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Mechanisms of spermiogenesis and spermiation and how they are disturbed

Liza O'Donnell[1,2,*]

[1]MIMR-PHI Institute of Medical Research; Clayton, Victoria, Australia; [2]Department of Anatomy and Developmental Biology; Monash University; Clayton, Victoria, Australia

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Abbreviations: IFT, Intraflagellar Transport; IMT, Intra-manchette Transport; PAS, Period Acid Schiffs; FAK, Focal Adhesion Kinase; TBC, Tubulobulbar complexes; ES, Ectoplasmic specialization

Haploid round spermatids undergo a remarkable transformation during spermiogenesis. The nucleus polarizes to one side of the cell as the nucleus condenses and elongates, and the microtubule-based manchette sculpts the nucleus into its species-specific head shape. The assembly of the central component of the sperm flagellum, known as the axoneme, begins early in spermiogenesis, and is followed by the assembly of secondary structures needed for normal flagella. The final remodelling of the mature elongated spermatid occurs during spermiation, when the spermatids line up along the luminal edge, shed their residual cytoplasm and are ultimately released into the lumen. Defects in spermiogenesis and spermiation are manifested as low sperm number, abnormal sperm morphology and poor motility and are commonly observed during reproductive toxicant administration, as well as in genetically modified mouse models of male infertility. This chapter summarizes the major physiological processes and the most commonly observed defects in spermiogenesis and spermiation, to aid in the diagnosis of the potential mechanisms that could be perturbed by experimental manipulation such as reproductive toxicant administration.

Signature Lesion

There are a number of signature lesions that could indicate a disturbance in spermiogenesis or spermiation. These would include misshapen heads and/or tails of elongating/elongated spermatids. Multinucleated round spermatids may reflect disturbances in spermiogenesis, but could also reflect altered Sertoli cell function. Disruption of spermiation could present itself as spermatid retention at the lumen of post stage VIII tubules, failure of elongated spermatids to ascend to the lumen of stage VII/VIII tubules, or phagocytosis of mature spermatids at the base of tubules in stages VII through to approximately XII (in mice) or XIV (in rats). The changes might occur in isolation or in combination with one another.

*Correspondence to: Liza O'Donnell; Email: liza.odonnell@mimr-phi.org
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Introduction

Spermiogenesis is the process by which haploid round spermatids complete an extraordinary series of events to become streamlined spermatozoa capable of motility. Spermiogenesis begins after spermatocytes complete 2 quick successive meiotic reductive divisions to produce haploid round spermatids. No further cell division occurs as spermatids undergo their complex cytodifferentiation, over a period of 2–3 weeks in mice and rats, to form mature elongated spermatids that will ultimately be released from the seminiferous epithelium via a process known as spermiation.

The different steps, or phases, of spermiogenesis are distinguished by the morphological appearance of the developing acrosome and the changing shape of the nucleus.[1] During steps 1–7 of rat and mouse spermiogenesis, round spermatids have a spherical, central nucleus, and begin to assemble the acrosome and the axoneme, structures needed for fertilization and motility, respectively. During step 8, the nucleus and acrosome polarize to one side of the cell, signaling the beginning of the elongation phase of spermiogenesis. At this time, the spermatid forms a unique intercellular junction with the supporting Sertoli cell[2] and the nucleus starts to change shape and compact, to achieve the dense, species-specific head shape that is important for motility. These changes involve nuclear compaction and chromatin condensation, as well as sculpting of the sperm head by a microtubule-based structure known as the manchette.[3]

As the spermatid nucleus compacts, nucleosomal chromatin is transformed into compacted chromatin fibers by the replacement of histones with transition proteins, which are subsequently replaced by protamines. The spermatid ceases active gene transcription as nucleosomes disappear and the chromatin is remodelled and compacted.[4,5] Accordingly, earlier round spermatids in steps 1–7 actively transcribe many mRNAs that are necessary for spermiogenesis,[6] however many of those mRNAs are subjected to translational delays until the protein is required later in spermiogenesis.[4,5]

Another major event occurring during spermiogenesis is the assembly of the sperm flagellum, reviewed in.[7-9] The central component of the flagellum, the microtubule-based axoneme, is assembled soon after the completion of meiosis. As spermatids elongate, the accessory structures needed for flagella function (outer dense fibers, fibrous sheath, mitochondrial sheath) are assembled around the central axoneme.

The final stage of spermiogenesis is known as spermiation, and is the process by which the elongated spermatids undergo their final remodelling and release from the seminiferous epithelium. Spermiation is a complex, multi-step process, which is particularly vulnerable to disruption.[10]

The following chapter gives a broad overview of the mechanisms governing the major events in spermiogenesis and spermiation. Wherever possible, recent review articles have been cited; the reader is encouraged to consult these reviews for a more detailed description of, and original references pertaining to, the mechanism of interest. To aid in the understanding of reproductive toxicant effects or phenotypic changes, the chapter is arranged according to the major morphological defects that are observed in these processes during reproductive toxicant administration, as well as those often observed in transgenic mouse models of male infertility.

Multinucleated Spermatids

Male germ cells develop clonally in a wide variety of species ranging from fruit flies to humans, remaining connected to one another during their development via intercellular bridges, reviewed in.[11] These bridges allow the passage of molecular signals, and are even large enough to allow the passage of small organelles such as mitochondria and chromatoid bodies.[11,12] In dividing somatic cells the intercellular bridge between daughter cells is transient, however in dividing germ cells, the bridge transforms into a stable structure that enables germ cells to remain connected to one another in a syncytium. These intercellular bridges facilitate synchronous development of hundreds of germ cells that are clonally derived from a single spermatogonial stem cell.[13] These bridges appear to be especially important in spermiogenesis, wherein round spermatids are haploid and thus some individual cells will lack single copy genes, e.g. those carried on sex chromosomes. The sharing of gene products across intercellular bridges thus enables haploid spermatids to be functionally diploid.[14,15] Interestingly the diameter of the bridges varies during spermiogenesis, with bridges being 1.8 μm between early step 1 spermatids, and up to 3μm between step 18 spermatids in rats.[16]

