Sperm-specific proteins: new implications for diagnostic development and cancer immunotherapy

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Sperm-specific proteins: new implications for diagnostic development and cancer immunotherapy

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Abstract

Spermatozoa are comprised of many unique proteins not expressed elsewhere. Sperm-specific proteins are first expressed at puberty, after the development of immune tolerance to self-antigens, and have been assumed to remain confined inside the seminiferous tubules, protected from immune cell recognition by various mechanisms of testicular immune privilege. However, new data has shown that sperm-specific proteins are released by the tubules into the surrounding interstitial fluid; from here they can contact immune cells, potentially promote immune tolerance, and enter the circulation. These new findings have clinical implications for diagnostics and therapeutics targeted at a specific class of proteins known as cancer-testis antigens (CTA), the opportunity to identify new communication pathways in the testis, and to discover new ways to monitor testis function.

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Introduction

Spermatozoa produced by the testis are one of the most highly specialized cells in the body. They contain a long, motile flagella to maneuver through the female tract, an acrosome that facilitates binding to, and fertilization of, the oocyte, and a compact nucleus containing highly condensed DNA that provides the paternal genetic code. Sperm develop within the seminiferous tubules supported by the somatic Sertoli cells, under the influence of endocrine stimulation from the pituitary and androgen production by the interstitial Leydig cells (LC) (Figure 1) [1]. Sperm develop from diploid spermatogonia that include cells committed to differentiation as well as self-renewing germline stem cells that enable life-long spermatogenesis. Spermatogonia divide to produce spermatocytes that undergo meiosis, involving homologous chromosome recombination to ensure genetic diversity of the gametes, and two reductive meiotic divisions to produce haploid round spermatids. These spermatids then undergo a complex cytodifferentiation process to produce the elongated spermatid that is released by the Sertoli cell at the end of spermatogenesis [2].

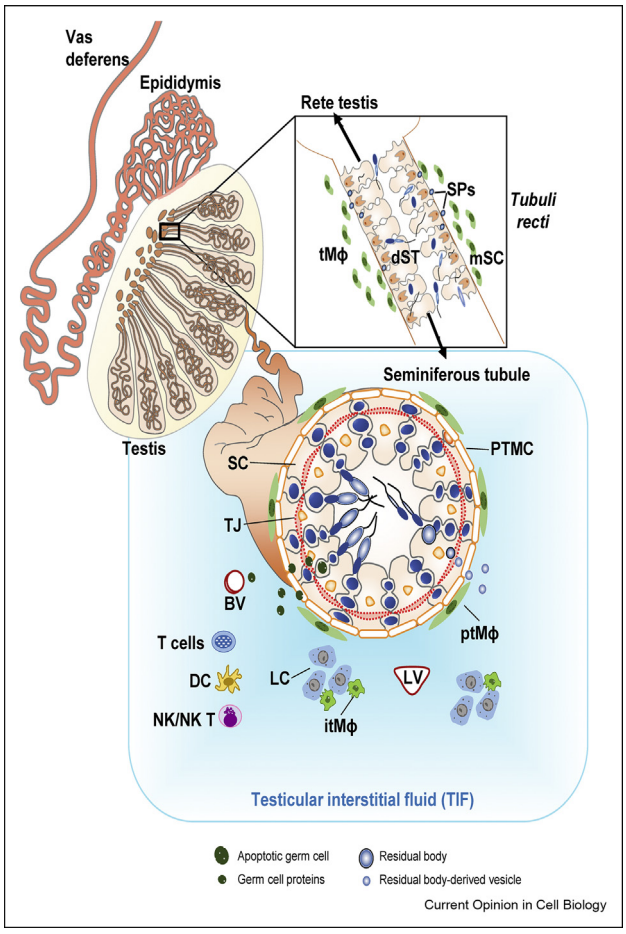
Because spermatids are so specialized, they are comprised of many unique proteins not found elsewhere in the body [3,4]. Sperm first form at puberty, long after the establishment of immune tolerance to self-antigens, and thus it has long been assumed that sperm-specific proteins must be protected from interaction with immune cells to prevent an autoimmune response. However, emerging data on sperm-specific proteins and their localization suggests the issue is more complex, with many sperm proteins visible to the immune system, changing the current understanding and offering potential applications of this newfound knowledge for clinical diagnoses and therapies.

