2.1 Mammalian Spermatogenesis

The term “spermatogenesis” is used to describe one of the most complex developmental processes that represents the core biological events required for the continuity of life. Biologically, it is a series of sequential cellular events taking place in the seminiferous tubules within the testis, that result in the production of functional haploid spermatozoa from diploid spermatogonial stem cells. During this process, testicular germ cells undergo complex morphological transformations involving the establishment of a stem cell pool, mitotic proliferation, meiosis and morphogenesis of haploid germ cells. Through mitotic proliferation, spermatogonial stem cells produce spermatocytes which enter meiosis to give rise to haploid round spermatids, and then round spermatids further undergo a complex transformation process to form mature elongated spermatids (Fig 2.1). These developmental changes are unique to male germ cells making them exceedingly fascinating for research. Being the process responsible for passing-on the paternal genome through the spermatozoon, spermatogenesis is well characterized at cellular and molecular levels. The following sections briefly provide an overview of molecular events underlying mammalian spermatogenesis.

![Figure 2.1. Process of spermatogenesis](image)

**Figure 2.1. Process of spermatogenesis.** Spermatogonial stem cells (SSC) proliferate to self-renew and produce more stem cells and give rise to spermatogonia. Differentiated spermatogonia enter meiosis to produce spermatocytes. After the completion of meiosis each spermatocyte generates four haploid round spermatids, each of which undergoes complex morphological transformations, called spermatid differentiation, to produce an elongated spermatid. Each of these processes are explained in more detail in section 2.4.

2.2 Organisation of the testis

Spermatogenesis in mammals occurs in the male reproductive organ called testis. The testis is encapsulated by an outer fibrous connective tissue layer called tunica albuginea (Fig 2.2A). The mammalian testis is composed of two structurally and morphologically distinct compartments, the interstitium and the seminiferous tubules, both of which
coordinate to support the process of sperm production (Fig 2.2B). Interstitial tissue contains vascular and lymphatic vessels, connective tissue, macrophages, and fibroblasts in addition to the most predominant cell type called Leydig cells (Bellve et al., 1977; Hess and Renato de Franca, 2008; Russell et al., 1990). The main function of interstitial Leydig cells is to produce the androgen hormone testosterone into the blood stream of males (Ge et al., 2008). Since seminiferous tubules constitute the actual site of sperm formation, the organisation of the seminiferous epithelium and different cell types present are discussed in more detail below.

The seminiferous tubules are intertwined within the testis in such a way that a single testis cross section depicts many individual seminiferous tubule cross sections (Fig 2.2B). Each seminiferous tubule is composed of a stratified seminiferous epithelium which contains developing germ cells and somatic Sertoli cells in close association with each other (Fig 2.2C). The epithelium is surrounded by peritubular myoid cells and basal lamina, which are contractile in nature and provide necessary forces required for the movement of spermatozoa along the epithelium, and also are involved in the paracrine regulation of spermatozoa (Russell et al., 1990). The seminiferous epithelium of mammals is highly organised involving interactions between Sertoli cells and different germ cells that traverse across the epithelium as they mature. This spatio-temporal arrangement of germ cells along the seminiferous epithelium divides the later into three compartments viz. basement membrane, adluminal compartment and lumen. The basement membrane or basal lamina is composed of extracellular matrix and peritubular myoid cells surrounding the seminiferous tubule (Clermont, 1972; Russell et al., 1990). Sertoli cells are arranged in a single layer towards the membrane with their cytoplasm extended spanning the entire width of the epithelium (Fig 2.2C and D). Throughout the adluminal compartment, all of the germ cells along the epithelium are embedded within the cytoplasm of Sertoli cells which provide physical and nutritional support to the developing germ cells (Russell et al., 1990). In an adult mouse testis, each Sertoli cell encompasses four to five germ cells by establishing Sertoli cell-germ cell contacts. Similarly, each Sertoli cell makes contacts with the adjacent Sertoli cells by establishing extensive inter-cell junctions that maintain the integrity of the epithelium and form the basis of the blood-testis barrier (Mruk and Cheng, 2004b). These Sertoli cell-Sertoli cell and Sertoli cell-germ cell junctions help in the movement of developing germ cells across
the seminiferous epithelium in addition to transducing the signals responsible for sperm maturation (Kopera et al., 2010; Russell et al., 1990).

**Figure 2.2. Organisation of the testis.** (A). Longitudinal view of mammalian testis showing intertwined seminiferous tubules covered externally by an outer protective covering called Tunica albuginea. Each testis is connected to the epididymis, that is the site for spermatozoa storage. (B). Diagrammatic representation of a testis cross section showing interstitial tissue and seminiferous tubules. Interstitial cells such as Leydig cells are present in the interstitium and germ cells such as spermatogonia and somatic Sertoli cells are present in seminiferous tubules. Several sections of seminiferous tubules can be viewed in a single transverse section of the testis. Yellow: Sertoli cell cytoplasm; green: germ cells. (C), Schematic representation of the seminiferous epithelium (SE) showing different types of developing germ cells embedded within the cytoplasm of a Sertoli cell. Basal lamina makes the outer covering of the seminiferous epithelium and, together with spermatogonia, forms the basal compartment of the SE. Germ cells, from spermatocytes to elongated spermatids, make up the adluminal compartment. Green indicates the Sertoli cell nucleus and the grey region represents its cytoplasm. The cytoplasm of germ cells is shown in yellow. (D), Haematoxylin and Eosin stained cross section of a single seminiferous tubule displaying all the germ cells making up the epithelium. Fig 2.2A has been reproduced from (Cooke and Saunders, 2002) with permission from Nature Publishing group (NPG) via copyright clearance centre (License No. 4165280466018). Fig 2.2B was modified from www.ck12.org under Creative Commons Attribution – Non-Commercial 3.0 (CC BY-NC) License (http://creativecommons.org/licenses/by-nc/3.0/). Fig 2.2C was modified from (Bettegowda and Wilkinson, 2010) under authorised institutional access that allows to use, copy, and share the content of the article for non-commercial purposes.
2.3 The cycle of the seminiferous epithelium

Seminiferous tubule cross sections of the murine testis display an intimate and complex interaction between Sertoli cells and different types of germ cells (Fig 2.2C). Analysis of these interactions reveals that germ cells within the testis are very well organised in the form of an epithelium, and a group of well-defined germ cells which are in different states of differentiation are always found together. Immature germ cells, spermatogonia, are always found towards the basement membrane whereas more advanced germ cells (spermatocytes, round spermatids, elongating spermatids and elongated spermatids) form successive layers and lie towards the lumen of the tubule (Fig 2.2 C and D). These recurrent cellular associations were construed for the first time in 1890 by Ebner and Regard to define a cyclic process called the spermatogenic cycle. However, a well-defined method which could precisely describe the different cross-sectional profiles of varying cell associations in the seminiferous epithelium of rat testis was developed by LeBlond and Clermont in 1952 (Leblond and Clermont, 1952). They also used the term “cycle of the seminiferous epithelium” to define a series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association. Later on in 1972, Clermont further stated that for any given species each “appearance of the same cellular association” has a constant duration and germ cell differentiation appears to be regulated by a rigid time-scaled program (Clermont, 1972). The “cycle” thus represents the recurring cellular associations at one point within the seminiferous tubule over time (Fig 2.3). LeBlond and Clermont (1952) were able to discern 14 distinct germ cell associations in rat seminiferous epithelium and called each germ cell association a stage of spermatogenesis. Soon it was found that spermatogenesis in all mammals can be morphologically divided into a number of different “stages” based on the cellular associations present in a given tubular cross section. In mice 12 such different stages of the seminiferous epithelium were described by Oakberg (1956) and then further classified in greater detail by Russell (1990) (Oakberg, 1956b; Russell et al., 1990). In mice, the seminiferous epithelium is divided into 12 stages represented by Roman numerals I – XII (Fig 2.4). Each stage consists of several germ cells which are at the same period of development, and depending on the specific stage of the cycle of the seminiferous epithelium, 3 to 4 distinct generations of germ cells occupy a particular tubular cross-section (stages I-VIII contain 4 germ cell generations and stages IX-XII contain 3 generations) (Russell et al., 1990). Thus, each stage represents a well-defined
germ cell association in which one generation of germ cells at a specific period of their development are always linked with other germ cell layers which are at their own particular period of development. In one complete cycle of the seminiferous epithelium in mice, all the 12 stages (I-XII) will sequentially occur over a period of time in a given area of the seminiferous tubule. The duration of one cycle of the seminiferous epithelium is constant in each species, and is 8.65 days in mice, 13 days in rat, and 16 days in humans.