Multinucleated spermatids are a common feature of abnormal spermiogenesis (**Fig. 1**). When considering possible mechanisms for this observation, it is important to consider the number and appearance of spermatid nuclei within the multi-nucleated cyst. If there are a large number of abnormal-appearing, densely stained nuclei, then it is highly likely that this arises due to spermatid death (**Fig. 1**). This is commonly observed when there is large scale cell death due to reproductive toxicant administration (see Vidal and Whiney, this issue). A direct action of an agent on intercellular bridges could also conceivably produce multi-nucleated spermatids, such as is observed after colchicine treatment.[17] These bridges can be visualized using immunohistochemical markers such as TEX14.[18]

However if there are only 2 nuclei or 4 nuclei per cell cytoplasm (**Fig. 1**), then a specific defect in meiotic cell cytokinesis is more likely. At the end of the long meiotic prophase, 2 reductive divisions rapidly follow one another to produce 4 haploid (N) spermatids from one 4N spermatocyte. Cytokinesis involves 3 major steps; cleavage furrow position, ingression, followed by the final abscission process to cleave the daughter cells in 2. During cytokinesis, the daughter cells remain tethered to one another via a microtubule-based structure known as the midbody, which acts as a scaffold for protein complexes required for abscission.[19] However in male germ cells, abscission is incomplete, and instead the midbody converts to intercellular bridges[11,18]; thus male meiotic cell cytokinesis differs to somatic cell cytokinesis. An example of failed meiotic cytokinesis is the binucleated spermatids observed in mice carrying a mutation in a subunit of a microtubule severing enzyme complex.[20]

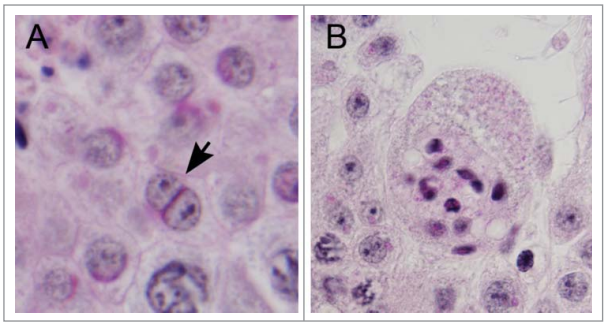

Figure 1. Bi- and multi-nucleated spermatids. (**A**). Bi-nucleated round spermatid, indicative of failure of cytokinesis during meiotic division. (**B**) Syncitia of multiple spermatids, indicative of marked degeneration.

Abnormal Acrosome Development

The acrosome is a membrane-bound organelle within the spermatid that participates in fertilization by releasing hydrolytic enzymes to facilitate sperm penetration through the zona pellucida. The acrosome can be easily visualized in histological sections by the Period Acid Schiffs (PAS) staining, see[21] for method, however analysis of acrosome structure is best visualised by electron microscopy. The acrosome begins developing in round spermatids soon after meiosis, and eventually spreads across the nuclear surface, ultimately covering up to a half of the anterior portion of the sperm head. The initiation of acrosome development begins with coated vesicles budding from the trans-Golgi network to produce pro-acrosomal granules that ultimately coalesce.[17] The Golgi sorts and traffics these vesicles to the nuclear surface, as the acrosome gradually spreads over the nuclear membrane.[22,23] Attachment of the developing acrosome to the nuclear surface appears to be mediated via the perinuclear theca, which is a thin layer of cytoskeletal elements between the acrosomal and nuclear membranes.[24] This structure has various functions, one of which is to facilitate attachment of the acrosome to the underlying nuclear membrane and the overlying plasma membrane as spermiogenesis proceeds.[24] Another structure involved in acrosome development is the acroplaxome,

an F-actin, myosin and keratin-containing structure that is attached to the nuclear lamina of the developing spermatid.[3] This structure appears to facilitate the aggregation and attachment of pro-acrosomal vesicles during acrosome biogenesis, thereby anchoring the developing acrosome to the nucleus.[3,24,25]

Inhibitors of microtubule dynamics interfere with the ability of these acrosomal vesicles to dock onto the nuclear surface and can result in abnormal spreading of the acrosome and/or dots of vesicles around the nuclear surface.[17,22] However abnormal deposition of acrosomal granules around the nucleus in early spermiogenesis can be apparent even when the acrosome appears normal in later stages (unpublished observations) suggesting that mild defects in the trafficking of acrosomal vesicles can eventually be overcome during spermiogenesis. Failure of the acrosome to form altogether is associated with a phenotype known collectively as globozoospermia.[26] This phenotype can arise due to the failure of acrosomal vesicles to form in the Golgi or to attach to the nuclear surface, or the subsequent detachment and degeneration of the acrosome later in spermiogenesis.[26] Studies in mice reveal a number of genes that are essential for acrosome development.[3,23,24,26,27] In terms of reproductive toxicant administration, abnormal acrosome development during early spermiogenesis could suggest an action on the round spermatid Golgi apparatus, vesicle-mediated trafficking,[23] F-actin and myosin-mediated vesicle trafficking[28] or microtubule dynamics.[29] Detachment of acrosomes later in spermatid elongation could reflect an action on the perinuclear theca.[24]

Abnormal Sperm Head Shape

During spermiogenesis, the round spermatid nucleus polarizes to one side of the cell and, soon after, begins to deviate from a spherical shape as nuclear condensation and sperm head shaping begin. Normal sperm nuclear morphology relies on sperm DNA compaction as well as specialized structures within spermatids that determine the characteristic species-specific sperm head shape.

The 2 major structures associated with sperm head shaping, known as the acroplaxome and manchette, are closely related to one another as spermatids proceed through the elongation phase.[3,24] The acroplaxome appears to be involved in sperm head shaping by acting as a mechanical scaffold that can transmit forces onto the nucleus. As the spermatid commences elongation, it associates with a junctional plaque, termed the ectoplasmic specialization (ES),[2] within the Sertoli cell. The ES contains "hoops" of actin bundles on the Sertoli cell side, which are proposed to confer "clutching forces" onto the spermatid; in turn the stress-resistant acroplaxome within the spermatid may transmit this force to the spermatid nucleus, participating in sperm head shaping.[3,25] The presence of keratin-5 in the acroplaxome may be important for the ability of these forces to be transmitted to the nucleus.[25]

