Published in final edited form as:

Mol Reprod Dev. 2014 October ; 81(10): 896–907. doi:10.1002/mrd.22358.

The acrosomal protein SP-10 (*Acrv1*) is an ideal marker for staging of the cycle of seminiferous epithelium in the mouse

Hari Prasad Osuru, Jennifer Monroe, Apoorv Chebolu, Joycelyn Akamune, Patcharin Pramoonjago, Sandeep Ranpura, and Prabhakara Reddi*

Department of Pathology, University of Virginia School of Medicine, Charlottesville, VA 22908

Abstract

The study of spermatogenesis in the mouse requires accurate identification of the cycle stage of seminiferous epithelium. A stage refers to the unique association of germ-cell types at a particular phase of development, as seen in a testicular cross section. The process of staging, however, is a daunting task. There are 12 stages represented in the mouse seminiferous tubule. Stages are typically identified on the basis of the morphology of the developing acrosome of spermatids. Although the characteristic features of the acrosome are well-documented in ultrastructure images, a reagent that can highlight the subtle differences in acrosome shape under the light microscope is lacking. Here we demonstrate that a polyclonal antibody raised against the mouse acrosomal protein SP-10 is extremely useful for stage identification. Immunohistochemistry showed that the anti-SP-10 antibody is highly specific for the acrosome of spermatids, as no other cell type in the epithelium showed immunoreactivity. At lower magnification, the gross shape of the acrosome and the increasing intensity of immunostaining served as a guide for the identification of stages 1–12. At higher magnification, characteristic morphological features –such as whether the part of the acrosome that contacts the nuclear surface is round (stage 3) or flat (stage 4) or curved (stage 6)– could be identified unambiguously. Overall, we present evidence that SP-10 is a useful marker for staging the cycle of a seminiferous epithelium. The anti-SP-10 antibody works well in different fixatives, on paraffin-embedded as well as cryosections; it has also been shown to be useful for characterizing spermatogenic defects in mutant mice.

Keywords

acrosomal marker; spermatogenesis; testis; round spermatids; staging

INTRODUCTION

Spermatogenesis progresses in continuous cycles along the seminiferous epithelium (Oakberg. 1956; Clermont. 1972; Hess and Renato de Franca. 2008). Cross sections of the seminiferous tubule show a defined group of germ-cell types at a particular phase of development; this grouping is referred to as cell association or stage. In the mouse, there are 12 such stages that, together, constitute the cycle of the seminiferous epithelium (Oakberg.

*Corresponding author, ppr5s@virginia.edu, Phone: 434-982-0007.

The authors have no conflict of interest to declare.

1956). Accurate staging of a cycle is important to study the biology of normal spermatogenesis as well as to understand the pathology of the testis (Russell et al. 1990), but assigning one of twelve stages to a seminiferous tubule in cross section is not trivial. Many excellent reviews and manuals have been written outlining the criteria to identify stages on the basis of spermatid morphology (Ahmed and de Rooij. 2009; Meistrich and Hess. 2013; Russell et al. 1990), which focus mainly on morphological changes in the acrosomal system of round spermatids and changes in the nuclear shape in the elongating spermatids. The task of staging becomes challenging because in hematoxylin and eosin (H&E)-stained histological sections of mouse testis, round spermatids of stages I through VIII appear very similar in morphology. Conventional methods of labeling the acrosome including Periodic Acid Schiff (PAS) (Langreth. 1969) and lectin staining have been in practice (Soderstrom et al. 1984; Wollina et al. 1989). Immunohistochemistry using antibodies to specific acrosomal proteins provides an alternative for staging the seminiferous cycle.

SP-10 is an evolutionarily conserved acrosomal protein first identified in human sperm, and subsequently shown to be present in all mammalian species tested, including the mouse (Herr et al. 1990; Kurth et al. 1993; Reddi et al. 1995). The SP-10 protein is specific to male germ cells, and is not expressed anywhere else in the body (Freemerman et al. 1994). Consistent with this, it was found that immunization with the SP-10 antigen does not lead to an autoimmune response in animal models (Kurth et al. 1997; Diekman and Herr. 1997; Kurth et al. 2008). It was therefore tested as a candidate reversible contraceptive vaccine in a number of animal models. Further, the gene coding for the SP-10 protein, *Acrv1*, served as an outstanding model to understand the regulation of male germ-cell-specific gene transcription (Reddi et al. 1999; Reddi et al. 2003; Acharya et al. 2006; Abhyankar et al. 2007; Lalmansingh et al. 2011).

SP-10 is an abundant protein present in both the acrosomal matrix as well as in the inner acrosomal membrane (Foster et al. 1994). We therefore reasoned that SP-10 antibodies might provide robust staining of the acrosome, thus permitting detection of subtle changes in the developing acrosome that might be helpful for distinguishing stages of the cycle of seminiferous epithelium. The human and mouse SP-10 proteins share homology in the carboxyl terminal region, but contain distinct amino termini (Reddi et al. 1995). In order to improve antibody specificity, we raised polyclonal antibodies in guinea pigs using the full-length recombinant mouse SP-10 protein as the immunogen. The resulting anti-mouse SP-10 polyclonal antibodies were characterized by Western blotting and then tested for immunohistochemistry of normal mouse testis cross sections. Our results show that the SP-10 polyclonal antibody stains the acrosome intensely and clearly accentuates differences in the shape of the developing acrosome, thus allowing for simple and unambiguous staging of the cycle of seminiferous epithelium.

