA immunoprecipitation of sperm preparations would be required to investigate this possibility.

At present, the roles of RBMY in the sperm are difficult to speculate. Beyond the mid-piece, RBMY was detected in the tail. This observation suggests that RBMY may play a role in regulation of sperm motility. To investigate into this possibility, spermatozoa were treated with RBMY antibodies and the motility was studied by CASA. The results revealed that in comparison to controls, treatment with N-RBMY antibody lead to significant decrease in number of motile and progressively motile sperm with a parallel increase in number of static spermatozoa. However, the I-RBMY antibody had no such effects despite the fact that in immunoflourescence experiments, the same antibody detected RBMY in both mid-piece and weakly in tail (similar to N-RBMY antibody). Also in cross immunopreceptation–Western blots, the protein immunopreceptated by the N-RBMY antibody was recognized by the I-RBMY antibody (not shown). This observation is novel as it suggests that RBMY in the tail may be controlling motility. Since the N-terminal-specific but not the internal-fragment-specific RBMY antibody reduced motility indicate that RBMY is surface-oriented in sperm tail with their N-termini exposed, the internal region may be buried. Alternately, it is likely that in the sperm N-terminal epitope may be functional and the internal region may not be functionally relevant for motility in spermatozoa.

How would RBMY regulate motility in spermatozoa is a matter of speculation. In somatic cells, RNA-binding proteins are reported to regulate motility by controlling the translation of motility-associated proteins (Boudoukha *et al.* 2010). However, as spermatozoa are translationally dormant, this mechanism seems to be unlikely. Since RBMY has a potential role in protein–protein interactions (Chang and Ramos 2005), it is possible that the binding of the antibody to RBMY may alter its conformation resulting in displacement of its protein partners along the sperm tail to inhibit motility. It will be of interest to identify the protein that RBMY may partner with in spermatozoa to determine if RBMY is indeed involved in regulation of sperm motility. It will also be relevant to study the levels of RBMY in spermatozoa of men with reduced motility to determine its *in vivo* relevance.

In summary, the results of the present study demonstrate that the RBMY protein show dynamic shifts in its subcellular distribution during spermatogenesis, suggesting its differential functions in the individual stages of germ cell maturation. The presence of the RBMY protein in post-meiotic germ cells and also in the transcriptionally quiescent spermatozoa implies its role beyond RNA editing. In spermatozoa, our results suggest a possible involvement of RBMY in regulation of motility. Such differentially regulated subcellular distribution of RBMY in the male gametes maybe of crucial importance in understanding of the regulation of spermatogenesis, spermiogenesis and sperm functions, and such information in the long term would be of clinical relevance in determining the molecular basis of male infertility and for development of sperm-based contraception.

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