The manchette is a microtubule-based structure that plays a major role in sperm head shaping, as well as in sperm tail development (see below). The sperm head shaping function has been revealed by the demonstration that abnormalities in the manchette induce deformations in sperm head shape.[3,24,27,30] The manchette may also play a role in the caudal positioning of the spermatid cytoplasm during the elongation phase.[24,31] The manchette is composed of up to 1000 microtubules, bundled together, in a "grass skirt" type structure that projects from just below the spermatid acrosome into the cytoplasm (see **Fig. 2**). The manchette shows a very precise timing of appearance and disappearance, coinciding with the period of nuclear shaping. Short microtubules first appear near the spermatid nucleus at step 7 of spermiogenesis and are then rapidly assembled into an extensive structure by step 8 [32,33] when the spermatid nucleus polarises to one side of the cell (**Fig. 3**). The manchette microtubules then remain closely applied to the nuclear surface[30] (**Figs. 2 and 3**) until manchette disassembly in ~step 14.[29]

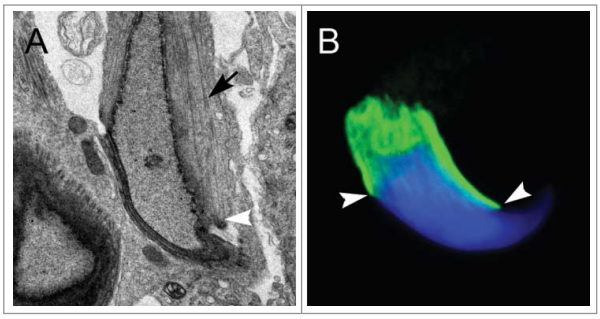

Figure 2. The spermatid manchette. (**A**). Electron micrograph of a step 9-10 spermatid with manchette. Manchette microtubules are indicated (black arrow). (**B**). Isolated elongating spermatid immunostained for microtubules (α-tubulin = green); nuclei are stained blue (DAPI). White arrowheads in (**A**) and (**B**) indicate the position of the perinuclear ring, from which the manchette microtubules emerge.

The manchette microtubules appear to emanate from the perinuclear ring at the base of the acrosome (**Fig. 2**), however whether the microtubules are nucleated in the perinuclear ring, or are nucleated elsewhere and are instead captured by this ring, is unclear.[29,34] Endoplasmic reticulum is aligned along the cytoplasmic face of the manchette,[27] whereas linkages are observed between the innermost microtubules and the nuclear membrane.[30] Dynein is present between the manchette microtubules and the nuclear membrane[35] and may facilitate movement of the manchette across the nuclear surface. During spermatid elongation, the manchette and perinuclear ring move caudally down the nuclear surface, sculpting the spermatid nucleus as it goes (**Fig. 3**). In the case of rodents with hooked shaped heads, the manchette appears to tilt as well as move, to create the dorsal and ventral nuclear surfaces. This movement and sculpting may be mediated by dynein and microtubule severing enzymes.[29]

Manchette abnormalities cause a failure of normal sculpting of the sperm head, with this phenotype persisting in elongated spermatids. Many agents have been shown to induce manchette abnormalities, including inhibitors of microtubule dynamics (such as carbendezim and taxol) and of RNA/DNA synthesis as well as alkylating agents.[30,36] Manchette dysfunction is also seen in a variety of genetically modified mouse models, reviewed

