

Phenotyping male infertility in the mouse: how to get the most out of a ‘non-performer’

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TABLE OF CONTENTS

- Background
 - Somatic cells in the testis
 - Spermatogenesis
 - The hormonal control of spermatogenesis
 - Post-testicular sperm maturation
 - Fertilization
 - Methods
 - Results
 - Breeding experiments
 - Histology
 - Defects in spermiogenesis
 - Sperm tail development and structure
 - Hormone analysis
 - Spermatogenic efficiency
 - Analyzing post-testicular sperm maturation and fertilizing ability
 - Conclusion
-

BACKGROUND: Functional male gametes are produced through complex processes that take place within the testis, epididymis and female reproductive tract. A breakdown at any of these phases can result in male infertility. The production of mutant mouse models often yields an unexpected male infertility phenotype. It is with this in mind that the current review has been written. The review aims to act as a guide to the ‘non-reproductive biologist’ to facilitate a systematic analysis of sterile or subfertile mice and to assist in extracting the maximum amount of information from each model.

METHODS: This is a review of the original literature on defects in the processes that take a mouse spermatogonial stem cell through to a fully functional spermatozoon, which result in male infertility. Based on literature searches and personal experience, we have outlined a step-by-step strategy for the analysis of an infertile male mouse line.

RESULTS: A wide range of methods can be used to define the phenotype of an infertile male mouse. These methods range from histological methods such as electron microscopy and immunohistochemistry, to hormone analyses and methods to assess sperm maturation status and functional competence.

CONCLUSION: With the increased rate of genetically modified mouse production, the generation of mouse models with unexpected male infertility is increasing. This manuscript will help to ensure that the maximum amount of information is obtained from each mouse model and, by extension, will facilitate the knowledge of both normal fertility processes and the causes of human infertility.

Key words: spermatogenesis / infertility / spermiogenesis / mouse models

Background

Male infertility affects 1 in 25 men in the Western world and is the cause of considerable social and financial burden (de Kretser and Baker, 1999; Holden et al., 2005). In several countries, children conceived with the aid of artificial reproductive technologies constitute more than 3% of annual births (Nyboe Andersen et al., 2008) and ~50% of such cases are, at least in part, caused by male infertility (de Kretser and Baker, 1999; Holden et al., 2005; Walsh et al., 2009). Despite this, and an increasing recognition of the value of male gamete-based contraceptives, there are still many uncertainties in the processes of sperm development (spermatogenesis) and maturation.

The establishment of male fertility in humans is not completed until puberty. In the mouse, full fertility can be seen by 6–7 weeks of age. The great majority of the genes and processes involved in sperm production appear to be conserved between mice and men, and thus, mice are excellent models of human infertility. An additional advantage of the mouse is that the key time points for the appearance of particular types of germ cells are well defined (Bellve et al., 1977; Russell et al., 1990a) (Table I), meaning that the temporal expression pattern of a gene product is frequently indicative of sites of cellular production.

Spermatogenesis is a complex series of events involving the establishment of a stem cell population, mitosis, meiosis and the morphogenesis of the haploid germ cell, which collectively involve the coordinated expression of >2300 different genes (Schultz et al., 2003). It is, therefore, not surprising that the production of mutated mouse models frequently results in male infertility. Spermatogenesis takes place in the seminiferous tubules of the testis, within the seminiferous epithelium, which is a stratified epithelium containing the developing germ cells and a fixed population of somatic Sertoli cells (Fig. 1A). The epithelium is surrounded by a layer of peritubular cells which are believed to be contractile and involved in the paracrine regulation of spermatozoa (Setchell, 1982; Burkitt et al., 1993).

This review takes the point of view of a researcher having a grossly normal-looking mouse with a XY genotype that fails to sire pups, or produces litters of reduced number, and aims to provide a structure to insightfully define a genotype–phenotype correlation. For a comprehensive list of infertile mouse models please see (Matzuk and Lamb, 2008; Naz et al., 2009).