Although one particular area of the seminiferous tubule represents one stage, the other stages are present sequentially along the length of the seminiferous tubule (Fig 2.4). This sequential arrangement of stages along the length of the seminiferous tubule is called the “wave of the seminiferous epithelium”. Thus, a wave of seminiferous epithelium is defined as the complete series of all the cell associations (stages) arranged in a succession along the seminiferous tubule. This means that cycle of the seminiferous epithelium is in time, but the wave is in space. The duration of one complete cycle of spermatogenesis in mice is 35 days during which spermatogonia undergo progressive transformations to produce elongated spermatids (Russell et al., 1990).

![Figure 2.3. Schematic representation of the spermatogenic wave.](image)

During postnatal testis development, various types of germ cells appear sequentially during the first wave of spermatogenesis, and then the wave becomes asynchronous. In mice, 8 day old testis contain only spermatogonia, meiosis begins by day 10 and is completed by day 18, round spermatids appear on day 18, elongating spermatids appear
by day 30 and then fully developed elongated spermatids arise by day 35 post-partum after which they are released into the tubule lumen to complete the first spermatogenic wave (Fig 2.5) (Bellve et al., 1977; Nebel et al., 1961).

What is interesting to note here is that although spermatogenesis is almost impossible to study without defining the staging technique, the stages described above only represent artificial delineations in a continuous process. Furthermore, the developing germ cells do not actually move along the seminiferous tubule, and any given tubular cross-sectional plane would temporally pass through each of the 12 stages before repeating the cycle. Also, only the first wave in a juvenile testis is synchronous and all the subsequent waves take place asynchronously along the seminiferous tubule.

**Figure 2.4. Stages of mouse spermatogenesis.** Each stage is representative of a particular tubular cross section depicting the cellular associations corresponding to that stage. Abbreviations.  I-XII, 12 stages of spermatogenesis; 1-16, 16 steps of spermiogenesis; In, intermediate spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes and m²m, secondary spermatocytes. Mouse spermatogenic cell staging map from Cache River Press was purchased from Quick Publishing, St. Louis, MO, USA.
2.4 Phases of spermatogenesis

Spermatogenesis is a complex yet tightly regulated process which takes place in the seminiferous epithelium of testis. It involves three different major processes during which cells that are pluripotent undergo a series of modifications to form highly differentiated spermatozoa. Each of these processes is called a phase, which include (i) mitosis, involving self-renewal of pluripotent stem cells and the production and expansion of progenitor cells; (ii) meiosis, comprising the reduction of chromosome content in each progenitor cell, exchange of genetic material due to crossing over between chromosomes, and ultimately giving rise to four haploid germ cells; and (iii) spermiogenesis, involving a unique differentiation and morphological transformation of haploid germ cells (Fig 2.6). In an adult testis, all the three phases are taking place simultaneously.
2.4.1 Mitotic or proliferative phase

This phase refers to the mitotic proliferation of spermatogonial stem cells (SSCs) to self-renew and to generate a population of spermatogonia committed to become spermatozoa. This way SSCs are able to provide a pool of stem cells to ensure their life long maintenance as well as to supply a large number of spermatogonial cells required for the production of millions of sperm every day. Interactions between the germ cells, testicular somatic cells and other growth factors determine the fate of SSCs. Failure of SSCs to properly self-renew or to generate other spermatogonia ultimately leads to defective spermatogenesis. The spermatogonia are located in the basal compartment of the seminiferous tubule in-between the Sertoli cells and can be of different types based on their shape and nuclear morphology and stage of the seminiferous epithelium (Fig 2.7). The developing spermatogonia either divide mitotically to maintain a pool of spermatogonial stem cells or become committed to differentiate into mature spermatozoa. These diploid germ cells are of three types; type A, intermediate, and type B spermatogonia. Type A spermatogonia divide to generate more type A spermatogonia (As, A_paired and A_aligned) by self-proliferation and also give rise to intermediate spermatogonia. Intermediate spermatogonia differentiate to type B spermatogonia (Fig 2.7) (Allen, 1918; Chiarini-Garcia and Russell, 2002; Kerr et al., 2006; Leblond and Clermont, 1952; Oakberg, 1956a, b; Russell et al., 1990). All of these cell types are actively engaged in proliferation and appear sequentially in time. Each cell type is characterised by a peculiar pattern of heterochromatin in the nucleus. Undifferentiated spermatogonia possess no chromatin along the inner side of nuclear membrane but their nuclei are elongated. Differentiated spermatogonia on the other hand possess increasingly more chromatin along the nuclear membrane and their nuclei are more or less ovoid in shape (Fig 2.7). The mitotic phase finally results in the production of differentiated type B spermatogonia which then enter the first meiotic prophase as preleptotene (PL) spermatocytes (Bellve et al., 1977; Clermont and Perey, 1957). Spermatogonia reside towards the basal lamina of the tubule and are connected to other spermatogonia of the same sub-type by intercellular bridges that may allow movement.
of RNA and proteins between cells, leading to synchronous development of cells within a clone. The association of spermatogonia with other cell types in testis can be used to determine the stage of the cycle of the seminiferous epithelium.

**Figure 2.7. Spermatogonial proliferation and differentiation, meiotic prophase, and spermiogenesis.** Spermatogonia proliferate to maintain the undifferentiated pool ($A_s, A_{pr}, A_{al}$) and then differentiate to types $A_{1-4}$, Intermediate and B. Meiotic prophase produces preleptotene (PL), leptotene (LS), zygote (ZS), pachytene (PS) and diplotene (DS) spermatocytes. During spermiogenesis, round spermatids (RS) assemble acrosome (magenta) and undergo nuclear elongation. Based on acrosome formation, 16 steps of spermiogenesis (1-16) can be discerned in mice (discussed later). Subscript numbers 1-16 in RS (round spermatids), eES (early elongating spermatids), CS (condensing spermatids) and ES (elongated spermatids) correspond to steps of spermiogenesis. This figure has been modified from (Mark et al., 2015) with permission from Elsevier via Copyright Clearance Centre (License No. 4172860871897).
2.4.2 Meiotic phase (Meiosis)

One of the unique features of germ cells is that they undergo meiosis. Meiosis takes place in the adluminal compartment of the seminiferous epithelium. The characteristic of meiosis is that it involves one round of DNA replication followed by two successive cell divisions and creates genetic diversity by the exchange of genetic information between homologous chromosomes due to crossing over (Fig 2.6) (Cobb and Handel, 1998; Hassold et al., 2000; Hunt and Hassold, 2002). Meiosis ensures that germ cells carry out DNA repair, synopsis of homologous chromosomes and exchange of genetic material enabling a diploid cell to give rise to four haploid cells with half the number of chromosomes. Primary spermatocytes produced from type B spermatogonia undergo the first division of meiosis called meiosis I. According to cytological features and chromosome movements, meiosis I can be divided into distinct phases: prophase, metaphase, anaphase, telophase and cytokinesis. In mammalian spermatogenesis, prophase of the first meiotic division (prophase I) is the longest phase and accounts for about 90% of time required to complete meiosis (almost 3 weeks). Based on interactions between pairs of homologous chromosomes and recombination events on each chromosome, prophase I is further divided into five periods: leptotene, zygotene, pachytene, diplotene and diakinesis (Fig 2.7). (Cobb and Handel, 1998; Russell et al., 1990). Prophase I starts when preleptotene spermatocytes (PL) undergo DNA replication to form leptotene spermatocytes (LS). During leptotene, DNA is relatively uncondensed and condensing chromosomes appear as long threads with two sister chromatids connected at the centromere to form a peculiar arrangement called bouquet. In mice, the centromeres cluster to form several DNA dense regions and axial elements start developing between the sister chromatids. DNA double strand breaks (DSBs) are introduced in leptotene spermatocytes by topoisomerase II-like protein SPO11 (Keeney, 2008; Milman et al., 2009). These DNA DSBs mark the beginning of meiotic recombination and are essential for normal pairing of homologous chromosomes. During zygotene, chromosomal pairing and synapsis is initiated wherein homologous chromosomes begin to align side by side and then synapse forming a proteinaceous zipper-like structure called synaptonemal complex (SC) (Parra et al., 2003). This cementing material which comprises a central axial element and two lateral elements joined by transverse filaments ensures that homologous chromosomes remain held together during prophase I. During pachytene, synapsis and pairing of homologous...
chromosomes is completed and chromosomes thicken and shorten. An exception to this is sex (X and Y) chromosomes, which are nonhomologous but contain certain similar regions called pseudoautosomal regions (PARs), and form a cytologically distinct structure called sex body (Solari, 1974). In pachytene spermatocytes (PS) autosomes are completely synapsed throughout their length by the SC but X and Y chromosomes remain unpaired along most of their length. SC assembly which begins in zygotene is completed in pachytene (Cohen et al., 2006). One of the key features of pachytene, and of meiosis as a whole is that at this stage homologous chromosomes undergo recombination (crossing over) to exchange genetic information at specific sites known as chiasmata or recombination nodules (Carpenter, 1987). In mice, meiotic recombination is initiated at 200-400 sites along the genome but only 10% of these ultimately end up in successful exchange of chromosomal regions (Baudat and de Massy, 2007). Male mice with mutations in genes required for synapsis or recombination, such as Dmc1, Msh5, Syce1-3, and Syce2 display meiotic arrest at pachytene and are therefore infertile, suggesting that synapsis and recombination are indispensable for meiotic progression (Bolcun-Filas et al., 2007; de Vries et al., 2005; de Vries et al., 1999; Edelmann et al., 1999; Pittman et al., 1998; Yang et al., 2006; Yoshida et al., 1998; Yuan et al., 2000). During diplotene, desynapsis takes place in diplotene spermatocytes (DS) and SC begins to disassemble, allowing homologous chromosomes to separate from each other except at chiasmata where the homologues remain connected to each other. This is followed by shortening of chromosomes at diakinesis, during which the centromeres move apart from each other, the nuclear membrane disintegrates and the meiotic spindle begins to form with the chromosomes aligned at metaphase plate and each chromosome comprised of two chromatids. Different phases of prophase I can be distinguished by following the development of the SC by staining with SC proteins such as synaptonemal complex protein 2 and 3 (SYCP2 and SYCP3) (Fig 2.8).