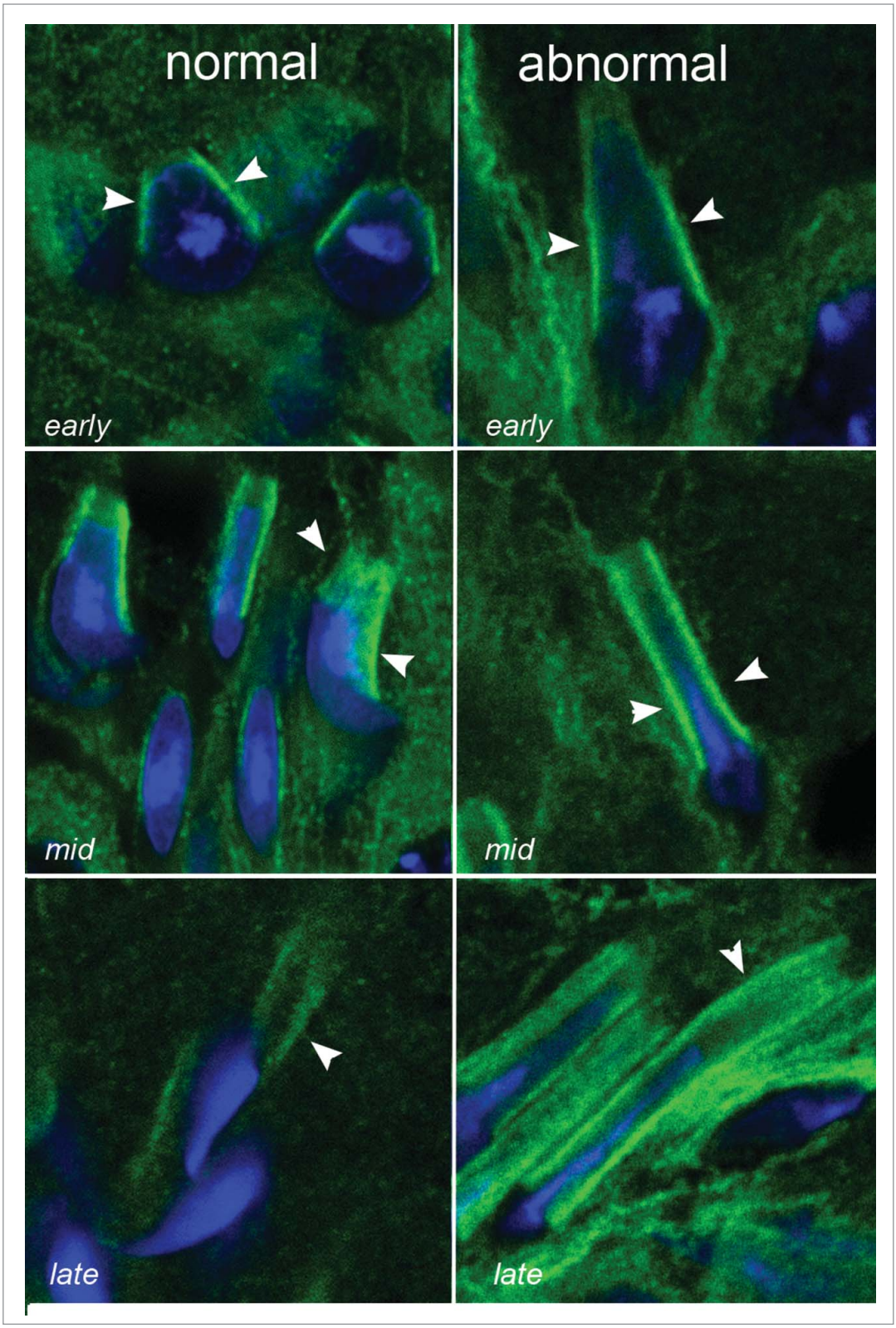

Figure 3. Visualization of normal and abnormal manchettes using α-tubulin immunostaining. Green = α-tubulin (microtubules), blue = DAPI (DNA). Arrowheads denote manchette microtubules. The normal progression of manchette development is shown on the left, and an example of abnormal development is shown on the right. Early indicates early manchette development in stage VIII-IX. Mid indicates manchette development around stages X-XII of spermatogenesis, whereas late indicates the morphology of the manchette prior to its removal. In the normal situation, the manchette moves caudally down the nucleus (compare the position of the manchette relative to the nucleus in early vs late, normal). The manchette also pivots, to facilitate the characteristic hook shape in mice (see mid, normal). The abnormal situation shows a manchette that does not move caudally (compare early to late, abnormal) and continues to lengthen, producing a long, thin nucleus lacking the characteristic hook.

in.[3,27,30] Ectopic manchettes are commonly observed (e.g.,[25,30,31,37]) and the position and direction of the microtubules then determine the type of deviations on nuclear surface. A spermatid nuclear phenotype that is observed in a variety of mouse models is an enlarged anterior portion yet a long tapered posterior portion (e.g.,[29,31,38-40]) (see **Fig. 3** for an example). An enlarged anterior portion of the nucleus could arise due to constriction caused by the failure of the perinuclear ring to expand and/or move, whereas abnormally long manchette microtubules could cause the posterior portion of the nucleus to attain a long and tapered shape. When assessing sperm head abnormalities, the manchette can be visualized by immunostaining of α-tubulin in sections or isolated cells (**Fig. 2 and 3**). A careful examination of the direction and length of

manchette microtubules during spermatid elongation in steps 9–13 may provide a better understanding of how such abnormal head shapes develop (**Fig. 3**).

Coincident with the formation of the manchette and shaping of the spermatid nucleus in mid-spermiogenesis, is the remodelling of sperm chromatin. Sperm chromatin compaction is required to dramatically reduce the nuclear volume and facilitate the development of a small nucleus that will not impede sperm motility. During nuclear compaction, the nucleosomes are disassembled and the histones are removed and replaced by transition proteins and, ultimately, protamines.[41-44] The protamines facilitate the compaction of the DNA into tightly compacted, toroidal structures, reviewed in.[41-43] The process of chromatin compaction is accompanied by DNA strand breaks and repair, reviewed in.[27] While the processes governing sperm DNA chromatin compaction are distinct from those involved in sperm head shaping, it is clear that defects in DNA compaction can ultimately impact on sperm nuclear morphology. For example, mice with reduced levels of protamines due to haploinsufficiency had defective chromatin condensation accompanied by narrowed sperm heads with a reduced curvature,[45] and mice lacking a novel histone-like protein involved in chromatin compaction showed abnormal nuclear morphology and "halos" within densely stained sperm nucleus.[46] While abnormalities in sperm chromatin packaging are more likely to manifest as altered quality, DNA damage and infertility in ejaculated sperm, rather than a difference in head shape,[47] relationships between sperm head morphology and DNA integrity in human sperm have been demonstrated.[48] Thus it is reasonable to assume that processes governing sperm DNA chromatin could impact on sperm head shape. Disordered sperm DNA condensation would be visible in mid-late spermiogenesis, which may appear as an abnormal head shape in these stages. If manchette morphology appears normal (see above), but nuclei show abnormalities in DNA staining, then DNA compaction could be assessed by acridine orange staining,[49] and the levels of transition proteins (TNP1 and 2) and protamines (PRM1 and 2) could be assessed to indicate potential defects in the DNA compaction process.

Abnormal Sperm Tail Development

Soon after the completion of meiosis, early round spermatids begin to assemble the central microtubule-based component of the flagella, known as the axoneme. Sperm axoneme assembly shares many similarities with the assembly of axonemes in motile cilia, and genes encoding axonemal components are highly conserved.[50] The axoneme consists of a central pair of microtubules surrounded by 9 outer doublet microtubules (the so-called "9+2" arrangement), and dynein motors on the outer doublets generate the forces required for antiparallel sliding to produce the waveform motion of the flagellum.[7] The axoneme is assembled within the round spermatid centrosome, consisting of a pair of centrioles; since spermatids do not undergo mitosis, the centrosome is now termed the basal body.[51] As the acrosome begins to form on one pole of the nucleus, the pair of centrioles move toward the opposite pole to initiate axoneme formation. The axoneme (also known as the axial filament) arises from the distal centriole and gradually extends out into the cytoplasm.[9] Various mutant or null mouse models have revealed many genes involved in axoneme formation and provide important insights into the evolutionarily conserved mechanisms governing the assembly of cilia and flagella.[8,9,50,52] It is also important to note that microtubules in the sperm axoneme are extensively post-translationally modified including acetylated, tyrosinated and polyglutamylated modifications.[34,52,53] Defects in tubulin modifications, such as the absence of tubulin-modifying enzymes, can cause abnormal axoneme assembly.[34,52,53] A failure in the earliest stages of axoneme assembly is manifested by abnormalities in the 9+2 arrangement of axonemal microtubules, such as an absence of the central microtubule pair and/or missing outer doublets. Axoneme structure is best visualised by electron microcopy (**Fig. 4A**), but can also be visualized by staining for acetylated tubulin (**Fig. 4B**), which can indicate absent or shorter axonemes in testicular and/or epididymal sperm.