Sperm are highly specialized cells that express many unique proteins

Newly formed haploid round spermatids transform over several weeks into elongated spermatids that are released by the Sertoli cells at the end of spermatogenesis. Spermatids are non-motile when released by Sertoli cells; they become functional, motile spermatozoa as they transit through the epididymis (Figure 1).

Given that the elongated spermatid is such a complex cell with a multitude of functional structures, it is

Figure 1



Sperm-proteins are released from the seminiferous tubules. The sperm-producing seminiferous tubules are coiled inside the testis and empty into the rete testis prior to their entry to the epididymis and ultimately the vas deferens. *Inset*: the *tubuli recti* are short segments of modified tubules at the end of the seminiferous tubules as they terminate at the rete testis. These tubules contain a narrow lumen and an epithelium of modified Sertoli cells (mSC). Fragments of degenerating sperm (dST) often appear in the epithelium and sperm proteins (SPs) are hypothesized to exit the tubules due to a weak epithelial barrier [45]. MHCII + testicular macrophages (tMϕ) are concentrated around this site in the normal testis. The seminiferous tubules are comprised of Sertoli cells (SC) and the developing male germ cells (in blue) surrounded by basement membrane and peritubular myoid cells (PTMC). Tight junctions (TJ) between Sertoli cells prevent the free passage of molecules and other cells in and out of the tubules. The testicular interstitial fluid (TIF) surrounds the tubules. The interstitium contains the steroidogenic Leydig cells (LC), blood vessels (BV), lymphatic vessels (LV), interstitial testicular macrophages (itMϕ), and other immune cells including a variety of T cells, dendritic cells (DC), and NK and NK-T cells. Elongated peritubular macrophages (ptMϕ) surround the seminiferous tubules. After mature sperm are released from the tubules, remnants of their cytoplasm known as residual bodies are phagocytosed and processed; at least one protein within residual bodies has been shown to be released from the tubules and promote peripheral tolerance. Germ cell proteins could also be released from particular sites within the tubules where there is continual apoptosis of a small percentage of spermatocytes and round spermatids. Figured adapted from Ref. [26].

perhaps not surprising that the process required to "build" the spermatid — known as spermiogenesis — involves the transcription of many novel genes, non-coding RNAs, and the post-translational regulation of protein expression [4–6]. There are waves of gene transcription, with little overlap, as spermatids proceed through spermiogenesis [7], highlighting that each of the cytoskeletal and nuclear structural changes requires different sets of genes. Because of their unique morphology and function, spermatids express a multitude of genes and proteins not expressed elsewhere in the body [3,4]. Sperm-specific proteins could be particularly attractive prospects as male contraceptive targets, as drugs that amend their function would be less likely to produce off-target effects. Example targets for contraception include lactate dehydrogenase C (LDHC) [8] and epididymal protease inhibitor (EPPIN) [9].

The protection of sperm-specific proteins within the seminiferous tubules

Spermatids first form at puberty, after the development of the immune system and systemic immune tolerance soon after birth. Thus, there is a risk of sperm-specific proteins being recognized by the immune system leading to an autoimmune response [10,11]. To prevent such a response, the seminiferous tubules and the testes are immune-privileged sites, with multiple mechanisms acting to protect the sperm from immune recognition [10,11]. Immune privilege is thought to be an evolutionary adaptation to protect vulnerable tissues with little capacity for regeneration, such as the testis [11].

The entire testis is considered an immune-privileged organ, and the interstitial space between the seminiferous tubules confers multiple levels of protection [11–13]. For example, the interstitium contains large numbers of specialized testicular macrophages with anti-inflammatory activities, such as via the production of anti-inflammatory cytokines TGFβ and IL10 [6]. Two distinct subtypes of testicular macrophages with immune–suppressive properties self-maintain their population in a steady state in the normal adult testis [14]. These macrophages reside in close contact with LC or with the basal aspect of the seminiferous tubules (Figure 1) and play important roles in testis development and adult testis function and homeostasis, as well as contributing to immune suppression and the maintenance of testicular immune privilege [12,15]. A variety of immune cells, including T cells, T memory cells, and dendritic cells, are also present in the interstitium (Figure 1) and contribute to local immune privilege, allowing a balance between local immunosuppressive mechanisms and an ability to respond to infection and inflammation when required [11,12,16,17].