Somatic cells in the testis

The Sertoli cells sit on the basement membrane of the seminiferous tubules and envelop all of the germ cells where they provide physical support, nutrients and paracrine signals (Setchell, 1982; Burkitt et al., 1993). Each adult Sertoli cell envelops four to five different germ cell types within its depth. Similarly, a single Sertoli cell is in contact with five to six other Sertoli cells and tight junctions between Sertoli cells form the basis of the blood–testis barrier (also known as the basal ectoplasmic specialization (ES)) (O'Donnell et al., 2006). The Sertoli

cells create a highly specialized microenvironment that separates the most immature (spermatogonia and pre-leptotene spermatocytes) cells in the basal compartment from the more mature (meiotic and post-meiotic) cells in the adluminal compartment (Kerr et al., 2006a, b). This barrier acts as an immunological barrier to protect the highly antigenic germ cells residing in the adluminal compartment. Many of the processes of blood–testis barrier structure remain unclear, however, recent studies have shown that gonadotrophins, cytokines and growth factors play a role in its function (Lui and Cheng, 2007; Yan et al., 2007; Wong et al., 2008).

The area surrounding the seminiferous tubules is known as the interstitial space. It contains the Leydig cells, blood vessels, immune cells and connective tissue. The most numerous cell type within the interstitium is the Leydig cell, which is the major source of androgens (Christensen and Mason, 1965; Cooke et al., 1972; Steinberger and Steinberger 1973; van der Molen et al., 1973). The interstitial compartment of the mouse also contains large numbers of resident macrophages. While the majority of research on testicular macrophages has been done in the rat, it is expected that the biology of the mouse will

Table I. Key time points for germ cell types

	Cell type	Time of first appearance ^a	
Process	Gonocyte	12d pc–1d pp	(Burgoyne, 1987)
Undifferentiated spermatogonia	Spermatogonia (A _s , A _{pr} and A _{al})	6d pp	(Bellve et al., 1977)
Differentiating spermatogonia	Spermatogonia (A _{1–4} , In- and B-)	8d pp	(Bellve et al., 1977)
Meiosis I (primary spermatocytes)	Pre-leptotene	10d pp	
	Leptotene	10d pp	
	Zygotene	12d pp	
Meiosis II	Pachytene	14d pp	(Bellve et al., 1977)
	Diploptene	17–18d pp	(Nebel et al., 1961)
	Secondary spermatocytes	18d pp	(Bellve et al., 1977)
Spermiogenesis	Round spermatids	20d pp	(Bellve et al., 1977)
	Condensing spermatids	30d pp	
	Spermatozoa	35d pp	(Kramer and Erickson, 1981)

^apc, post-coitum; pp, post-partum.