RESULTS

Characterization of the mouse SP-10 polyclonal antibodies

Sera from three guinea pigs immunized with recombinant mouse SP-10 were tested for reactivity against the endogenous SP-10 protein in mouse testis and sperm extracts by Western blotting. RIPA-soluble extracts from testis and sperm obtained from adult C57BL/6

males were separated by SDS-PAGE and immunoblotted. The recombinant mouse SP-10 protein used for immunization was included as a positive control. Immunoblotting with the pre-immune sera did not show any reactivity with the extracts (Fig.1, panel: control) whereas immune sera from guinea pigs B, C, and D showed strong reactivity with the histidine-tagged, recombinant mouse SP-10 protein (Fig.1, lanes: Re).

In testis extracts, the SP-10 protein migrates at 45–50 kDa (Foster et al. 1994). All three sera showed immunoreactivity to a single band in this region (Fig.1, lanes: T). In sperm extracts, immunoreactivity was observed with multiple bands between 32–15 kDa (Herr et al. 1992). The 32-kDa band represents the full-length SP-10 protein, while the 30–15-kDa bands correspond to proteolytically cleaved products; this molecular heterogeneity of SP-10 protein was previously documented by N-terminal amino-acid sequencing (Herr et al. 1992). The three guinea pig immune sera showed identical reactivity with the SP-10 antigen in testis and sperm extracts. In contrast, the pre-immune sera (control panel, Fig.1) as well as the “no-primary antibody” control showed no background reactivity (data not shown), thus supporting the specificity of the anti-mouse SP-10 polyclonal antibodies.

Staging of the cycle of seminiferous epithelium using the anti-SP-10 antibody

We next performed immunohistochemistry on mouse testis cross sections to localize the SP-10 antigen in germ cells, and to determine the utility of these antisera in staging the cycle of the seminiferous epithelium. Immunohistochemistry of testis cross sections obtained from wild-type C57Bl/6 males was performed using polyclonal antibodies against mouse SP-10 protein generated in each guinea pig (B, C, and D), as described in Materials and Methods. The pre-immune sera showed no reactivity with mouse testis cross sections, thus confirming the specificity of the immune sera (not shown). Immune sera B, C, and D, on the other hand, showed highly specific reactivity in the acrosome of round and elongated spermatids. No other cells in the seminiferous epithelium or in the interstitial space reacted against any of the antibodies. As the immunohistochemical staining pattern was identical for all the sera, here we present data obtained using “antibody D”.

The SP-10 polyclonal antibody marked and defined the changing shape of the acrosome so clearly at each stage that identification of the stages of the cycle of seminiferous epithelium was relatively simple. We performed PAS staining, which labels the glycoproteins within the acrosome (Langreth, 1969), in parallel with immunohistochemistry to mark the acrosome by a conventional method. Serial cross sections of testis were used for antibody-D labeling and PAS staining. The images are arranged in adjacent panels in Figure 2–Figure 7. PAS staining confirmed the acrosome-specific reactivity of the anti-SP-10 sera. In testis cross sections, immunoreactivity against SP-10 was strong, appearing first as a dot and then gradually increased as acrosome formation advanced in round spermatids. The shape of the acrosome was clearly defined, aiding in the identification of stages.

The following is a description of SP-10 reactivity characteristics for each stage of the cycle, followed by confirmation of those stages by conventional morphological and histological criteria as well as by PAS-staining characteristics. In Figures 2–7, Panels A and B show immunoreactivity with anti-SP-10 antibody D while panels C and D show the corresponding serial sections stained by PAS. These lower magnification images showing the entire cross

section of the tubule, captured with a 40x objective, not only depict the changing shape of the acrosome but also to provide a complete picture of the characteristic cellular associations at each stage (Figs. 2–7). The higher magnification images, captured with 100x objective, more clearly show the morphological characteristics of the developing acrosome at each stage (Fig. 8).

Stage I

Newly formed Step-1 round spermatids can be seen at this stage, which immediately follows the second round of meiotic division of spermatocytes in stage XII. SP-10 immunoreactivity is not seen in Step-1 round spermatids because the acrosomal granule has not yet formed in these cells. The older generation of spermatids at this stage, Step-13 elongating spermatids, exhibit SP-10 staining in the acrosome (Fig. 2A). Another distinguishing feature of stage I is the presence of only one generation of spermatocytes (early pachytene spermatocytes), which remain close to the basement membrane. A few type A spermatogonia in mitotic division can be found at stage I. Note that elongating Step-13 spermatids (St13) show acrosomal staining by PAS (Fig. 2C).

Stage II

SP-10 immunoreactivity begins to appear as faint dots in Step-2 round spermatids, consistent with the fact that one or more proacrosomal granules are first formed at this stage (Fig. 2B). Careful examination reveals one or two separate SP-10-stained dots on top of the nucleus, representing the proacrosomal granules. The pachytene spermatocytes at this stage are more advanced. The A4 spermatogonia have divided and differentiated into Intermediate spermatogonia (In) (Fig. 2B and D). A rim of chromatin lines the inner nuclear membrane of the Intermediate spermatogonia, which makes these cells prominent. PAS staining is faintly visible over the proacrosomal granules of Step-2 spermatids (Fig. 2D). The elongated Step-14 spermatids stained equally well with both reagents.