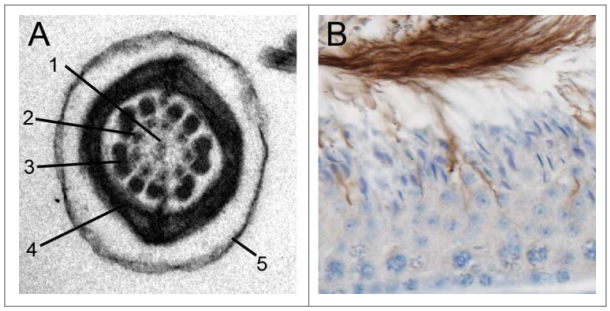

Figure 4. Sperm flagella. (**A**). Electron micrograph of a cross section of the sperm flagella (taken from the principal piece). 1 = inner microtubule doublet of the axoneme, 2 = outer microtubule doublets of the axoneme (9 outer doublets in total), 3 = Outer Dense Fibers (ODF), 4 = fibrous sheath, 5 = plasma membrane. (**B**). Cross section of seminiferous epithelium immunostained with antibody to acetylated tubulin (brown) to detect sperm axonemes, nuclei are stained blue (haematoxylin).

After the initiation of axoneme formation in early spermiogenesis, secondary structures necessary for flagella function are assembled during the elongation phase of spermiogenesis. These sperm-specific structures are not found in motile cilia in other cells. These secondary structures include the outer dense fibers, the fibrous sheath and the mitochondrial sheath,[7,9,54] the latter of which is involved in ATP generation needed for sperm motility. The outer dense fibers (ODF) (**Fig. 4A**) are rich in keratins[55] and function to facilitate motility as well as to impart a rigidity to the flagella that enables it to withstand shear forces during its passage through the female reproductive tract, reviewed in.[9] The fibrous sheath (**Fig. 4A**) also provides a rigidity to the flagella that may be important for determining the shape of the flagella beat, see.[9,56] This structure is rich in A-kinase anchoring proteins (AKAPs)[57], contains a variety of phosphorylated and signaling-related proteins[9] including components of the Rho signaling pathway[58] and is thought to act as a scaffold for signaling proteins that may regulate sperm motility and hyperactivation.[56]

Both axoneme extension and sperm secondary structure assembly rely on protein and vesicle-mediated trafficking pathways. Intraflagellar Transport (IFT) is a process that is conserved in primary cilia[59] and involves the transport of cargos from the basal body along the developing axoneme (anterograde) and back again (retrograde) via the microtubule-associated motor proteins kinesins and dynein. The second pathway is specific to developing sperm, and is termed the Intra-manchette Transport (IMT) pathway.[3,23,34] This pathway involves the delivery of Golgi-derived proteins destined for the developing flagella via the acroplaxome beneath the acrosome, along the manchette and to the head tail-coupling apparatus (HTCA) between the base of the sperm nucleus and the flagellum.[3,23,28] Rafts of proteins are thought to be transported to the flagella via F-actin tracks within the acroplaxome as well as along microtubules of the manchette.[23,28] Thus, defects in the development of sperm flagella could arise due to disruptions to protein trafficking, such as via the IFT and IMT pathways. Since the manchette appears to be integrally involved in the delivery of proteins to the developing tail,[3,23] agents or genetic modifications that disrupt manchette development and/or function often also cause defects in flagella assembly and hence can impair sperm motility.

Disordered Spermatid Orientation

As round spermatids enter the elongation phase of spermiogenesis, the round spermatid nucleus and acrosome becomes polarized to one side of the cell (step 8 of spermiogenesis in rats and mice, tubule stage VIII in both species). At this time, the spermatid associates with a specialized adhesion junction termed the ectoplasmic specialization. The development and function of, and defects associated with, the ES are covered in a separate chapter (see Cheng, this issue). It is worth briefly mentioning here that the ES functions to orient the spermatid nucleus toward the base of the tubule from steps 8–16 (mouse) and 8–19 (rat) of spermiogenesis, and that the orientation and translocation of spermatids in the epithelium via the ES involves microtubules and associated motor proteins.[60,61] Therefore the abnormal positioning of sperm heads within the epithelium is likely due to defects in either the ES (also see Cheng, this issue) and/or its ability to be translocated along Sertoli cell microtubules[60,61] (also see Johnson, this issue).

Disruptions to Spermiation

Spermiation is the process by which elongated spermatids undergo their final remodelling and ultimate release from the seminiferous epithelium into the tubule lumen prior to their passage to the epididymis, reviewed in.[10,62,63] Spermiation occurs in the mid-spermatogenic stages (stages VII-VIII in mice and rats and stage II in humans) and is initiated when elongated spermatids are rapidly translocated to the luminal edge of the epithelium (at the beginning of stage VII in rodents). While at the luminal edge, the spermatids undergo quite extensive remodelling, whereby their large cytoplasm is condensed and ultimately shed as the residual body. This remodelling also involves the removal of the extensive ES junction that was required for positioning the developing spermatid within the epithelium. Once this remodelling is complete, the spermatids are rapidly released by the Sertoli cell, in a process known as disengagement.

From the above description it is obvious that spermiation is actually a multi-step process. It encompasses several different processes, including the removal and regulation of intercellular adhesion junctions, the remodelling of the spermatid cytoplasm, and encompasses processes involving the initiation of spermiation, as well as the final disengagement event. These processes are accomplished by the "spermiation machinery," the various structures within the Sertoli cell and between the spermatid and the Sertoli cell, that contribute to successful spermiation. The following will present a very brief overview of the different processes involved in spermiation; the reader is referred to a recent review on this topic for more detailed information.[10] An overview of the common abnormalities seen during spermiation, and their possible causes, will then follow.

An overview of spermiation

Spermiation is initiated when the majority of late spermatids align along the luminal edge at the beginning of stage VII in rats and mice. Prior to initiation, the spermatids are rapidly translocated to the luminal edge by the Sertoli cell. This translocation is facilitated by the Sertoli cell cytoskeleton (also see Johnson, this issue) and the ES junctional plaque (also see Cheng, this issue) which confers a tight intercellular adhesion between developing spermatids and Sertoli cells throughout the elongation phase of spermiogenesis. The Sertoli cell face of the ES binds to microtubules within the central Sertoli cell cytoplasm, and the microtubule motor protein dynein moves the ES plaque along these microtubules,[60,61] effectively translocating the spermatid from the deep crypts within the epithelium, to the luminal edge to commence spermiation.