Subsequent to prophase I follows metaphase I during which the paired chromatids of a homologous chromosome are attached at equator of the spindle to form what is called the metaphase plate. During anaphase I individual homologous chromosomes with paired chromatids move to opposite poles of the spindle. Telophase I divides the cell to produce two secondary spermatocytes with each cell containing one chromosome from each homologous pair, with two sister chromatids. Metaphase, anaphase and telophase of meiosis I are very short and take place very quickly. Each secondary spermatocyte is
short lived and contains a haploid number of chromosomes although their DNA content is diploid. The two secondary spermatocytes enter meiosis II during which sister chromatids are separated to produce four round spermatids (RS) with haploid DNA content and half the number of chromosomes (Russell et al., 1990). Round spermatids then enter the final phase of spermatogenesis called spermiogenesis.

2.4.3 Spermiogenesis

This final phase of spermatogenesis is characterised by drastic morphological transformation from a round spermatid to the uniquely shaped elongated spermatid without involving any further cell divisions. Spermiogenesis begins in round spermatids after the completion of meiosis II and continues for 2-3 weeks in mice, producing mature elongated spermatids (Figs 2.6 and 2.7). Some of the remarkable changes that take place during spermiogenesis include: (1) formation of acrosome; (2) formation of sperm tail;
(3) elongation and condensation of sperm nucleus; (4) formation of chromatoid bodies; (5) removal of excess cytoplasm as residual body; and (6) formation of apical ectoplasmic specialisation between elongated spermatids and Sertoli cells and spermiation (Hermo et al., 2010). Each of these processes is briefly detailed below:

### 2.4.3.1 Acrosome formation

Acrosome is a structure that is attached to the anterior portion of the sperm nucleus. It acts as a bag of enzymes containing many proteolytic enzymes that dissolve the zona pellucida and help sperm enter the egg during fertilization. Soon after the meiosis II, the acrosome begins to develop in round spermatids spreading along their nuclei until it covers up to more than half of the anterior portion of the spermatid head (Fig 2.7). Attachment of the developing acrosome to the nuclear envelope is facilitated by the perinuclear theca, a thin layer of cytoskeleton between the acrosome and the nuclear envelope. Acrosome formation involves three consecutive phases: (a) Golgi phase, (b) cap phase, and (c) acrosome phase. During the Golgi phase, numerous vesicle like proacrosomal granules bud off from the trans-Golgi network and fuse to form a single large acrosomal granule that translocates to the anterior pole of the spermatid nucleus to associate with it (Abou-Haila and Tulsiani, 2000). During the cap phase, additional Golgi-derived granules combine with the growing acrosome so that it increases in size and covers more of the nucleus. During the acrosome phase, the acrosome elongates to become arc shaped and covers two-thirds of the anterior sperm nucleus (Fig 2.7) (Clermont and Leblond, 1955).

Any defects in the fusion of proacrosomal granules, such as in the *Zphpl1* mouse (Lin et al., 2007), or defects in the attachment of the acrosome to the nuclear membrane, such as in *Hrb* (Kang-Decker et al., 2001), *Gopc* (Yao et al., 2002) and *Pick1* knockout mice (Xiao et al., 2009), result in a phenotype called globozoospermia (Dam et al., 2007). Defects in acrosome biogenesis can be detected either by performing PAS (Periodic Acid Schiff) staining on testis sections or transmission electron microscopy on testis and sperm samples, or a combination these.

Based on the morphological appearance of the developing acrosome in round spermatids and the changing shape of the nucleus, spermiogenesis in mouse is further divided into sixteen steps (designated 1-16) (Fig 2.7) (Leblond and Clermont, 1952). The steps 1-12
correspond to spermatids present at stages I-XII and steps 13 -16 correspond to elongated spermatids present in stages I-VIII (Fig 2.4) (Ahmed and de Rooij, 2009). The nuclei in round spermatids, which are at steps 1-7 of spermiogenesis, are central and spherical in shape, and progressively assemble the acrosome and axoneme structures. However, during step 8, the nucleus and acrosome separate to one side of the spermatid, which marks the start of the elongation phase of spermiogenesis. At this time, the nucleus begins to condense to form a typical head shape that is unique to each species and in the mice, is falciform (sickle-shaped).

2.4.3.2 Formation of sperm tail and manchette

For fertilisation to occur, sperm need to travel long distances in order to penetrate the zona pellucida of the egg. Sperm motility, which is an essential element of male fertility, is generated by sperm flagella (sperm tail). The basic and functional component of sperm flagella is called axoneme, which is a microtubule-based structure composed of motor proteins that function in a coordinated manner to produce movement in the form of waves (Fig 2.9A) (Haimo and Rosenbaum, 1981; Mortimer, 1997; Porter and Sale, 2000). The sperm axoneme is composed of a central pair of microtubules surrounded by nine outer doublet microtubules and associated proteins to form a “9+2” arrangement (Fig 2.9B and C) (Fawcett, 1975). The forces required for antiparallel sliding of the flagella to generate the wave motion are provided by dynein motors associated with outer doublet microtubules (Hermo et al., 2010). In round spermatids, the axoneme is assembled from the basal body involving a pair of centrioles (centrosomes in round spermatids are called basal body because they do not undergo any further division or mitosis) (Kierszenbaum, 2002). As the acrosome starts assembling on the pole of the round spermatid nucleus, the two centrioles migrate to the opposite pole to form the axoneme. Sperm axoneme microtubules have been shown to undergo various post-transcriptional modifications such as acetylation, tyrosination and polyglutamylation, and any deficiencies in these modifications can lead to an abnormal axoneme and infertility (Ikegami and Setou, 2010; Kierszenbaum, 2002; Sperry, 2012). Furthermore, defects in the assembly of the axoneme can lead to abnormalities in the “9+2” arrangement of microtubules or can even lead to absence of either the central pair or outer doublets of microtubules. Axoneme structure can be visualised and studied by
electron microscopy to reveal any abnormalities in its assembly or in the arrangement of microtubules.