Stage III

The proacrosomal granules have coalesced and formed one big granule that stains intensely by the anti-SP-10 antibody (Fig. 3A). Intermediate spermatogonia are smaller in size compared to stage II, and contain more heterochromatin at the nuclear periphery. PAS staining could also be seen over the acrosomal granule of Step-3 spermatids (Fig. 3C).

Stage IV

The shape of the acrosomal vesicle changed at this stage as the round acrosome flattens upon contacting the nuclear envelope in Step-4 spermatids. The resulting triangular shape of the acrosome, typical of stage IV, can be appreciated well following immunohistochemistry with the anti-SP-10 antibody at both lower (Fig. 3B) and higher magnifications (Fig. 8). The mid-pachytene spermatocytes move away from the basement membrane towards the lumen. The Intermediate spermatogonia at this stage are slightly bigger because they are undergoing mitosis to give rise to type B spermatogonia. PAS staining of the acrosome of Step-4 spermatids can be seen in Fig. 3D.

Stage V

SP-10 immunoreactivity became stronger in Step-5 spermatids. The shape of the acrosome flattens as it spread over the surface of the nucleus of Step-5 spermatid. The contact area between the vesicle and nuclear surface remained flat (Fig. 4A). The pachytene spermatocytes at this stage displayed a darkly stained sex body located near the inner nuclear boundary. The spermatogonia are type B, which are similar in appearance to the Intermediate spermatogonia of stage IV. The PAS staining in Step-5 spermatids is darker compared to stage IV (Fig.4C).

Stage VI

SP-10 immunoreactivity showed a crescent-shaped acrosome in Step-6 spermatids. The acrosome spread out more, with the edges of the vesicle now conforming to the contour of the nucleus, covering at least a third of its surface. The acrosome formed a cap over the nucleus of Step-6 spermatids (Fig. 4B). At this stage, the type B spermatogonia can be seen along the basement membrane. The elongating spermatids move toward the lumen of the tubule. SP-10 immunoreactivity can also be seen on top of the heads in these Step-16 spermatids. PAS staining of the acrosome is appreciable in Step-6 spermatids (Fig. 4D).

Stage VII

Immunoreactivity with the anti-SP-10 antibody showed an expanded acrosome that occupies more than one-third of the surface of the nucleus of Step-7 spermatids (Fig. 5A). The characteristic feature of this stage is that the Step-16 elongated spermatids line the luminal surface of the seminiferous epithelium, ready for release into the lumen at stage VIII. Type B spermatogonia have divided and given rise to preleptotene spermatocytes, which are smaller in size. The dark heterochromatin within the nuclei of preleptotene spermatocytes can be seen lining the inner nuclear membrane. Spreading of the acrosome of Step-7 spermatids can be appreciated in PAS stained sections as well (Fig. 5C).

Stage VIII

The acrosome spreading over the nucleus is maximal in Step-8 spermatids, and covers one-half of the nuclear surface. A notable feature with respect to Step-8 spermatids is that they exhibit a polarity such that the acrosome is pointing towards the basement membrane. The intensity of anti-SP-10 antibody staining is maximal at this stage, making it easy to identify the polarized Step-8 round spermatids (Fig. 5B). This stage represents the time point at which the elongated spermatozoa (Step 16) are released from the seminiferous epithelium into the lumen of the tubule. Several preleptotene spermatocytes, which can be distinguished from spermatogonia because of their small size and round nucleus, can be seen lining the basement membrane. The orientation of Step-8 spermatid heads towards the basement membrane can also be appreciated in the PAS stained panel (Fig. 5D). This is the last stage that one can see round spermatids.

Stage IX

The round shape of the nucleus of Step-8 spermatids changes as the nucleus begins to elongate length-wise while flattening bilaterally. There is a curved dorsal side and a flat

ventral side to the sperm head. Overall, the nucleus of Step-9 spermatids appears asymmetrical. The acrosome lies over the rounded dorsal surface of the nucleus. Both anti-SP-10 antibody as well as PAS labeled the acrosome on the dorsal surface of the Step 9-spermatids (Fig. 6A, C). At this stage, mature spermatozoa are missing because they have been released into the lumen in the previous stage; their remaining residual bodies, however, are still prominent at this stage. The preleptotene spermatocytes have become leptotene spermatocytes, which can be identified by the thread-like appearance of chromatin. Thus, at stage IX, there are two generations of spermatocytes: the newly formed leptotenes and the pachytene spermatocytes, which are already in meiotic prophase (Fig. 6A, C).

Stage X

The sperm head looks more elongated and flattened from the sides of Step-10 spermatids. The acrosome, which is stained intensely by the anti-SP-10 antibody, expands over the dorsal surface of the head reaching towards the caudal aspect of the head (Fig. 6B). Type A spermatogonia can be spotted at this stage. The leptotene spermatocytes have given rise to zygotene spermatocytes. The pachytene spermatocytes are bigger in size compared to stage IX. PAS staining is comparable to the SP-10 immunostaining of the acrosome (Fig. 6D).

Stage XI

The entire length of the sperm head appears to be stained by the anti-SP-10 antibody in Step-11 spermatids (Fig. 7A) because the acrosome moves along the dorsal surface of the head, reaching back to the caudal aspect (compare the acrosomal staining in stage X and XI) (Fig. 8). The shape of the sperm is more elongated, slender, and variable compared to the previous stage.