Soon after the commencement of spermiation, specialized structures called Tubulobulbar Complexes (TBCs) appear between the spermatid and the Sertoli cell, see[64,65] for recent review. These structures appear to be a modified form of endocytic machinery (**Fig. 5**).[64,65] They first appear as a bristle coated pit containing clathrin, and then elongate into the classic TBC structure, containing a tubular region surrounded by actin, a bulbous portion surrounded by endoplasmic reticulum, and a clathrin-coated tip (**Fig. 5**). This bulbous portion eventually "buds off" and fuses with lysosomes which are ultimately degraded within the Sertoli cells. TBCs almost invariably form in regions where the ES is absent,[62] which is consistent with the proposition that TBCs participate in ES removal.[64-66] Numerous proteins have been localized to specific sites within TBCs.[64,65] The formation of TBCs likely involves regulators of clathrin coated pit assembly, local regulation of actin polymerization and drivers of dendritic actin assembly, such as cortactin,[67] N-WASP and the Arp2/3 complex,[67] whereas the budding off of the bulbous portion may involve dynamin,[68] reviewed in.[64,65] The failure of normal TBC formation causes spermiation failure.[69] As well as a

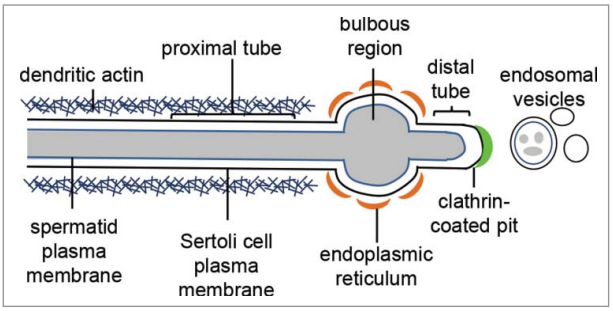

Figure 5. Diagram of tubulobulbar complex (TBC) structure. This tubular structure is surrounded by dendritic actin, the polymerization of which may help to drive TBC formation. The Sertoli cell plasma membrane forms the outer wall of the tube, whereas the spermatid plasma membrane and some cytoplasmic contents are within the tube. The distal portion of the TBC is tipped with clathrin. The bulbous portion is surrounded by endoplasmic reticulum but not actin; this bulbous region eventually buds off and fuses with vesicles that are labeled with early endosome markers.

role in intercellular adhesion remodelling during spermiation, TBCs have been proposed to play a role in final sperm head and acrosome remodelling that occurs during spermiation, reviewed in.[65] TBCs are hypothesized to be involved in the reduction in the volume of the spermatid cytoplasm during spermiation.[62,70] This is an attractive proposition, given that TBCs are present while the spermatid cytoplasm undergoes an approximate 70% reduction in volume[71] and that there appears to be a direct link between the spermatid cytoplasm and the contents of TBCs when visualised by electron microscopy.[70] However, direct evidence for a role for TBCs in spermatid cytoplasm removal is lacking.

As spermiation proceeds, major changes occur in the position of the spermatid head and in the spermatid cytoplasm. The spermatid head and, consequently, the flagella are pushed out into the tubule lumen via the extension of a microtubule-rich Sertoli cell cytoplasmic stalk.[62] As the spermatid head and flagella are extended into the lumen, the spermatid cytoplasm remains anchored to the Sertoli cell by unknown mechanisms. The net effect is that the spermatid cytoplasm is repositioned until it is below the level of the head, where it becomes concentrated and ultimately separated from the spermatid and remains with the Sertoli cell as the residual body, reviewed in.[10,62] Simultaneously, the Sertoli cell cytoplasm that has surrounded the spermatid during its elongation phase, recedes until it only contacts a small portion of the spermatid head.[10,62] Thus toward the end of spermiation, the spermatid head and tail are extended out into the tubule lumen, the spermatid cytoplasm has been "stripped away" and the Sertoli cell remains in contact with only a small portion of the spermatid head.

Dynamic changes in the adhesion complex between the Sertoli cell and the spermatid accompany the progression of spermiation.[10] Many adhesion and signaling proteins are present at this site.[72,73] At the beginning of spermiation, the ES, as characterized by actin bundles sandwiched between the Sertoli cell plasma membrane and cisternae of endoplasmic reticulum[60] encompasses the entire spermatid head. Also at this time, integrins and integrin-related molecules become concentrated on the outer dorsal curvature of the spermatid head, suggesting that there may be clustering of integrins to form a focal adhesion-like complex.[10] As spermiation proceeds, TBCs form primarily in the inner ventral curvature of the spermatid head[65] whereas many adhesion proteins cluster on the outer dorsal curvature,[10,74] presumably conferring tight adhesion between the Sertoli cell and the spermatid as it is extended into the lumen.[10] Toward the end of spermiation, the classic ES structure has been removed, yet an integrin-based focal-adhesion type structure remains.[10,75,76,] The overall goal of these dynamic changes in adhesion junctions during spermiation appears to be the removal of the ES junctional plaque that has conferred tight adhesion and facilitated spermatid translocation during the elongation phase, while maintaining a tight adhesion between the Sertoli cell and the spermatid as it is likely subjected to considerable shear forces in its relatively precarious position at the edge of the epithelium.[10]

The adhesion complex that is present between spermatids and Sertoli cells just prior to disengagement seems a likely candidate for mediating the final disengagement process. This complex must facilitate the rapid "loss of adhesion" event that results in the near-simultaneous release of all spermatids in the same portion of seminiferous tubule[10,62] and thus it likely "holds on" to the spermatid until the precise moment when it is ready for release. A variety of proteins, including integrins and Focal Adhesion Kinase (FAK) are likely components of this complex.[10,72,73] The signals for disengagement appear to come from within the Sertoli cell,[10] and a variety of pharmacological agents can interfere with the disengagement process.[72]

The following examples represent the major morphological defects associated with defective spermiation. It is important to note, however, that several defects are often observed simultaneously. See[21] for methods to examine and diagnose spermiation abnormalities.