Although there are structural and functional similarities between flagella and motile cilia, certain specific flagellar accessory structures, such as outer dense fibres (ODF), fibrous sheath and mitochondrial sheath, are only assembled during the elongation phase of spermiogenesis (Fig 2.9A) (Borg et al., 2010). The ODF are abundant in keratins and impart motility and rigidity to the tail and enable it to resist the shearing forces during its passage through the female reproductive tract (Nakamura et al., 1999; O’Bryan et al., 1998). Fibrous sheath, in addition to providing rigidity to the tail, also helps in the generation of ATP via glycolysis to facilitate the sliding of microtubules and axoneme motility (Eddy et al., 2003; Miki et al., 2004). The fibrous sheath is a home for A-kinase anchoring proteins, various phosphorylated proteins and Rho signalling pathway components and is considered to function as a scaffold for signal transduction cascades regulating motility and sperm hyperactivation (Eddy et al., 2003; Moos et al., 1998; Nakamura et al., 1999). Mitochondrial sheath that is composed of ATP rich mitochondria provides energy for the sliding of axonemal microtubules and thus helps in sperm

Figure 2.9. Structure of sperm flagella. (A). Schematic describing structural components of sperm flagella; mitochondria, outer dense fibres and mitochondrial sheath in mid-piece; fibrous sheath, longitudinal column and transverse rib in principal piece, and axoneme. (B). Schematic diagram showing the ultrastructure of sperm axoneme. Sperm axoneme, like ciliary axoneme, is composed of a central microtubular pair; outer doublet microtubules, consisting of alpha (A) and beta (B) microtubules; outer and inner dynein arms; and radial spokes. (C). Transmission electron micrograph of axoneme showing inner singlet and outer doublet microtubules. Fig 2.9A has been reproduced form (Vadnais et al., 2014) which is an open access article and allows the reuse of the content under terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0). Fig 2.9 B and C have been reproduced from (Horani et al., 2014) with permission from Nature Publishing Group (NPG) via copyright clearance centre (License No. 4174580912861).
motility (Ford, 2006). Teratospermia is a phenotype arising due to defects in sperm tail development and/or sperm motility. Electron microscopy of axoneme or its accessory structures is the best available methodology to assess these defects (Fig 2.9C).

**Figure 2.10. Structure of sperm manchette.** Manchette is transiently visualised in steps 9-11 elongating spermatids as a microtubular structure originating from the base of acrosome. Depiction of Manchette in elongating spermatids by schematic diagram (left) and transmission electron micrograph (B).

The other structure which is involved in the sperm nuclear shaping and sperm tail formation is called manchette. Manchette is a transient microtubule-based structure in elongating spermatids, composed of about 1000 microtubules arranged together in the form of a “grass skirt” arrangement projecting from just below the acrosome into the spermatid cytoplasm (Fig 2.10). Being a transitory structure, the manchette displays a precise timing of appearance and disappearance, which coincides with spermatid condensation and elongation during spermiogenesis. In mouse spermatids the manchette can first be visualised in step 9 spermatids, when it is assembled in the form a microtubular platform with microtubules originating from the perinuclear ring at the base of the acrosome around the spermatid nucleus (O'Donnell and O'Bryan, 2014). The disintegration of manchette takes place during steps 13-14 of spermiogenesis after which it no longer remains visible (O'Donnell and O'Bryan, 2014). During spermatid elongation, the manchette moves along the nuclear surface and shapes the spermatid nucleus as it moves down the nuclear surface, creating the dorsal and ventral surfaces for microtubule nucleation. Both manchette and sperm axoneme are microtubular
structures consisting of α- and β-tubulin heterodimers with α-tubulin exposed towards the minus-end and β-tubulin towards the plus-end. Defects in manchette assembly result in abnormal spermatid head shaping and elongation. While examining sperm head elongation, manchette can be visualised in testis sections or in isolated cells either by immunostaining for α-tubulin or by performing transmission electron microscopy on elongating spermatids (Fig 2.10).

2.4.3.3 Sperm head elongation and nuclear condensation

One of the most remarkable and perhaps the most important process that takes place during spermiogenesis is that the round spermatids change their nuclear shape from round to elongate by undergoing chromatin condensation (Fig 2.7). During this process, the sperm DNA undergoes a specific type of chromatin remodelling and compaction by associating with sperm specific proteins to form a highly condensed sperm head. Although the mechanism of this sperm-specific chromatin remodelling is conserved among humans, mice and drosophila, the shape of the sperm head resulting from it varies among different species (Fabian and Brill, 2012; Gaucher et al., 2010; Oliva and Dixon, 1991; Toshimori and Ito, 2003). In mice, the spermatid nuclei begin to elongate at step 9 of spermiogenesis to form elongating spermatids (spermatids corresponding to steps 9-11 of spermiogenesis). However, this condensation is completed in step 15-16 spermatids (elongated spermatids) which exhibit fully condensed, falciform or hook-shaped nuclei and are ready for release into the lumen of the seminiferous tubule (Fig 2.4) (Meistrich and Hess, 2013; Russell et al., 1991). This extensive chromatin condensation occurring during the final steps of spermiogenesis brings about tenfold compaction in sperm chromatin by packing it into a toroid structure (Miller et al., 2010). This enables the sperm nucleus to drastically reduce its volume (~ 5% of the size of a somatic cell nucleus (Brewer et al., 1999) to generate a more compact and hydrodynamic nucleus which is resistant to DNA damage, and thus helps in free and unobstructed movement of the sperm during fertilization (Oliva, 2006). In elongating spermatids, nucleosome-based chromatin is changed to sperm-specific chromatin composed of more basic proteins called protamines. During this chromatin reorganisation, the nucleosomes are disassembled and somatic histones are removed from the sperm DNA and replaced initially by small basic proteins called transition proteins, and subsequently by protamines (Fig 2.11) (Braun, 2001; Miller et al., 2010; Oliva, 2006). While the complete
mechanism of how the spermiogenic reorganisation is orchestrated is not fully understood, the molecular basis of histone-to-protamine transition in spermatids is now being sought.

**Figure 2.11. Histone to protamine replacement during spermiogenesis condensation.** Schematic displaying the key molecular events that take place during chromatin remodelling in round spermatids. During postmeiotic spermatid differentiation, histone are hyperacetylated by histone acetyltransferases (HATs) and DNA double strand breaks are transiently generated in elongating spermatids. H4 hyperacetylation loosens up the chromatin, acetylated nucleosomes are evicted from the chromatin and degraded through polyubiquitination and proteasomal activity. In elongating spermatids (steps 9-12), more basic proteins called transition proteins (TNP1 and TNP2) are transiently incorporated into the DNA to replace the histones. These transition proteins are in turn replaced by even more basic proteins called protamines (PRM1 and PRM2). The association of sperm DNA with protamines brings about tenfold compaction in sperm chromatin by packing it into a toroidal structure. Acetylated histones are in blue, transition proteins (TNPs) are in green, and protamines (PRMs) are in black.

Although the complete mechanism of this chromatin reorganization is not known, a number of histone variants and histone modifications have been shown to play essential roles. During spermiogenesis, high levels of testis-specific histone variants are expressed and associated with DNA. This association of histone variants destabilizes the nucleosomes and facilitates histone displacement (Govin et al., 2004). Genetic ablation of certain testis-specific histone variants is associated with defective spermiogenesis and hence male infertility (Martianov et al., 2005; Tanaka et al., 2005). The replacement of canonical histones by protamines (Boissonneault, 2002) requires DNA double strand breaks (DSBs) to be generated during mammalian spermiogenesis in order to remove nucleosomal DNA supercoiling and relieve torsional stress (Boissonneault, 2002; Laberge and Boissonneault, 2005; Leduc et al., 2008; Marcon and Boissonneault, 2004).
Marcon and Boissonneault (2004) reported that during mouse spermiogenesis, endogenous DNA breaks are generated in elongating spermatids by Topoisomerase II beta (TOP2β) as a natural developmental programme. TOP2β first causes the DSBs and then reseals them with the help of another enzyme called tyrosyl-DNA phosphodiesterase 1 (TDP1), which is known to resolve topoisomerase-mediated DNA damage (Leduc et al., 2008). These DSBs are transient and normally appear in stage IX to stage XI elongating spermatids. These DSBs can be detected by the presence of the DNA damage response marker γ-H2AX in steps 9-11 elongating spermatids. Additionally, epigenetic modifications (acetylation, methylation, and ubiquitylation) on certain histones (H2A, H2B, H3 and H4) are also important for regulating gene expression in germ cells and condensation of spermatid chromatin. Histone H4 hyperacetylation has been shown to be an important prerequisite for initiation of DSBs, histone displacement and chromatin condensation in early elongating spermatids (Govin et al., 2007; Hazzouri et al., 2000; Meistrich et al., 1992). Histone methylation is also tightly regulated during spermatogenesis and is observed at specific stages of spermatogenesis (Peters et al., 2001). Although it is not known how histone modifications are involved in the regulation of gene expression and histone displacement, it is possible that epigenetic modifications of histones may alter chromatin architecture and induce the gene expression program required for normal spermatogenesis (Peters et al., 2001). Some of the steps in spermiogenic chromatin remodelling are briefly summarised below.