Stage XII

The spermatid head appeared the longest, and the SP-10 immunoreactive acrosome can be seen covering the entire length of the head of Step-12 spermatids (Fig. 7B). Although the shape of the elongating spermatids appears similar at stages XI and XII, the latter stage can be easily identified because of the meiotic figures displaying metaphase, anaphase, or telophase chromosomes (Fig. 7B, D). The secondary spermatocytes provide additional diagnostic markers of stage XII, but one has to carefully distinguish them from Step-1 spermatids based on their larger size. The zygotene spermatocytes are in the process of transitioning into pachytene spermatocytes at stage XII. In both stages XI and XII, comparable staining was obtained with the anti-SP-10 antibody and PAS staining (Fig. 7D).

Stages at a glance

Higher magnification images showing a portion of the epithelium with characteristic cellular associations from each stage are assembled as a composite in Figure 8. This montage illustrates the usefulness of the anti-SP-10 antibody in identifying subtle differences in the shape of the developing acrosome (Steps-1 to -12 spermatids of stages I-XII are marked as St1-12, and the arrows point to the acrosome).

The proacrosomal granule, lightly stained in Step-2 spermatids, coalesced to form a single, round acrosomal vesicle that made contact with the nucleus in Step 3. Flattening of the

acrosomal vesicle over the surface of the nucleus, giving the distinct triangular shape at Step 4, and the gradual spreading out at Step 5 can be appreciated because of the antibody staining. The difference between Steps 5 and 6 is that the acrosomal vesicle in contact with the nuclear surface is flat at Step 5, whereas it is expanded and curves with the contour of the nucleus at Step 6. Continued expansion over the nucleus in Steps 6 and 7, before the acrosome eventually occupies more than a third of the nuclear surface, is also captured well due to SP-10 immunoreactivity. The difference between Step-7 and -8 spermatids, in addition to the extent of the spread of the acrosome, is that the acrosomes face the Sertoli cells and the basal aspect of epithelium at Step 8. The asymmetrical shape of the nucleus, with the acrosome covering its dorsal surface, can be seen in Step-9 spermatids. Steps 10 to 12 are readily identifiable by a lack of round spermatids. The spermatid head is more elongate in Step-11 compared to Step-10 spermatids, and the acrosome covers the dorsal surface of the nucleus in both. Step 12 shows the longest nucleus, with the most accentuated acrosomal SP-10 staining.

DISCUSSION

Determining which stage of the seminiferous cycle is represented by a given cross section of the testis is challenging (Meistrich and Hess. 2013, Ahmed and de Rooij. 2009). The textbook method is to use spermatid morphology as a guide to identify stages. The problem, however, is that at the light-microscope level, round spermatids at stages I-VIII appear very similar by H&E-staining of histological sections. Yet, it is critical to distinguish among subtle changes in the shape of the acrosome as it develops in round spermatids. Thus, a robust reagent that stains the acrosome from the point of its biogenesis to complete formation of the organelle is required. Here, we report that polyclonal antibodies raised against the mouse acrosomal protein SP-10 stain the acrosome intensely, and are ideal for staging the cycle of seminiferous epithelium in mouse testis cross sections. The anti-SP-10 antibody accentuated the characteristic morphological features of the acrosome pertaining to each stage so well that it has made staging of the cycle relatively simple. Based on the data presented above, we have established the following criteria for staging of the cycle of seminiferous epithelium using immunohistochemistry with the SP-10 polyclonal antibody.

Guidelines for staging using the anti-SP-10 antibody

The SP-10 staining is quite robust making it easy to apply the following diagnostic measures for the identification of stages. After performing immunohistochemistry on mouse testis cross sections with the anti-SP-10 antibody, first separate tubule sections into those containing round spermatids (I-VIII) and those that do not (IX-XII). By focusing on tubules containing round spermatids (I-VIII), separate them further based on immunoreactivity with the developing acrosome structure on top of the nucleus: none, low, medium, or high. The tubule cross sections in which round spermatids show no immunoreactivity correspond to stage I of the cycle; at this stage, no acrosomal granule is present in round spermatids (Fig. 2A). Closer examination shows light staining of a region corresponding to the juxtanuclear Golgi apparatus in some Step-1 spermatids (Fig. 2A). Next, tubules with low immunoreactivity show one or two proacrosomal granules weakly reactive with the anti-SP-10 antibody; these correspond to stage II (Fig. 2B). From this point onwards, the

acrosome in round spermatids is stained very clearly with the anti-SP-10 antibody; it goes from medium intensity in stages III-IV to high intensity in stages V-VIII (Figs 3–8). The shape of the developing acrosome changes from round in Step 3 (stage III) to triangular in Step 4 (stage IV) spermatids (Fig. 3A, B). The SP-10 immunoreactivity enhances the distinction between round spermatids at Stages V and VI. In Step-5 spermatids (stage V), the acrosome begins to spread, but the plane that is in contact with the nuclear surface still remains flat whereas the edges of the acrosome form a bend conforming to the nuclear surface in Step-6 spermatids. This distinction in morphology can be made by SP-10 immunohistochemistry (Fig. 4A,B; Fig. 8). Stages VII and VIII can be identified by the fully developed, crescent-shaped acrosome stained strongly by the anti-SP-10 antibody. Compared to Step 6, the acrosome spreads out more and occupies nearly one-half of the nuclear surface in Step-7 spermatids (Fig.5A). In Step-8 spermatids, all the acrosomes are oriented towards the basement membrane of the tubule (Fig. 5B).