Another mechanism governing testicular immune privilege is that the seminiferous tubules restrict the entry of immune cells to the adluminal compartment. Sertoli cells form specialized intercellular tight junctions that prevent the free passage of molecules and cells into the tubules (Figure 1); these junctions form part of the "blood-testis barrier" that protects developing sperm from immune cell recognition [10]. Sertoli cells also express immunoregulatory factors to create an immune-privileged environment [10,11,18], as evidenced by the protection of Sertoli cell grafts from immune rejection when they are transplanted into other sites [10]. In mice with Sertoli cells lacking the androgen receptor, the permeability of tight junctions between Sertoli cells is increased, their sera contain antibodies that recognize spermatid antigens, and inflammatory immune cells infiltrate the interstitium, indicating that the immune-privileged status of the seminiferous tubules is compromised [19]. However, the seminiferous tubules of mice in which Sertoli cells have been ablated for up to 1 year show very little infiltration of immune cells into the tubules, suggesting that other cells and factors, such as peritubular myoid cells and the interstitial environment are significant contributors to seminiferous tubule immune privilege [20]. Thus, Sertoli cells are only one factor contributing to immune privilege within the seminiferous tubules.

Another major mechanism that was widely believed to confer immune privilege within the testis was the assumed protection of sperm-specific proteins from immune system recognition via sequestration within the seminiferous tubules (Figure 1). While immature germ cells "below" the blood-testis barrier (basal) can be recognized by the immune system [21], proteins from developing sperm "above" the barrier (adluminal) have long been thought to be actively prevented from being released into the interstitium where they could contact immune cells [10]. This sequestration is thought to be facilitated by the blood-testis barrier [10,11]. However, reproductive immunologists noted early on that this sequestration may not be complete, and that some sperm-specific antigens may be able to induce immune tolerance [22,23] via mechanisms involving T regulatory (Treg) cells [24]. This suggests that the sequestration of sperm-specific proteins within the seminiferous tubules is not complete.

Some sperm-specific proteins are released from the seminiferous tubules

A recent study showed that a protein highly specific to sperm, LDHC, was able to promote Treg-mediated peripheral immune tolerance in mice [25]. These elegant studies proved that LDHC was able to "egress" from the seminiferous tubules into the interstitial space (Figure 1) and contact nearby immune cells to promote tolerance, whereas another sperm-specific protein (zonadhesin) was unable to tolerize, suggesting it remained sequestered inside the seminiferous tubules. This study proved that sperm-specific proteins under normal circumstances could be released by the seminiferous tubules for the purpose of promoting peripheral immune tolerance [25].

However, the extent to which sperm proteins are released by the seminiferous tubules into the testicular interstitial fluid (TIF, see Figure 1) was unknown until a high-resolution mass spectrometry approach was used to resolve the TIF proteome. The analysis showed that hundreds of sperm proteins are released by the seminiferous tubules into TIF in both mice and men [26]. From this fluid, these proteins are free to contact resident testicular immune cells, and enter the lymphatics and blood vessels (BV) (Figure 1). Many of these proteins were highly specific to sperm and not expressed elsewhere in the body — thus they would be particularly vulnerable to immune system recognition. The abundance of sperm-specific proteins in TIF is relatively low, and these proteins are only detectable using a high-resolution mass spectrometry method, explaining why they have not been detected using less sensitive methods [26,27]. The fact that many of the sperm-specific proteins released into TIF are conserved between mice and men [26] suggests that this process is a physiologically important phenomenon.

Sertoli cells deposit the sperm proteins into TIF as evidenced by a highly significant decrease in sperm-specific proteins in TIF within one week of Sertoli cell ablation from mouse seminiferous tubules. Only a subset of sperm-specific proteins in the seminiferous tubules was detected in TIF; approximately 40% of mouse sperm-specific proteins were detected but the remaining 60% were not [26]. This supports the previous observation that a sperm-specific protein LDHC was able to promote peripheral tolerance, but another protein, zonadhesin, which is not detectable in TIF [26], was unable to promote peripheral tolerance [25]. The fact that only a proportion of sperm-specific proteins are released in TIF likely explains the induction of autoimmune orchitis (testicular infection in response to the presence of anti-sperm antibodies) under various circumstances and also why there is an unexpectedly narrow repertoire of anti-sperm antibodies generated after immune-breach of the reproductive tract induced by vasectomy [24,28].