Failure to commence spermiation

In control testes with normal spermiation, tubule cross sections in stages VII and VIII will have spermatid heads lined along the luminal edge ready for spermiation.[77,78] However a failure to commence spermiation will result in a failure of many, if not all, elongated spermatids (step 16 in mice, step 19 in rats) to align along the luminal edge at the beginning of stage VII through VIII (**Fig. 6C and 7A**). Instead, spermatid heads will be retained within the epithelium (**Fig. 6C**) and eventually be phagocytosed by the Sertoli cell (commonly referred to as spermatid retention). These basally-located spermatids will retain ES structures, which can be immunolabelled by ES-specific markers, e.g., espin.[79] The presence of basally-located, ES-positive spermatid heads in stage VII (**Fig. 6C**) is indicative of a failure to initiate spermiation, as opposed to the presence of basally-located, ES-negative spermatid heads in stages VIII-IX that is indicative of failure later in the spermiation process (see below). Agents that interfere with microtubule dynamics[80] or microtubule motor proteins could cause this phenotype, as they will prevent the spermatid

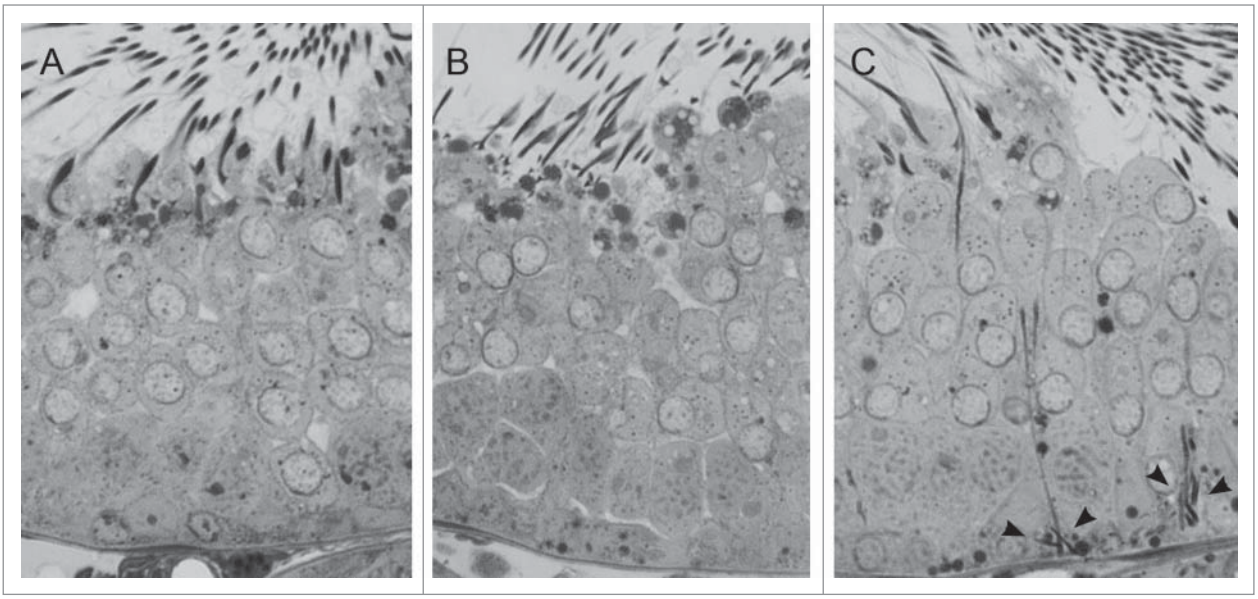

Figure 6. Histology of normal spermiation vs. spermiation failure. (**A**). Normal spermiation in stage VII, showing mature elongated spermatid heads lined along the tubule lumen. (**B**). Normal spermiation in stage VIII; mature spermatids are aligned along the luminal edge just prior to their release. (**C**). Spermiation failure in stage VIII. Instead of being released, mature elongated spermatid heads are seen at the base of the seminiferous epithelium (black arrowheads).

translocation to the luminal edge on the microtubule tracks of the Sertoli cell cytoskeleton.[60,61]

Premature spermatid release

As is evident from the above discussion, the adhesion complex between spermatids and Sertoli cells during spermiation involves many adhesion and signaling molecules. Pharmacological agents that interfere with kinase signaling can stimulate spermatid disengagement in vitro[72] and thus have the potential to promote the premature dissolution of junctions during spermiation. This would be manifested by an absence of elongated spermatids lined up along the luminal edge in stages VII and/or VIII[77,78] (**Fig. 7B**), as well as an absence ofof spermatid heads retained at the base of the epithelium (**Fig. 7B**). In addition, spermatids in the epididymis would likely have excess cytoplasm around the tails, since this cytoplasm is normally removed during stages VII/VIII. See[10,64,72,73,81] for lists of the many adhesion and signaling proteins that are present during spermiation, and could thus contribute to premature spermatid release.

Abnormalities of spermatid cytoplasm removal

A major goal of spermiation is to remove the extensive cytoplasm from the spermatid, to reveal its streamlined form that will eventually be capable of motility. The volume of the spermatid cytoplasm is reduced by approximately 70% during spermiation,[71] a process that could conceivably be regulated by aquaporins,[82] and/or by TBCs.[10,65,70] The failure of normal spermatid cytoplasm removal could lead to an absence or a reduction in the number of residual bodies in stage VIII/IX, the presence of retained spermatids with abnormal cytoplasm in stages IX/X (**Fig. 7C**), and/or the presence of spermatids with cytoplasm in the epididymis. A major failure in cytoplasmic removal would likely cause the spermatids to remain in the epithelium and be phagocytosed, as their normal disengagement would likely be impaired. Defective spermatid cytoplasm removal could be a consequence of disturbed Sertoli cell function, such as an inability of the Sertoli cell to extend the spermatid into the lumen while "stripping away" its cytoplasm, and potentially due to failure of TBC formation and function (see above). Histologic markers of TBC formation are available, e.g. clathrin, cortactin, N-WASP, actin, and EEA1, and can be combined to assess the formation of the various substructures within TBCs.[65-67] It is important to note that transgenic mouse models demonstrate that defects within the spermatid cytoplasm itself could lead to an inability of the cytoplasm to be effectively removed, reviewed in.[10]

Failure to disengage/phagocytosis of spermatids

When step 19 spermatids fail to be released from the Sertoli cell during spermiation, they may remain at the luminal edge of tubules in stages IX-XI (**Fig. 7D**) or be rapid phagocytosed and appear as spermatid heads at the base of the epithelium in stages VII-XII (**Figs. 6C, and 7E**). It should be noted that if phagocytosed spermatid heads are seen in all stages, then this is likely due to an inability of sperm to exit the testis, such as abnormal efferent ductule function or a marked reduction in testicular testosterone levels (**Fig. 7F**); spermatids that are released into the lumen yet cannot escape the testis and are phagocytosed by Sertoli cells.