2.4.3.3.1 Incorporation of histone variants

Spermiogenic chromatin remodelling involves the incorporation of a number of noncanonical histone variants. In mice, except histone H4, variants for all other core histones (H2A, H2B, H3) and linker histone (H1) have been identified (Govin et al., 2005; Govin et al., 2007). Some of these histone variants that are expressed in postmeiotic germ cells and are involved in spermiogenic chromatin reorganisation include:

(i) **H1 variants:**
H1 variants that are specifically expressed in testis are H1t, H1T2 and HILS (Happel and Doenecke, 2009). H1t is expressed in pachytene spermatocytes and round spermatids, and is eliminated in elongating spermatids during chromatin remodelling (Brock et al.,
1980; Drabent et al., 1996). H1t knockout mice are fertile and do not display any defects with regards to spermatogenesis (Drabent et al., 2000). H1T2 is expressed in round and elongating spermatids, and like H1t, is degraded during chromatin remodelling in elongating spermatids. Loss of H1T2 in mice leads to abnormal nuclear condensation and subfertility (Martianov et al., 2005; Tanaka et al., 2005). HILS1 is highly expressed in elongating and elongated spermatids and is essential for chromatin reorganisation (Fig 2.12) (Iguchi et al., 2004; Yan et al., 2003).

(ii) **H2A and H2B variants:**

Some of these variants which are specifically expressed in testis and present in round spermatids include TH2A and TH2B (Trostle-Weige et al., 1982; Unni et al., 1995a). Both of these histone variants disappear after chromatin condensation. Other variants which are present in round and elongating spermatids, and help in chromatin destabilisation during spermiogenesis include spermatid-specific H2B (ssH2B), H2A.B.bd, H2AL1, H2AL2, H2AL3, H2BL1, H2BL2, H2AL1, H2AL2, H2AL3, and H2BL (Fig 2.12) (Chadwick and Willard, 2001; Eirin-Lopez et al., 2008; Govin et al., 2007; Ishibashi et al., 2010; Moss et al., 1989; Unni et al., 1995b).

(iii) **H3 variants:**

H3 histone variants that are specifically expressed in mammalian germ cells include H3.5, H3.3A, H3.3B and H3t (Schenk et al., 2011; Tachiwana et al., 2008; Trostle-Weige et al., 1984). H3t is expressed in pre-meiotic and post meiotic germ cells and disappears in condensed chromatids (Fig 2.12) (Trostle-Weige et al., 1984). H3.3A is expressed in all germ cells except elongated spermatids whereas H3.3B is present only in spermatocytes (Bramlage et al., 1997).

### 2.4.3.3.2 Histone modifications that take place before histone removal

Histone to transition protein to protamine replacement during spermiogenesis simultaneously requires the removal of somatic nucleosomes and initial incorporation of transition proteins in elongating spermatids. Transition proteins are finally replaced by protamines. This phenomenon is facilitated by specific histone modifications that destabilise the higher-order chromatin structure in nucleosomes making DNA more accessible to different factors and ultimately resulting in more open chromatin (Voigt and Reinberg, 2011). The most common post-translational modifications in histones
include acetylation, methylation, phosphorylation, ubiquitination and SUMOylation (Sims et al., 2003; Strahl and Allis, 2000; Turner et al., 1992).

In mammals, histone H4 hyperacetylation is the most predominant modification taking place in elongating spermatids just before the removal of histones (Figs 2.11 and 2.12) (Grimes and Henderson, 1984; Sonnack et al., 2002). Histone H4 hyperacetylation in mammalian spermatids is thought to induce the histone displacement by loosening up the chromatin (Braun, 2001; Sassone-Corsi, 2002). Reduced H4 acetylation in elongating spermatids has been reported to impair fertility in mammals (Fenic et al., 2004; Sonnack et al., 2002). Although hyperacetylation appears to be an important prerequisite for spermiogenic chromatin remodelling, prematurely induced hyperacetylation does not induce premature condensation in drosophila spermatids, suggesting that H4 hyperacetylation is not the only chromatin remodelled (Awe and Renkawitz-Pohl, 2010). Furthermore, premature expression of either protamine 1 (PRM1) or TNP2 in mouse round spermatids leads to male infertility due to abnormal spermatid condensation (Lee et al., 1995; Tseden et al., 2007), indicating that these proteins can be incorporated into spermatid chromatin without initiating the histone hyperacetylation. In elongating spermatids of mice, H4 can be acetylated at lysines K5, K8, K12 and K16 (Govin et al., 2007; Hazzouri et al., 2000; Moriniere et al., 2009; van der Heijden et al., 2006). In mouse and human, elongating spermatids also display H3K9 hyperacetylation (Nair et al., 2008; Song et al., 2011; Steilmann et al., 2011). These histone modifications are lost along with the histones when the latter are replaced by transition proteins. In addition to histone hyperacetylation, other modifications which have been reported to occur in elongating spermatids include H3K4 mono-, di- and tri-methylation, and H2A, H2B and H3 ubiquitination (Baarends et al., 1999; Chen et al., 1998; Godmann et al., 2007).
Figure 2.12. Schematic showing the major events of spermiogenic chromatin remodelling in mice. Various histone variants are incorporated into the chromatin of haploid spermatids. Some of these variants are then removed while others are retained. The first trigger for histone replacement is the hyperacetylation of histone H4 in early elongating spermatids, which is followed by DNA DSBs by TOP2B with the help of TDP1. Histones are removed, and transition proteins are incorporated, which are finally replaced by protamines 1 and 2. Abbreviations. RS (1-8), round spermatids at steps 1-8; eES (9), step 9 early elongating spermatids; ES (10-11), elongating spermatids at steps 10-11; CS (12-13), condensing spermatids at steps 12-13; CDs (14-16), elongated fully condensed spermatids at steps 14-16; TOP2B, Topoisomerase II beta; TDP1, tyrosyl-DNA phosphodiesterase 1; TP1 and TP2, Transition protein 1 and 2. This image has been modified from (Rathke et al., 2014), which is an open access article and allows the reuse of the content under terms of the Creative Commons Attribution License (CC BY 3.0) (https://creativecommons.org/licenses/by/3.0/).
2.4.3.3.3 Transient DNA double strand breaks in elongating spermatids

In addition to histone modifications, among other essential changes that take place in elongating spermatids is the appearance of transient DNA double strand breaks that are thought to remove positive DNA supercoiling during nucleosome eviction (Fig 2.12) (Laberge and Boissonneault, 2005; Marcon and Boissonneault, 2004; McPherson and Longo, 1993). It is reported that these breaks, in mice, are generated by topoisomerase II beta (TOP2B) which is co-expressed in elongating spermatids along with the DNA DSB marker γH2AX and tyrosyl-DNA phosphodiesterase 1 (TDP1) (Fig 2.12) (Laberge and Boissonneault, 2005; Leduc et al., 2008). Associated with the repair of DNA DSBs in elongating spermatids is the poly(ADP-ribosyl)ation of proteins, the inhibition of which leads to defective chromatin reorganisation in spermatids (Meyer-Ficca et al., 2009; Meyer-Ficca et al., 2005; Quenet et al., 2009). Since the spermatids are haploid in DNA content, the entire processes of DNA repair in these have to rely on nonhomologous end joining (NHEJ) (Leduc et al., 2008).

2.4.3.3.4 Transition proteins

Transition proteins are basic non-histone chromatin components that are transiently expressed and recruited on DNA in elongating spermatids, constituting almost 90% of chromatin in these spermatids (Figs 2.11 and 2.12) (Meistrich et al., 2003). Just after H4 hyperacetylation in elongating spermatids, histones are removed from DNA and replaced by transition proteins, which bind to DNA more strongly than histones due to their arginine- and lysine-rich sequence (Brewer et al., 2002; Kistler et al., 1975). In mice, two transition proteins, transition protein 1 (TNP1) and transition protein 2 (TNP2) are expressed (Grimes et al., 1975; Heidaran and Kistler, 1987; Kleene et al., 1988; Kleene and Flynn, 1987). It is proposed that transition proteins help in reducing the melting temperature of supercoiled DNA and facilitate chromatin remodelling and condensation by making the DNA more flexible and accessible to the action of topoisomerases (Akama et al., 1999; Akama et al., 1998; Brewer et al., 2002; Singh and Rao, 1988). In mice, genetic deletion of Tnp1 leads to subfertility and reduced sperm motility whereas TNP2 deficient mice are fertile (Yu et al., 2000; Zhao et al., 2001). However, double knockout mice with TNP1 and TNP2 deletion are sterile and display defective chromatin condensation and abnormal head morphology, indicating that transition proteins are
essential for chromatin condensation, but can be functionally redundant (Zhao et al., 2004).