Stages IX to XII can be set apart based on the fact that they do not contain round spermatids. The acrosomes of Steps-9 to –12 spermatids show SP-10 immunoreactivity on the dorsal surface (Steps 9–10) and on the apical surface (Steps 11–12) of the sperm heads (Fig. 6A,B; Fig. 8). Step-9 spermatids are easily identified because of their irregular shape as the round spermatid begins to elongate. Step-10 spermatids are fully elongated, but the nucleus is not as condensed compared to the Step-11 spermatids of stage XI (Fig. 7A). Finally, stage XII is the easiest stage to identify independent of spermatid morphology because spermatocytes have completed meiotic divisions I and II by this stage, and therefore meiotic figures showing metaphase spreads (chromosomes lined up at the equatorial region of spermatocytes) can be spotted easily (Fig.7B).

The mouse is the most widely used animal model for studies on mammalian spermatogenesis and fertility. To analyze the effect of treatment with a pharmacological reagent or gene knockout on spermatogenesis, researchers typically fix the testes (*e.g.*, in Bouin's solution or paraformaldehyde), cut sections of the paraffin embedded or frozen tissue, and perform H&E, immunohistochemistry, and PAS staining on the sections. The present study suggests that using the anti-SP-10 antibody for immunohistochemistry will be extremely useful for phenotype analysis of mouse models with genetic mutations that present with a failure in spermatogenesis. Testis cross sections of mutant mice with germ-cell defects often show maturation arrest at various points. Since the morphological differences characteristic of Steps-1 to –8 round spermatids are easily discernible using the anti-SP-10 antibody (Fig. 8), this reagent will be extremely useful to determine the precise point at which the differentiation of round spermatids fails to progress further in the testis of the mutant mouse. For example, Pan and colleagues used our anti-SP-10 antibody on 4% paraformaldehyde-fixed, frozen sections of the testis, and performed immunofluorescence to characterize the effect of the *Rnf17*-knockout on spermatogenesis (Pan et al. 2005). Bi and colleagues used our anti-SP-10 antibody on both Bouin's and formaldehyde-fixed and paraffin-embedded testis for immunohistochemistry to study spermatid maturation arrest due to the *Basigin* knockout. Similarly, VanGompel and Xu documented the utility of our anti-SP-10 antibody in determining the stage at which spermatid arrest took place in their *Boule*-knockout mice (VanGompel and Xu. 2010). Thus, the anti-SP-10 antibody works well in

different fixatives and cryosections, and its utility in characterizing the testis phenotype of knockout mice has been demonstrated by several laboratories.

There are other well-characterized acrosomal proteins in addition to SP-10, such as acrosin and sp56 (Kallajoki et al. 1986; Kim et al. 2001a, 2001b; Roqueta-Rivera et al. 2011). A comprehensive study on the utility of anti-acrosin or anti-sp56 antibodies, in terms of staging the cycle, has yet to be documented though. Such information will be beneficial to the field because some of the genetic models that exhibit spermatid arrest may express one, but not the other, acrosomal antigen, so it will be advantageous to use a battery of acrosomal markers to achieve a thorough analysis.

Finally, since the SP-10 protein is evolutionarily conserved, the polyclonal antibodies raised against the full-length mouse SP-10 protein should cross-react with other species, including human, monkey, and rat. Thus, the anti-SP10 antibody D reported here will be useful for staging the seminiferous cycle in these species as well. The antibody could also be useful in the infertility clinic using assisted reproductive technologies. In cases where round spermatid injections are performed for in vitro fertilization, for example, prior screening of a portion of the extracted testicular sample with the anti-SP-10 antibody will inform the progression of spermiogenesis in a patient. This information will be valuable for both the clinician and the infertile patient for decision-making. We will continue to make available aliquots of the anti-SP10 antibody D reported here to investigators for use in academic research.

MATERIALS AND METHODS

Generation of polyclonal antibodies to mouse SP-10 protein

The coding sequence for mouse SP-10 was cloned in pET22b+ vector, and this plasmid was then used to produce and purify histidine-tagged recombinant SP-10 protein, as previously described (Reddi et al. 1994; Reddi et al. 1995). The recombinant protein consisted of murine SP-10 protein, spanning amino acids 17 through 264, with a carboxyl-terminal six-histidine tag. The purified recombinant protein was mixed with the Incomplete Freund's Adjuvant (Sigma), and three separate guinea pigs (B, C, and D) were immunized as described previously (Acharya et al. 2006). After two booster injections at one month intervals, final bleeds were collected and aliquots were stored at -80°C .

Immunoblot analysis

Decapsulated testes from C57Bl/6 males (11 weeks of age) were snap frozen in liquid nitrogen before transferring to -80°C . Cauda epididymides were carefully dissected out, teased, and suspended in sterile phosphate-buffered saline (PBS) for 15 min at 37°C to release sperm. Sperm were collected without letting the pipet tip touch the minced tissue at the bottom of the tube, counted, and pelleted at 6000 rpm at 4°C . Protein extraction from the frozen testis and sperm samples was performed by brief homogenization followed by extraction with RIPA buffer. Testis and sperm protein extracts as well as recombinant mouse SP-10 protein used for immunization of guinea pigs were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membrane (Laemmli. 1970; Towbin and

Gordon. 1984). The blots were incubated with guinea pig anti-SP10 antibodies B, C, and D or preimmune sera at a 1:5000 dilution. Goat anti-guinea pig HRP-conjugated secondary antibodies (Peroxidase-AffiniPure Goat Anti-Guinea Pig IgG (H+L) Code: 106-035-003, Jackson Immuno Research Laboratories) were used at a 1:7000 dilution. Immunoreactivity was visualized by chemiluminescence (Amersham Pharmacia, NJ).