Analysis of the localization of sperm proteins released into TIF revealed some details about possible mechanisms by which they are released. Sperm-specific proteins in TIF were particularly associated with sperm flagella and cytoplasm, but not with nuclear and mitochondrial proteins [26]. When mature sperm are released by the Sertoli cells during the process of spermiation, their cytoplasm is removed, condensed into structures termed residual bodies, and phagocytosed by the Sertoli cells [29]. This suggests the elimination of

sperm proteins by Sertoli cells during spermiation (Figure 1). In support, Tung and colleagues found antibodies to the released protein LDHC accumulated around areas of tubules undergoing spermiation [25]. Other likely sites of sperm protein release include the tubuli recti where the seminiferous tubules empty into the rete testes and from the apoptosis of spermatocytes and spermatids in the mid-spermatogenic stages [26] (Figure 1). Sperm proteins may also be released into TIF via extracellular vesicles from Sertoli cells [30].

What could sperm-specific proteins be doing outside the seminiferous tubules?

From the above information, the most obvious role for sperm-specific proteins released into the interstitium is to provide another layer of peripheral immune tolerance. As discussed above, multiple mechanisms contribute to the maintenance of immune privilege in the testis [10,12]. By depositing hundreds of sperm-specific proteins into the interstitium, the seminiferous tubules may be able to promote peripheral immune tolerance to a wide array of anti-sperm antigens, thus providing another mechanism to prevent the induction of autoimmune inflammatory disorders when the immune privilege status of the reproductive tract is breached by surgery, injury or infection. However, it's important to note that not all sperm-specific proteins released may be capable of promoting tolerance, as has been shown for LDHC [25].

Although the ablation of germ cells from the testis in mice did not influence LC steroidogenesis [31], there is some evidence that the spermatogenic capacity (i.e. number and type of germ cell populations in the tubules) influences optimal steroidogenesis. For example, germ cell transplantation into mice lacking germ cells since birth stimulates LC steroidogenesis [32]. LC and testicular macrophages differ in their transcriptional signature depending on the type of germ cells present in adjacent seminiferous tubules [33] and Leydig stem cell localization after regeneration differs depending on the presence of germ cells in the tubules [34]. These observations could be of clinical relevance because LC function is reduced in men with infertility and poor spermatogenesis compared to their fertile counterparts after long-term follow-up [35]. Thus, it is possible that sperm proteins (or perhaps the release of extracellular vesicles containing sperm proteins as well as other proteins) could support long-term LC health and androgen production.

The clinical implications of sperm-specific proteins being released by the seminiferous tubules

The finding that sperm-specific proteins are released by the seminiferous tubules into the interstitial fluid and can be detected in human plasma [26] challenges the dogma that these proteins only exist within the confines of the blood-testis barrier. The demonstration that sperm-specific LDHC, present at high levels in TIF [26], can promote peripheral immune tolerance in mice [25] suggests that at least some of these sperm-specific proteins may have been previously recognized by the immune system, and could have induced immune tolerance, presumably around the time of puberty when sperm first appear in the body. These findings have a number of highly significant clinical implications.

The first is for CTA biology. CTA are defined in different ways, but in pure terms are defined as proteins specific to germ cells within the blood-testis barrier, not expressed elsewhere in the body, but expressed in cancer [36,37]. The reason why they are commonly expressed in cancer is not entirely known but may be due to the fact that proteins that build the unique spermatozoa provide a competitive advantage when repurposed by cancer cells. Many CTAs are intrinsically disordered proteins with the potential to have multiple functions [38] that could be advantageous to cancer cell proliferation and migration. Indeed, recent studies suggest that CTA expression is a hallmark of cancer [39], highlighting their importance in cancer biology.

Because CTAs are routinely considered to be absent in the circulation of normal, healthy individuals, but are turned on in cancer, they are considered to be excellent targets for biomarker and therapeutic development. However, we have shown that certain CTAs are released into the interstitial fluid and are detectable in human plasma [26] and thus their presence in the circulation of healthy men would be expected. Whether or not a particular CTA is released from the seminiferous tubules into the circulation in normal men, and whether the CTA has the ability to tolerize the immune system, will have a major impact on its clinical utility as a biomarker or immunotherapy target.