2.4.3.3.5 Protamines as the final components of sperm chromatin

After all the chromatin modifications have taken place and histones have been removed, transition proteins are finally replaced by protamines in condensing spermatids (steps 12-13) (Figs 2.11 and 2.12). Protamines are arginine and cysteine rich highly basic proteins that constitute almost 95% of chromatin in mature elongated spermatids (Balhorn et al., 2000; Wouters-Tyrou et al., 1998). Mice and humans express two types of protamine, protamine 1 (PRM1) and protamine 2 (PRM2) while other species synthesise only one protamine (Balhorn et al., 1988; Braun, 2001; Yelick et al., 1987). In mice, both PRM1 and PRM2 are indispensable for male fertility and haploinsufficiency of either of these genes leads to infertility (Cho et al., 2001). However, in humans the ratio of PRM1/PRM2 (which is equal to 1) is an important factor in male fertility and any deviation from this ratio can lead to male infertility (Mengual et al., 2003; Torregrosa et al., 2006). It is usually thought that protamines stabilise the chromatin architecture and bring about higher chromatin compaction in the sperm head by binding minor groove of sperm DNA (Cho et al., 2003). High amounts of arginine in protamines mediate their strong DNA binding, and disulphide bonds between cysteine residues facilitate the establishment of highly compact chromatin (Carrell et al., 2007). Reduced amounts of protamines cause abnormal sperm head morphology and disorganised acrosome (Cho et al., 2001; Cho et al., 2003). Histone to protamine replacement requires assistance from other accessory factors, such as chromatin remodelling complexes (Choi et al., 2008), readers of histone modifications (such as testis specific bromodomain-containing proteins, BRDT) (Berkovits and Wolgemuth, 2013; Moriniere et al., 2009), and heat shock proteins (Park and Luger, 2008).

Protamination of sperm genome results in hypercondensation of sperm chromatin, making sperm DNA unfeasible for transcription. During step 8 of spermiogenesis, spermatid nuclei stop all transcriptional activity and the chromatin begins to condense (Hecht, 1998). Therefore, to regulate protamine protein expression, germ cells have developed mechanisms that uncouple the transcription and translation of these proteins (Kleene, 2003). For example, in drosophila testis, protamine mRNAs are transcribed in
primary spermatocytes and kept in a repressed state until late spermiogenesis (Barckmann et al., 2013). Similarly in mammals, early round spermatids engage in extensive transcription of spermiogenic genes, and transcriptional activity completely ceases in condensing spermatids towards the end of spermiogenesis (Kierszenbaum and Tres, 1975). Protamine and transition protein transcripts are synthesised in round spermatids and then stored in a translationally repressed state (Steger, 1999). This suggests that chromatin condensation in elongated spermatids necessitates the existence of a spatiotemporal control of gene expression peculiar to germ cells.

2.5 Posttranscriptional regulation of gene expression during spermiogenesis

Since post-meiotic chromatin condensation in elongating spermatids makes them transcriptionally inactive (Braun, 1998; Kimmins and Sassone-Corsi, 2005), these cell types have to rely on ready-made mRNA for protein synthesis. Therefore, mRNAs for many proteins that are required during later steps of spermiogenesis are already transcribed in round spermatids and then temporarily stored in a translationally repressed state until needed during spermatid differentiation (Fig 2.13). This is best characterised by protamine 1 (Prm1) and sperm-mitochondria cysteine-rich protein (Smcp) mRNAs that are transcribed and stored in round spermatids for several days in the form of translationally inactive messenger ribonucleoprotein particles (free-mRNPs) before their translation in elongating spermatids (Kleene, 1989, 2013).

This uncoupling of transcription and translation lowers the requirement of condensing spermatids for new transcripts by allowing them to rely on presynthesised mRNA (Kierszenbaum and Tres, 1975). During spermatid differentiation many mRNAs undergo translational repression by binding to RNA-binding proteins in meiotic and post-meiotic cells (Idler and Yan, 2012; Paronetto and Sette, 2010) (Fig 2.13). The most extensively studied transcripts that undergo translational repression in spermatids include Prm1, Prm2, Tnp1, Tnp2 and Smcp (Braun et al., 1989; Cullinane et al., 2015; Hawthorne et al., 2006; Kleene, 1989; Zhong et al., 2001). Disruption of this translational control in the case of Prm1 and Tnp2 mRNAs due to the induction of their premature translation in round spermatids leads to male sterility resulting from abnormal sperm head morphogenesis (Lee et al., 1995; Tseden et al., 2007). Therefore,
posttranscriptional mRNA regulation in spermatids constitutes an important mechanism to control sperm development.

Figure 2.13. Translational repression in round spermatids, and transcriptional inhibition in elongating and elongated spermatids. Schematic displaying active rounds of transcription taking place in spermatogonia, spermatocytes and round spermatids. Round spermatids actively engage in transcription until silencing of the haploid genome due to chromatin compaction occurs in elongating spermatids. Due to transcriptional inhibition in elongating and elongated spermatids, mRNAs that are required for chromatin condensation during late spermiogenesis are transcribed, stored and translationally repressed in round spermatids. mRNA storage and translational regulation is mediated by many RNA-binding proteins that play an important role in the synthesis of many spermatid specific proteins. Chromatoid body (CB) material is first apparent in late pachytene spermatocytes as several cytoplasmic fibrous-granular structures (red dots) that disperse during meiotic division. The CB is condensed to its final shape immediately after meiosis, and remains active as a single distinctive perinuclear granule in the cytoplasm of round spermatids (red dot). It diminishes in size and then disappears in elongating spermatids. Sg, spermatogonia; LS, leptotene spermatocyte; ZS, zygotene spermatocyte; PS, pachytene spermatocyte; DS, diplotene spermatocyte; RS, round spermatid; eES, early elongating spermatid; ES, elongated spermatid.

The formation of ribonucleoprotein (RNP) particles (called RNA granules) by the binding of RNA-binding proteins to mRNA in the cytoplasm of male germ cells provides an important mechanism for the posttranscriptional regulation of gene expression.
Germ cells in mammalian and non-mammalian organisms possess some special type of cytoplasmic granules called germ granules that are involved in germ cell RNA control. Different types of germ granules have been identified (Chuma et al., 2009). The most conspicuous of RNA granules found in late pachytene and round spermatids is called chromatoid body (CB). It is a prominent perinuclear RNP granule with ~1µm diameter and consisting of thousands of mRNA and noncoding RNA species associated with RNA-binding proteins (Meikar et al., 2014). The CB first appears in pachytene spermatocytes as cytoplasmic fibrous-granular structures which may vary in number, and remains active as a single lobulated perinuclear granule in round spermatids until they begin to elongate (Fig 2.13) (Kotaja and Sassone-Corsi, 2007). In round spermatids, the CB is in dynamic motion, moving caudally opposite to the acrosome towards the base of the sperm flagellum (Fawcett et al., 1970). The CB can be easily visualised by phase contrast microscopy during all the steps (1-8) of round spermatid differentiation, before it is disintegrated in elongating spermatids. Till date all the available evidence suggests that it has a role in mRNA regulation and small RNA mediated gene control (Kotaja and Sassone-Corsi, 2007; Meikar et al., 2011; Nagamori and Sassone-Corsi, 2008). Recent studies on the CB suggest that it acts as a main controlling and organising centre for different RNA processing pathways and a site for the accumulation of micro RNAs, piRNAs and mRNAs (Meikar et al., 2011; Nguyen Chi et al., 2009). The CB contains many proteins that are involved in RNA processing, and are indispensable for CB assembly, the most important of which include ATP-dependent DEAD-box RNA helicase VASA (also called MVH-mouse homolog of VASA), the mouse homolog of PIWI (MIWI), tudor domain containing 6 (TDRD6), tudor domain containing 7 (TDRD7), gonadotropin regulated testicular RNA helicase (GRTH/DDX25) and poly(A) binding protein PABPC3 (Meikar et al., 2010). Gene deletion of these components leads to defects in assembly of the CB and male infertility in mice (Deng and Lin, 2002; Kotaja et al., 2006; Tanaka et al., 2011; Toyooka et al., 2000; Tsai-Morris et al., 2004; Vasileva et al., 2009), suggesting that the CB plays an important role in sperm development. Based on its composition (protein and RNA), localisation and the timing of its appearance, the CB is thought to have a role in RNA regulation during male haploid germ cell differentiation.
2.6 The CB and mRNA regulation in round spermatids

Germ cell-specific mRNA control has long been thought to be mediated by the CB in round spermatids (Kotaja and Sassone-Corsi, 2007; Parvinen, 2005). This was supported by tritium labelling in testis when high resolution autoradiography analysis revealed the accumulation of tritiated uridine in CB (Soderstrom and Parvinen, 1976). Furthermore, it was reported that the highest rate of transcriptional activity of round spermatids is essential for the maintenance of CB morphology (Parvinen et al., 1978; Soderstrom, 1977). The CB was also found to accumulate many poly(A)-containing RNA molecules (Kotaja et al., 2006; Nguyen Chi et al., 2009), poly(A)-binding proteins (PABP) (Meikar et al. 2010), and other components of CB (such as MIWI, DDX2, DDX4) that bind mRNAs of protein coding genes (Deng & Lin 2002, Tsai-Morris et al. 2004, Nguyen Chi et al. 2009). Likewise, the need for posttranscriptional control in round spermatids perfectly coincides with the appearance of the CB in these cells, allowing the CB to suppress the translation of spermiogenic genes until required in elongating spermatids (Fig 2.13). However, what is not clear is whether the CB acts as a remodelling centre by regulating the interactions of RBPs with mRNAs, or as a realisation centre by bringing about translational repression as a consequence of these interactions. Therefore, what remains to be determined is whether translationally repressed mRNP granules are formed and stored in the CB, or only formed in the CB but stored in the general cytoplasm. Till date only Tnp2 mRNA has been unambiguously shown to be stored in a translationally repressed state in the CB of rat testis (Saunders et al., 1992).