Immunohistochemistry

C57Bl/6 males (11 weeks of age) were euthanized as per the guidelines of the University of Virginia Institutional Animal Care and Use Committee. Testes were harvested and fixed overnight in Bouin's fixative (Sigma), followed by embedding in paraffin (Hogarth and Griswold. 2013). Five-micron-thick sections were deparaffinized in xylene, and rehydrated in a graded series of ethanol baths. Immunohistochemistry was performed on a robotic platform (Autostainer, Dako, Glostrup, Denmark). Endogenous peroxidases were blocked using Peroxidase and Alkaline Phosphatase Blocking Reagent (Dako). No antigen retrieval step was performed. Guinea pig anti-mouse SP-10 primary antibodies (B, C, and D) were used at a 1:1000 dilution. Goat anti-guinea pig-HRP conjugated secondary antibodies (Peroxidase-AffiniPure Goat Anti-Guinea Pig IgG (H+L) Code: 106-035-003, Jackson Immuno Research Laboratories) were used at a 1:200 dilution. The antigen-antibody reaction was assessed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB+) chromogen (Dako), as per the manufacturer's instructions. All the slides were counterstained with hematoxylin, and were then dehydrated, cleared, and mounted for assessment and imaging. Periodic acid-Schiff histology was performed using a PAS kit (Sigma-Aldrich), following the instructions provided by the manufacturer.

Imaging

Brightfield images shown in Figures 2–7 were captured using an Olympus BX41 microscope with the 40x objective via Olympus Microsuite™ Five image processing software. Images in Figure 8 were captured using an Olympus UPlanSApo 100x/1.4 Oil / 0.17/FN 26.5 objective with a Dage-MTI DC330 camera and Scion Image software.

Acknowledgments

The authors thank the anonymous reviewers for their helpful suggestions. We are grateful to Drs. Ammasi Periasamy and David Shook of the Department of Biology, University of Virginia, for use of the Olympus microscope with 100x oil immersion objective. This work was supported by NIH R01HD36239 (PPR).

Abbreviations

H&E	hematoxylin and eosin
PAS	Periodic Acid-Schiff
SP-10	sperm protein 10

REFERENCES

- Abhyankar MM, Urekar C, Reddi PP. A novel CpG-free vertebrate insulator silences the testis-specific SP-10 gene in somatic tissues: role for TDP-43 in insulator function. *J Biol Chem.* 2007; 282:36143–36154. [PubMed: 17932037]
- Acharya KK, Govind CK, Shore AN, Stoler MH, Reddi PP. Cis-Requirement for the Maintenance of Round Spermatid-Specific Transcription. *Dev Biol.* 2006; 295:781–790. [PubMed: 16730344]
- Ahmed EA, de Rooij DG. Staging of mouse seminiferous tubule cross-sections. *Methods Mol Biol.* 2009; 558:263–277. [PubMed: 19685330]
- Bi J, Li Y, Sun F, Saalbach A, Klein C, Miller DJ, Hess R, Nowak RA. Basigin null mutant male mice are sterile and exhibit impaired interactions between germ cells and Sertoli cells. *Dev Biol.* 2013; 380:145–156. [PubMed: 23727514]
- Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev.* 1972; 52:198–236. [PubMed: 4621362]
- Diekmann AB, Herr JC. Sperm antigens and their use in the development of an immunocontraceptive. *Am J Reprod Immunol.* 1997; 37:111–117. [PubMed: 9138444]
- Foster JA, Klotz KL, Flickinger CJ, Thomas TS, Wright RM, Castillo JR, Herr JC. Human SP-10: acrosomal distribution, processing, and fate after the acrosome reaction. *Biol Reprod.* 1994; 51:1222–1231. [PubMed: 7888499]
- Freemerman AJ, Wright RM, Flickinger CJ, Herr JC. Tissue specificity of the acrosomal protein SP-10: a contraceptive vaccine candidate molecule. *Biol Reprod.* 1994; 50:615–621. [PubMed: 8167233]
- Herr JC, Klotz K, Shannon J, Wright RM, Flickinger CJ. Purification and microsequencing of the intra-acrosomal protein SP-10. Evidence that SP-10 heterogeneity results from endoproteolytic processes. *Biol Reprod.* 1992; 47:11–20. [PubMed: 1637938]
- Herr JC, Wright RM, John E, Foster J, Kays T, Flickinger CJ. Identification of human acrosomal antigen SP-10 in primates and pigs. *Biol Reprod.* 1990; 42:377–382. [PubMed: 2337631]
- Hess RA, Renato de Franca L. Spermatogenesis and cycle of the seminiferous epithelium. *Adv Exp Med Biol.* 2008; 636:1–15. [PubMed: 19856159]
- Hogarth CA, Griswold MD. Immunohistochemical approaches for the study of spermatogenesis. *Methods Mol Biol.* 2013; 927:309–320. [PubMed: 22992925]
- Kallajoki M, Parvinen M, Suominen JJ. Expression of acrosin during mouse spermatogenesis: a biochemical and immunocytochemical analysis by a monoclonal antibody C 11 H. *Biol Reprod.* 1986; 35:157–165. [PubMed: 3527284]
- Kim KS, Cha MC, Gerton GL. Mouse sperm protein sp56 is a component of the acrosomal matrix. *Biol Reprod.* 2001a; 64:36–43. [PubMed: 11133656]
- Kim KS, Foster JA, Gerton GL. Differential release of guinea pig sperm acrosomal components during exocytosis. *Biol Reprod.* 2001b; 64:148–156. [PubMed: 11133669]
- Kurth BE, Digilio L, Snow P, Bush LA, Wolkowicz M, Shetty J, Mandal A, Hao Z, Reddi PP, Flickinger CJ, Herr JC. Immunogenicity of a multi-component recombinant human acrosomal protein vaccine in female *Macaca fascicularis*. *J Reprod Immunol.* 2008; 77:126–141. [PubMed: 17643494]
- Kurth BE, Weston C, Reddi PP, Bryant D, Bhattacharya R, Flickinger CJ, Herr JC. Oviductal antibody response to a defined recombinant sperm antigen in macaques. *Biol Reprod.* 1997; 57:981–989. [PubMed: 9369161]
- Kurth BE, Wright RM, Flickinger CJ, Herr JC. Stage-specific detection of mRNA for the sperm antigen SP-10 in human testes. *Anat Rec.* 1993; 236:619–625. [PubMed: 8379586]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Lalmansingh AS, Urekar CJ, Reddi PP. TDP-43 is a transcriptional repressor; the testis-specific mouse ACRV1 gene is a TDP-43 target in vivo. *J Biol Chem.* 2011
- Langreth SG. Spermiogenesis in Cancer crabs. *J Cell Biol.* 1969; 43:575–603. [PubMed: 4187136]