Defects at various points during spermiation can cause spermatid nuclei to be retained within the epithelium. As described

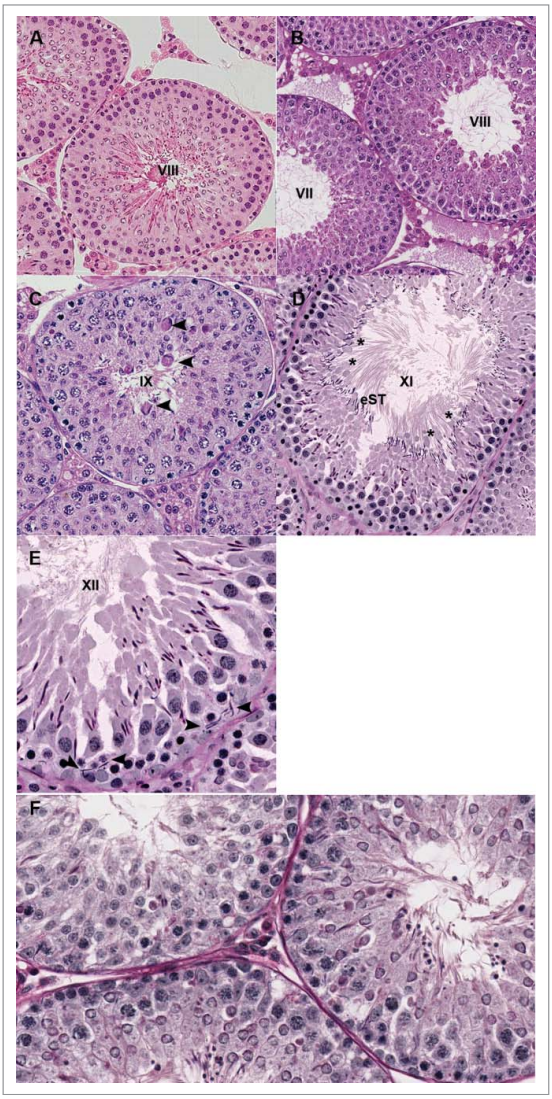

Figure 7. Various defects in spermiation. (**A**). Failure to commence spermiation in a stage VIII tubule. At this stage, elongated spermatid heads are normally lined up along the edge of the epithelium, however in this tubule elongated spermatids remain deep within the epithelium instead of being translocated to the luminal edge. (**B**). Premature release of mature spermatids, as evidenced by an absence of mature elongated spermatids at the luminal edge in stage VII and VIII tubules. (**C**). Abnormal removal of cytoplasm from elongated spermatids (arrowheads) in stage IX. (**D**). Failure of spermatids to be released during spermiation following dosing with 9000ppm boric acid for 14 d. Spermatids that fail to spermiate are often rapidly phagocytosed by Sertoli cells in stage onwards, however in this example phagocytosis has not occurred and elongated spermatids (eST) that should have been released instead remain at the luminal edge in stage XI. These spermatids also display evidence of abnormal cytoplasm removal (asterix). (**E**). Phagocytosis of spermatids that fail to be released during spermiation following dosing with 9000ppm boric acid for 14 d. Retained elongated spermatid heads (arrowheads) are located within the Sertoli cell cytoplasm at the base of this stage XII tubule. The stage of spermatogenesis is indicated in each micrograph. (**F**). Retained spermatid heads in the Sertoli cell cytoplasm of multiple stages throughout the cycle following administration of an LHRH antagonist for 14 d. The spermatids are being phagocytosed due to progressive degeneration of elongating spermatids. All images are from rat, except for C which is mouse.

in point b, failure to initiate spermiation is associated with a failure to line up along the luminal edge. However, if many spermatids are assembled at the luminal edge in stage VII[77,78] yet the number of sperm in the epididymis is reduced[21] and retained spermatids appear at the base of the tubule, this suggests the failure of adhesion complex removal or failure in the final disengagement event.

If spermiation failure arises due to abnormal ES removal, then retained spermatids in stages VII and VIII will be immunolabelled with ES markers such as espin.[79] This could signal defects in TBC development or function, since TBCs have a defined role in ES removal (see point a). TBC formation could be assessed by careful observation of TBC marker proteins within the ventral curvature of the elongated spermatids in stage VII, see.[65-67] Vizualization of the actin-associated tubular region of TBCs (see **Fig. 5**) can be facilitated by F-actin or cortactin staining. The ability of TBCs to remove ES components could be assessed by co-labeling actin or cortactin with the ES-associated protein nectin 2 and the ability to remove ES components via the lysosomal pathway could be assessed by co-labeling ES components such as espin or nectin 2 with the early endosomal marker EEA1.[66]

If, however, retained spermatids are negative for ES markers such as espin, and appear in late VIII/early IX, rather than predominantly in stage VII, then spermiation failure is more likely due to a defect in the dissolution of an integrin-based, focal adhesion-like junction present between Sertoli cells and spermatids just prior to sperm release.[10,75,76] The failure of this final disengagement event likely arises from changes in signaling and/or adhesion-related pathways within the Sertoli cells.[10]

It is worth highlighting the role of protein phosphorylation in the final disengagement of spermatids from Sertoli cells. Various phosphorylated proteins are present at the site of spermiation, and antibodies against serine and threonine phosphorylated proteins also label the Sertoli cell –spermatid interaction during spermiation.[10,72,73] Agents that interfere with kinase and/or phosphatase actions can stimulate or inhibit spermiation in vitro.[72] Therefore the regulation of phosphorylation cascades within the Sertoli cell, and the regulation of protein phosphorylation within the adhesion complex itself, is likely to be essential for both the successful release of sperm and for the timing of disengagement.

On a final note, it is worthwhile mentioning that spermiation failure and the appearance of retained spermatids in the seminiferous epithelium is a common response to a range of pharmacological agents, such as boron[83] and chemotherapeutic agents,[84] as well as environmental insults, reviewed in.[63] This reflects the complexity of the overall process, and the varied structures and protein complexes that are required for the final remodelling and release of the mature spermatid. Careful analysis of when spermiation fails, as indicated above, could provide important clues as to the specific pathways and molecular components affected.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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