2.6.1 Structural and functional components of the CB

With the advancement in immunofluorescence techniques, roughly more than fifty different proteins have been reported to localise to the CB, the majority of which function in RNA-binding and RNA processing (Fig 2.14) (Kotaja and Sassone-Corsi, 2007; Meikar et al., 2011). The ATP-dependent DEAD-box RNA helicase (MVH) constitutes one of the major components of the CB. MVH, in addition to localising to the CB is also present in the cytoplasm of spermatogonia to round spermatids, is critical for spermatogenesis, and is used as a general CB maker (Chuma et al., 2009; Tanaka et al., 2000; Toyooka et al., 2000). Mvh null mice display meiotic arrest at the diplotene stage of meiosis I (Tanaka et al., 2000).
Figure 2.14. **Structure and functions of chromatoid body (CB)**. Schematic displaying CB components and functions. CB consists of various components including mouse VASA homolog (MVH), mouse homolog of PIWI (MIWI), components of microRNA (miRNA) pathway (such as Argonaute proteins and the endonuclease Dicer) and the RNA-decay pathway (such as the decapping enzyme DCP1a). miRNAs and mRNAs that are transcribed in the nucleus are exported to the cytoplasm with the help of kinesin protein KIF17b through nuclear pores and assembled directly in the CB. In the CB, mRNAs are subjected either to decay or undergo translational repression. The CB is also involved in piRNA (PIWI-interacting RNAs) production, gene silencing through small RNA biogenesis and mRNA scanning. piRNAs regulate posttranscriptional gene expression during spermatogenesis and silencing of LINE1 transposon. CB is important for posttranscriptional regulation in round spermatids. Due to the functional relationship of the CB with the active translation apparatus, it is suggested that mRNAs that are temporarily stored in the CB undergo translational derepression and engage in active translation. CB, chromatoid body; RNP, ribonucleoprotein particle.

In the CB, MVH interacts with small RNA pathway components such as DICER and members of the Argonaute or PIWI family (Kotaja et al., 2006a; Kuramochi-Miyagawa et al., 2004). Another CB member which is used as a CB marker is MIWI, a member of the PIWI family, present in spermatocytes and round spermatids. Miwi null mice exhibit arrest at round spermatids, which show a diffuse/disrupted CB (Kotaja et al., 2006b). Tudor domain containing proteins such TDRD1, TDRD6, TDRD7, and TDRD9, are also known to localise to the CB and are considered well established CB components. Other proteins that are found in the CB include: Kinesin motor protein 17; the Argonaute 1
subfamily members, AGO2 and AGO3 (Kotaja et al., 2006a); RanBPM, a RanGTP-binding protein involved in microtubule nucleation (Shibata et al., 2004); DCP1a, a decapping enzyme; GW182, an RNA-binding protein (Kotaja et al., 2006a); actin (Walt and Armbruster, 1984); histone H4 (Werner and Werner, 1995); mRNA-binding protein p48 and germ cell-specific RNA-binding protein p52 (Oko et al., 1996); small nuclear RNA associated proteins (snRNPs), a large group of essential proteins of the spliceosome complex involved in pre-mRNA processing (Biggiogera et al., 1990; Moussa et al., 1994); cytochrome c isozymes (Hess et al., 1993); and GRTH, Gonadotrophin-regulated testicular RNA helicase (DDX25) (Fig 2.14) (Tsai-Morris et al., 2004). Meikar et al. (2014) performed a full proteomic analysis of the CB and identified many additional proteins, majority of which are either RNA-binding or are involved in RNA regulation.

2.6.2 CB and piRNA

PIWI-interacting RNAs (piRNAs) are a group ~26-31 nucleotide long small RNA that are specifically produced in male germ cells of many species including flies and mammals. Two types of piRNAs are produced in germ cells of mice: pre-pachytene and pachytene piRNAs. Pre-pachytene piRNAs are expressed in prospermatogonia and premature postnatal spermatogonia. These piRNAs are involved in the silencing of transposable elements by repressing their expression and protect the male germ line genomic integrity (Ender and Meister, 2010). Pachytene piRNAs are expressed in pachytene spermatocytes during meiosis and in round spermatids. The production and function of this class of piRNAs are dependent on the CB (Fig 2.14). In mice, three different PIWI proteins that have been identified to interact with piRNAs include MIWI (PIWIL1), MILI (PWIL2) and MIWI2 (PWIL4). Pre-pachytene piRNAs are highly repetitive and are known to associate with MILI and MIWI2, whereas non-repetitive pachytene piRNAs interact with MILI and MIWI (Aravin et al., 2008). Pachytene piRNAs are involved in the posttranscriptional regulation of gene expression during spermatogenesis (Goh et al., 2015), silencing of LINE1 transposon (Reuter et al., 2011), and the progression of meiotic and postmeiotic phases of spermatogenesis (Fig 2.14) (Zheng and Wang, 2012). Mice with homozygous mutations for Miwi (Deng and Lin, 2002), Mili (Kuramochi-Miyagawa et al., 2004), or Miwi2 (Carmell et al., 2007) all display male specific sterility. Genetic deletion of Mili and Miwi2 causes early arrest of meiosis at the zygotene stage, but deletion of Miwi results in the block of...
spermatogenesis at round spermatids. Since these PIWI proteins form essential components of the CB, the piRNAs that they associate with are always enriched in the CB. Therefore, these small RNAs are able to exert their effect through the CB (Meikar et al., 2014).

2.7 Cell-cell junctions in the testis

In all epithelial tissues, cells communicate with each other through different types of cell-cell junctions. The seminiferous epithelium in the testis is a unique type of epithelium composed of Sertoli cells and germ cells which are at different stages of development (spermatocytes and spermatids). Throughout the process of spermatogenesis, Sertoli cells remain closely attached to each other and with different types of developing germ cells through multiple cell-cell junctions (Mruk and Cheng, 2004b). These testicular junctions can be broadly divided into three types: (a) tight junctions (TJ), (b) gap junctions (GJ) and (c) anchoring junctions. Anchoring junctions can further be subdivided into: (i) actin filament-based adherens junctions (AJ) and focal adhesions, and (ii) intermediate filament-based desmosome-like junctions (Yan et al., 2008). In contrast to somatic tissues where cells at a particular location are connected by only one type of junctional complex, testicular junctions are heterogeneous where two or more types of junctions coexist in close proximity within the seminiferous epithelium (Lee et al., 2009). The best example of testicular heterogeneity is provided by the blood-testis-barrier (BTB), which is a junctional complex between two adjacent Sertoli cells (Fig 2.15). The BTB is composed of tight junctions (TJ), adherens junctions (AJ), gap junctions (GJ) and desmosome-like junctions, all of which are present together in the epithelium (Wong and Cheng, 2005). The BTB acts as an impermeable barrier dividing the seminiferous epithelium into basal and adluminal compartments, thereby creating a unique microenvironment suitable for postmeiotic germ cell development. As an immunological barrier, it excludes the postmeiotic germ cells present within the adluminal compartment from systemic circulation and prevents the triggering of autoimmune response towards spermatid antigens (Mruk and Cheng, 2004b). In order to allow the passage of preleptotene spermatocytes from the basal to the adluminal compartment, which takes place at stage VIII of spermatogenesis, the BTB has to disassemble. During the course of germ cell development, intense assembly and disassembly of inter Sertoli cell and Sertoli cell -
germ cell junctions take place to promote the proper orientation and migration of germ cells along the seminiferous epithelium. Based on the cell types that are in contact with each other, testicular junctions can be of two types: (i) Sertoli cell-Sertoli cell junctions, and (ii) Sertoli cell-germ cell junctions.