Since many (but not all) CTAs can be released from the testis [26] and at least one has been shown to be able to promote peripheral immune tolerance [25], one cannot assume that the presence of particular CTA (or antibodies against it) is indicative of cancer. For example, AKAP4 is a highly sperm-specific protein [40] detected in TIF and human plasma [26], yet it has been suggested to be an excellent target for cancer biomarker and therapeutic development [41]. CTAs are often considered attractive targets for immunotherapy as they are assumed to be neoantigens (i.e. proteins not previously recognized by the immune system) that could be harnessed to generate a strong immune response against a CTA-positive cancer, as has been suggested for LDHC [42,43]. However, CTAs released by the seminiferous tubules are less likely to be neoantigens in men [25,26].

The above observations suggest that whether a CTA is likely to be a neoantigen that could be harnessed for immunotherapy for cancer treatment depends on a) whether the CTA remains sequestered within the

seminiferous tubules or is released into the interstitium, b) the sex of the patient, i.e. presumably men are more likely than women to have male-specific CTAs in their circulation which could reduce the efficacy of particular immunotherapies, and c) the fertility status of the male patient; if he has a congenital or prepubertal form of infertility and has never produced sperm, then certain CTAs could be a neoantigen in this individual, but not in his fertile counterparts. Indeed, we provided the first evidence that the levels of a CTA (LDHC) in TIF correlate with fertility status in men, with a significant reduction seen in men with infertility [26]. Thus, certain CTAs that are released into TIF could show sex- and fertility-dependent differences in terms of their levels in circulation that would influence their ability to generate a strong immune response during immunotherapy.

The fact that certain CTAs are released into TIF in men could limit their utility as biomarkers or immunotherapy targets for all individuals, but also offers an opportunity to better design CTA-based diagnostics and therapeutics. For example, the CTAs NY-ESO-1 (*CTAG1B*), MAGE-A (*MAGEA1*), and IL-3Rα (*IL13RA2*) are not detected in human TIF using pre-fractionation and high-resolution mass spectrometry [26] suggesting they could be useful targets for cancer immunotherapy, as is currently being investigated [44]. Some male-specific CTAs that appear in circulation in men could still be utilized as immunotherapy targets for female-specific cancers, assuming that such proteins are not taken up by the female reproductive tract, such as in sperm-derived exosomes. Thus, knowledge of which male-specific CTAs are released by the seminiferous tubules offers an opportunity to refine CTA selection for biomarker and immunotherapy development.

Finally, the presence of sperm-specific proteins in the circulation presents the opportunity to develop a blood test for male fertility. This would be particularly beneficial for the clinical assessment of men with azoospermia (zero sperm count) and could potentially be used to discriminate which men have sperm in their testes for the purpose of surgical sperm retrieval prior to IVF. Sperm-specific LDHC is decreased in TIF in infertile men, indicating that sperm proteins are released from the seminiferous tubules in a fertility-dependent manner [26]. This provides proof of the concept that the level of sperm-specific proteins could be used to discriminate the spermatogenic capacity of the testis, which is particularly important for men who have azoospermia but may still have sperm present in their testes.

Conclusions

Sperm are highly specialized cells expressing many unique proteins. Because sperm first form at puberty, after immune tolerance has been acquired, sperm-specific proteins were assumed to require protection from immune system recognition by remaining sequestered inside the seminiferous tubules. These proteins, including proteins classified as CTAs, were assumed to be neoantigens and to not be found elsewhere in the healthy male. However new data now show that a proportion of sperm-specific proteins are released by the seminiferous tubules where they can encounter immune cells and enter the circulation [26], and at least one sperm-specific protein and CTA can promote peripheral immune tolerance [25]. These data have important new implications for understanding mechanisms of cell—cell communication in the testis, for optimizing the development of CTA-based cancer biomarkers and immunotherapies, and for the prospects of monitoring sperm production in the testis via a blood test.

Author credit statement

Liza O'Donnell: Conceptualization, Writing- Original draft preparation; **Lee B. Smith**: Conceptualization, Writing- Reviewing and Editing; **Diane Rebourcet**: Conceptualization, Writing- Reviewing and Editing**.**

Conflicts of interest

Nothing declared.

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