- Meistrich ML, Hess RA. Assessment of spermatogenesis through staging of seminiferous tubules. *Methods Mol Biol.* 2013; 927:299–307. [PubMed: 22992924]
- Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat.* 1956; 99:507–516. [PubMed: 13402729]
- Pan J, Goodheart M, Chuma S, Nakatsuji N, Page DC, Wang PJ. RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development.* 2005; 132:4029–4039. [PubMed: 16093322]
- Reddi PP, Castillo JR, Klotz K, Flickinger CJ, Herr JC. Production in *Escherichia coli*, purification and immunogenicity of acrosomal protein SP-10, a candidate contraceptive vaccine. *Gene.* 1994; 147:189–195. [PubMed: 7926798]
- Reddi PP, Flickinger CJ, Herr JC. Round spermatid-specific transcription of the mouse SP-10 gene is mediated by a 294-base pair proximal promoter. *Biol Reprod.* 1999; 61:1256–1266. [PubMed: 10529272]
- Reddi PP, Naaby-Hansen S, Aguolnik I, Tsai JY, Silver LM, Flickinger CJ, Herr JC. Complementary deoxyribonucleic acid cloning and characterization of mSP-10: the mouse homologue of human acrosomal protein SP-10. *Biol Reprod.* 1995; 53:873–881. [PubMed: 8547483]
- Reddi PP, Shore AN, Shapiro JA, Anderson A, Stoler MH, Acharya KK. Spermatid-specific promoter of the SP-10 gene functions as an insulator in somatic cells. *Dev Biol.* 2003; 262:173–182. [PubMed: 14512027]
- Roqueta-Rivera M, Abbott TL, Sivaguru M, Hess RA, Nakamura MT. Deficiency in the omega-3 fatty acid pathway results in failure of acrosome biogenesis in mice. *Biol Reprod.* 2011; 85:721–732. [PubMed: 21653892]
- Russell, LD.; Ettlin, RA.; Sinha Hikim, AP.; Clegg, EC. *Histological and histopathological evaluation of the testis.* USA: Cache River Press; 1990.
- Soderstrom KO, Malmi R, Karjalainen K. Binding of fluorescein isothiocyanate conjugated lectins to rat spermatogenic cells in tissue sections. Enhancement of lectin fluorescence obtained by fixation in Bouin's fluid. *Histochemistry.* 1984; 80:575–579. [PubMed: 6206032]
- Towbin H, Gordon J. Immunoblotting and dot immunobinding--current status and outlook. *J Immunol Methods.* 1984; 72:313–340. [PubMed: 6206159]
- VanGompel MJ, Xu EY. A novel requirement in mammalian spermatid differentiation for the DAZ-family protein Boule. *Hum Mol Genet.* 2010; 19:2360–2369. [PubMed: 20335278]
- Wollina U, Schreiber G, Zollmann C, Hipler C, Gunther E. Lectin-binding sites in normal human testis. *Andrologia.* 1989; 21:127–130. [PubMed: 2469362]

Figure 1. Characterization of the anti-mouse SP-10 polyclonal antibodies raised in guinea pigs Immunoblots containing recombinant mouse SP-10 (Re), mouse testis extract (T), and mouse sperm extract (S) were probed with 1:7000 dilution of immune sera from guinea pigs B, C, and D (Ab-B, Ab-C, and Ab-D, respectively). The preimmune sera did not show any background reactivity (control). Note the identical immunoreactivity by sera from the three individual guinea pigs. Positive signals above 50 kD (Re), approximately 45 kD (T), and bands ranging from 32-14 kD (S) match the expected pattern for recombinant SP-10, SP-10 in the testis, and sperm, respectively (see text).