2.7.1 Tight junctions

TJs are points of close contact between two adjacent cells in mammalian epithelial cells. They perform several important functions in the tissue. TJs help in cell compartmentalisation by establishing a strong barrier that can restrict the movement of solute molecules through the cellular passage. TJs also generate cell polarity by creating a boundary between the two opposing interfaces of a cell. TJs also form an immunological barrier that isolates germ cell antigens from systemic circulation and also blocks the entry of immunoglobulins and lymphocytes into the adluminal compartment of the seminiferous epithelium. In mice, testicular TJs are assembled at the age of 12 days after birth. In testis, the TJs are formed between two adjacent Sertoli cells towards the basement membrane (Fig 2.15). These Sertoli cell tight junctions exist in the form a large inter-Sertoli cell junctional complex consisting of TJs, gap junctions, desmosome-like junctions and adherens junction complex, all of which are collectively called BTB (Fig 2.16) (Mruk and Cheng, 2004b). The BTB, which unlike other epithelial junctions is formed near the basement membrane, partitions the seminiferous epithelium into two compartments. The basal compartment is occupied by all types of interstitial cells, blood vessels and early germ cells (spermatogonia and preleptotene spermatocytes) while in the adluminal part, meiotic and postmeiotic germ cells grow (Figs 2.15 and 2.16). The BTB prevents nutrients from reaching germ cells; therefore, developing germ cells depend on Sertoli cells for their nutrients and other biological factors. Finally, the BTB is highly dynamic in nature and undergoes constant restructuring to allow the transit of preleptone spermatocytes towards the adluminal compartment. Some of the important tight junction proteins that are known to be present in Sertoli cells tight junctions include: claudins - claudin-1, claudin-3, claudin-5, claudin-11, caludin-12 and claudin-13; occludin; junctional adhesion molecules (JAMs); coxsackievirus and adenovirus receptor (CAR); and zona occludins-1, -2, and -3 (ZO-1, ZO-2, ZO-3). Of all the TJ proteins, deletion of only claudin-11 leads to infertility, indicating that it is essential for spermatogenesis (Stanton, 2016).
Figure 2.15. Different types of junctions between two adjacent Sertoli cells and between Sertoli cells and germ cells. A schematic showing tight junctions (TJ), adherens junctions (AJ) and desmosome-like junctions formed at the interfaces of Sertoli-Sertoli cells and Sertoli–germ cells in testis. Tight junctions between two Sertoli cells towards the basement membrane form the blood-testis-barrier (BTB), which is a junctional complex between two adjacent Sertoli cells. BTB consists of tight junctions (TJ), adherens junctions (AJ), gap junctions (GJ) and desmosome-like junctions, all of which are present together in the seminiferous epithelium, dividing it into basal and adluminal compartments. Pachytene spermatocytes and round spermatids are connected to Sertoli cells via both adherens junctions and desmosome-like junctions while elongating and elongated spermatids are connected to Sertoli cells via adherens junctions only. This image has been reproduced from (Cheng and Mruk, 2002) which freely allows to reproduce the content without the permission.
Chapter 2

Spermatogenesis

Figure 2.16. A schematic showing the different components of BTB and assembly/disassembly of apical ectoplasmic specialisation (ES). Blood-testis barrier (BTB) is composed of tight junction, gap junction, basal ectoplasmic specialization and desmosome-like junction. Towards the lumen of the seminiferous tubule, apical ES is established between elongating / elongated spermatids and Sertoli cells. Apical ES is assembled and disassembled in a cyclic manner and is finally disrupted during spermiation to allow sperm release. This image has been reproduced from (Berruti and Paiardi, 2014) which is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

2.7.2 Adherens junctions (AJ)

Mammalian seminiferous epithelium contains two types of testis-specific anchoring junctions: (i) desmosome-like-junctions, and (ii) ectoplasmic specialisation (ES) (Kopera et al., 2010). Actin-based cytoskeleton or intermediate filaments, actin-based AJs are usually found at the two specific locations in the seminiferous epithelium: (i) the
BTB at the Sertoli cell–Sertoli cell junction, and (ii) the ectoplasmic specialization (ES) and the tubulobulbar complex (TBC) at the Sertoli cell–spermatid junction (Yan et al., 2008). Desmosome-like-junctions in testis are intermediate filament cytoskeleton containing cell junctions that mediate contacts between Sertoli cells at the BTB as well as between Sertoli cells and germ cells. These junctions are mainly composed of cadherin family of proteins. A distinguishing feature of testicular desmosome-like-junctions is that they are deficient in an obvious dense midline, indicating that they unable to produce stronger adhesion (Kopera et al., 2010). At the Sertoli cell-germ cell interface, the desmosome-like-junctions are replaced by ES and these two anchoring junctions cannot coexist together at this interface.

### 2.7.3 Ectoplasmic specialisation (ES)

ES is the best characterized AJ type in the testis. It is a unique actin-based atypical adherens junction that is found between Sertoli cells at the BTB towards the basement membrane and at the Sertoli cell-germ cell interface at the adluminal compartment. Based on their localisation, two types of ES are present in the seminiferous epithelium: basal ES, and apical ES. Basal ES is present at the BTB between the two adjacent Sertoli cells, while apical ES is restricted to Sertoli cell-elongating/elongated spermatid (beyond step 8) interface towards the luminal surface of the tubule (Fig 2.16). However, contacts between Sertoli cells and step 1-7 (round) spermatids and at Sertoli cell-spermatocyte interface are mediated by other AJs and desmosome–like junctions (Wong et al., 2008). Basal ES at the BTB coexists along with TJ, tubulobulbar complex (TBC), desmosome-like junctions and gap junctions, and is formed throughout the seminiferous epithelium cycle (Fig 2.16). Although both the ESs look structurally identical, the apical ES, unlike the basal ES, lacks any TJs. In rat and mouse testis, the apical ES begins to form between Sertoli cells and stage VII round spermatids, and by stage IX, it is completely established at the interface of Sertoli cells and elongating spermatids. The ES is mostly composed of three different classes of transmembrane proteins- cadherins, nectins and integrins. However, additional anchoring junction protein complexes, such as integrin–laminin and nectin-afadin complexes, also exist at the ES.
Figure 2.17. Structure of the apical ectoplasmic specialization (ES). Schematic displaying the apical ES. The apical ES consists of hexagonal bundles of actin filaments that are sandwiched between the Sertoli cell plasma membrane and cisternae of the endoplasmic reticulum, covering the entire head region of the elongating/elongated spermatids. Also shown is the tubulobulbar complex (TBC) which is a testis-specific anchoring junction found between Sertoli cells and elongated spermatids, but it is restricted only to the concave side of the spermatid head. This image has been modified from (Mruk and Cheng, 2004a) with permission from Elsevier via copyright clearance centre (License No. 4178891104214).

Although tight junctions are not present at the apical ES, several TJ proteins such as coxsackie and adenovirus receptor (CAR) and junctional adhesion molecule-C (JAM-C), and gap junction (GJ) proteins such as Connexin 43 have recently been localised to the apical ES. The presence of these TJ, GJ and AJ proteins at the apical ES is believed to enhance the intense junction restructuring that takes place at the apical ES to facilitate the movement of germ cells along the epithelium (Wong et al., 2008). Since postmeiotic germ cells constitute the majority of germ cells embedded in the seminiferous epithelium, the apical ES is the principle AJ type in the seminiferous epithelium. Once the apical ES is assembled at the Sertoli cell-germ cell interface, other types of junctional complexes such as desmosome-like junctions and GJs are disassembled. Ultrastructurally the apical ES consists of hexagonal bundles of actin filaments that are sandwiched between the Sertoli cell plasma membrane and cisternae of the endoplasmic reticulum, covering the entire head region of the elongating/elongated spermatids (Wong et al., 2008) (Fig 2.17). Just before the release of spermatozoa into the tubule lumen
(spermiation), the apical ES is disrupted and is replaced by the TBC (a testis-specific anchoring junction found between Sertoli cells and elongated spermatids, restricted to the concave side of the spermatid head). The apical ES is believed to have three important functions: (i) since disruption of the apical ES leads to release of elongated spermatids from the epithelium, it is believed to act as an adhesive between Sertoli cells and elongating/elongated spermatids; (ii) the apical ES orients as well as positions the elongating/elongated spermatids along the seminiferous epithelium by anchoring the spermatids to the Sertoli cell; and (iii) it is believed to take part in formation of sperm head shape since it contracts the spermatids by holding them up in the seminiferous epithelium (Mruk and Cheng, 2004a).