Figure 2. Stages I and II: Dot-like appearance of SP-10 in proacrosomal granules of round spermatids at stage II

The anti-SP-10 antibody marked the proacrosomal granules of the Step-2 round spermatids at stage II (St2 in B). The Golgi-phase acrosome vesicles are stained brown in a few Step-1 round spermatids at stage I, but not by PAS (St1 in A and C). A and B represent immunohistochemistry using the anti anti-SP-10 antibody (brown staining) while C and D are adjacent sections stained with PAS reagent (purple staining). A, Type A spermatogonium; In, intermediate spermatogonium; P, pachytene spermatocyte; Ser, Sertoli cell; St1 and St2, Step-1 and Step-2 round spermatids; St13 and St14, Step-13 and Step-14 elongated spermatids. Scale bars, 50 μ m.

Figure 3. Stages III and IV: The anti-SP-10 antibody staining distinguishes round spermatids of stages III and IV

The anti-SP-10 antibody shows the round shape of the acrosome of round spermatids at stage III (St3 in A), and its triangular shape at stage IV (St4 in B). In contrast, PAS staining can only be seen in round spermatids at stage IV (St4 in D). In, intermediate spermatogonium; P, pachytene spermatocyte; Ser, Sertoli cell; St3 and St4, Step-3 and Step-4 round spermatids; St15, Step-15 elongated spermatids. Scale bars, 50 μm.

Figure 4. Stages V and VI: The anti-SP-10 antibody staining shows the flattening and spreading of the acrosome over the nucleus of round spermatids

Note that in round spermatids (St5 in A) at stage V, the part of the acrosome that comes in contact with the nucleus has flattened. At stage VI, the spreading of the acrosome over the nucleus can be seen (St6 in B). These morphological changes characteristic of stages V and VI can be appreciated with the anti-SP-10 antibody staining (St5-6 in A-B). B, Type B spermatogonium; P, pachytene spermatocyte; Ser, Sertoli cell; St5 and St6, Step-5 and Step-6 spermatids; St15, Step-15 elongated spermatids. Scale bars, 50 µm.

Figure 5. Stages VII and VIII: The acrosome spreads over half the surface of the nucleus in round spermatids at VII and the acrosomes face the basal aspect of the epithelium at VIII
 The anti-SP-10 antibody prominently demarcates the crescent-shaped acrosome of Step-7 spermatids in stage VII (St7 in A). In the antibody-stained panel B, one can appreciate that the acrosomes of a majority of round spermatids at stage VIII face the basement membrane at stage VIII. Large numbers of spermatozoa (St16) lining the lumen is another diagnostic feature of stage VII. A majority of spermatozoa have been released at spermiation in stage VIII. L, leptotene spermatocyte; P, pachytene spermatocyte; pL, preleptotene spermatocyte; Ser, Sertoli cell; St7 and St8, Step-7 and Step-8 spermatids; St16, Step-16 spermatozoa. Scale bars, 50 µm.

Figure 6. The absence of round spermatids and the distinct shape of spermatid nuclei are diagnostic features of stages IX and X

The acrosome lies over the rounded dorsal surface of the nucleus of spermatids at stage IX (St9 in A and C). As the spermatids elongate at stage X, the acrosome expands and over the dorsal surface and extends to the caudal aspect of the head (St10 in C and D). Note here that the acrosome is stained equally well by the anti-SP-10 antibody (A-B) as well as PAS (C-D). L, leptotene spermatocyte; P, pachytene spermatocyte; Ser, Sertoli cell; St9 and St10, Step-9 and Step-10 spermatids. Scale bars, 50 μm.

Figure 7. Stages XI and XII

The acrosome covers the entire length and reaches the caudal aspect of the head of the elongated spermatid at these stages (St11-12 in A-B). While the shape of the elongated spermatids is identical at both stages, the spermatocytes at anaphase of meiosis aid in identifying stage XII (MII in C and D). Also, secondary spermatocytes (sSc in C-D) can be seen at stage XII. As in stage IX and X, the acrosome is stained equally well by the anti-SP-10 antibody (A-B) as well as PAS (C-D). Di, diplotene spermatocyte; MII, meiotically dividing spermatocyte in metaphase; P, pachytene spermatocyte; Ser, Sertoli cell; sSc, secondary spermatocyte; St11 and St12, Step-11 and Step-12 elongated spermatids; Z, zygotene spermatocyte. Scale bars, 50 μm.

Figure 8. Stages at a glance: higher resolution images of the seminiferous epithelium at all 12 stages, highlighting the morphology of the acrosome in spermatids

Roman numerals on the top indicate cycle stages of the epithelium. Round spermatids of stages I-VIII are designated as St1-8 and elongated spermatids of stages IX-XII as St9-12. The more advanced elongated spermatids (Steps 13-16) of stages I-VIII are not marked here. In these higher-resolution images, captured using 100x oil immersion objective, the SP-10 immunoreactivity makes it easy to distinguish the morphological features of the acrosome that are characteristic for spermatids at each stage. (See Results for description). Other cell types seen in the epithelium are also marked at each stage. G, spermatogonia; L, leptotene spermatocytes, PL, preleptotene spermatocytes; P, pachytene spermatocytes; S, Sertoli cells; St1-St12, spermatids steps 1-12.