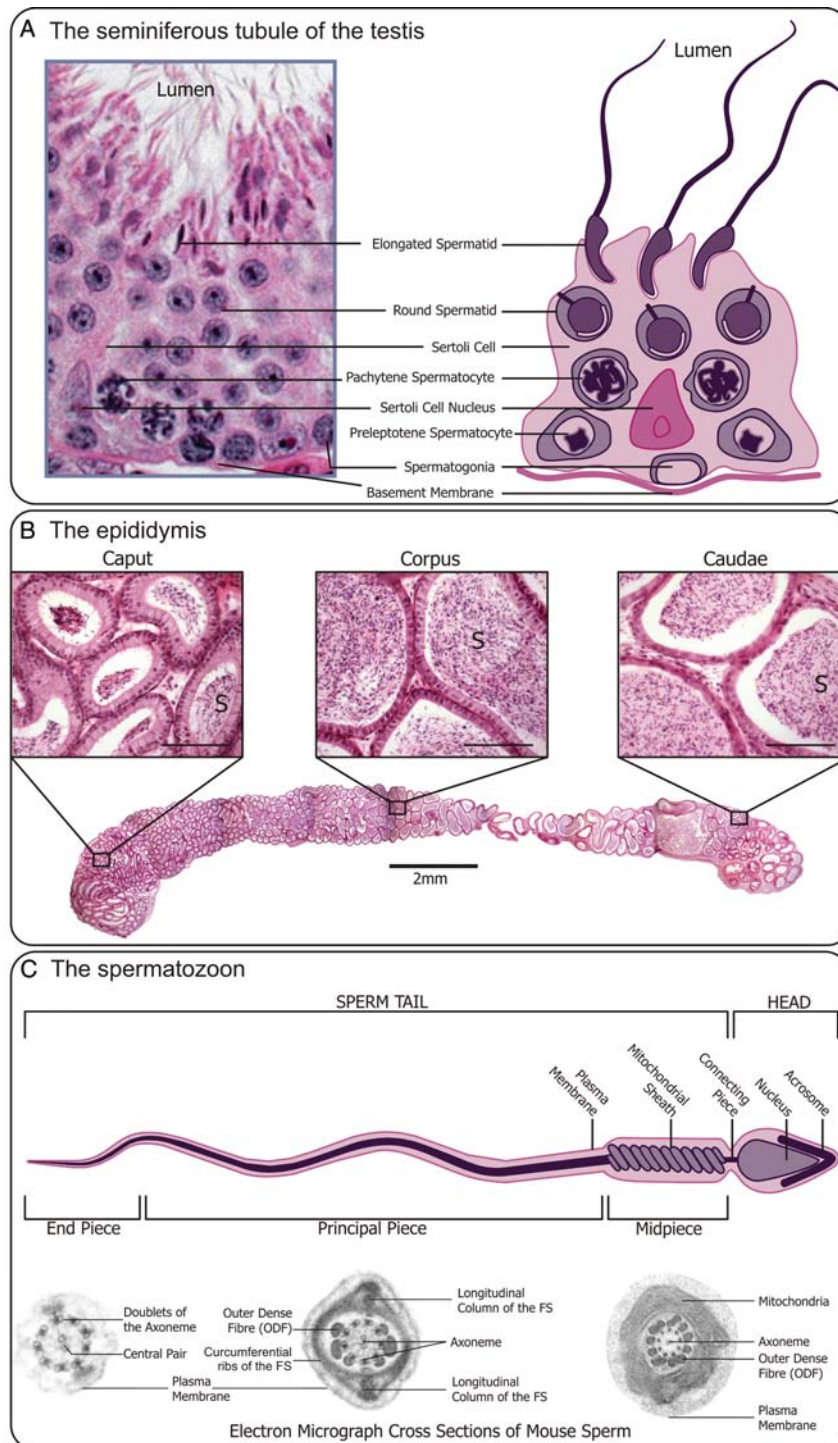


Figure 1: (A) The seminiferous tubule of the testis (a cross-section): the Sertoli cells provide support and nutrients for the developing germ cells. Sertoli cells also form the blood–testis barrier between adjacent Sertoli cells, which functionally divide the seminiferous epithelium into the basal and luminal compartments. Spermatogonia, the self-renewing stem cells of the testis, are associated with the basement membrane of the tubule. As the germ cells develop, from spermatocytes to spermatids, they move progressively closer to the lumen of the tubule where they are released in a process known as spermiation. (B) The epididymis: periodic Acid Schiff (PAS) stained epididymal sections, scale bar = 100 μm . Sperm released from the rete testis enter the efferent ducts, travel through the caput, corpus and caudal epididymal regions, during which time they undergo epididymal maturation. The epididymis ends at the vas deferens (not shown). S=spermatozoa. (C) The Spermatozoon: the spermatozoon is made up of two main regions, the head and the tail. The anterior portion of the head is covered by the acrosomal cap and the head is joined to the tail by the connecting piece. The tail is divided into three regions: the midpiece; principal piece; and the end-piece. The electron micrographs showing cross-sections (not to scale) of each region highlights the main components of the tail structure: the axoneme; outer dense fibers (ODF); and the mitochondrial sheath (midpiece) and fibrous sheath (FS) (principal piece). The end-piece consists solely of the axoneme and plasma membrane.

be closely aligned. Macrophages are powerful regulators of immune and inflammatory responses and though they are in low numbers prior to puberty, they begin to populate the interstitium of mice (and rats) around the time that spermatogenesis begins (Hardy et al., 1989; Ariyaratne and Chamindrani Mendis-Handagama, 2000). For an extensive review of the immunophysiology of the male reproductive tract, see (Hedger and Hales, 2006). The interstitial space also contains smaller numbers of dendritic cells, T cells and natural killer cells (Niemi et al., 1986; Hutson, 1994; Itoh et al., 1995; Tompkins et al., 1998).

Spermatogenesis

The stem cells of the testis, the spermatogonia, are situated in the basal compartment of the seminiferous tubule, between the Sertoli

cells and the basement membrane and divide mitotically to replace themselves and to provide a population of spermatogonia committed to becoming spermatozoa (Fig. 1A). These diploid cells are classified as type A, intermediate and type B spermatogonia based on the characteristics of the nucleus (Allen, 1918; Leblond and Clermont, 1952; Oakberg, 1956a, b; Chiarini-Garcia and Russell, 2002; Kerr et al., 2006a, b). Type A spermatogonia divide to produce further type A spermatogonia (self-proliferation) and intermediate spermatogonia, which in turn differentiate into type B spermatogonia.

Primary spermatocytes arise from type B spermatogonia. Meiosis is characterized by two cell divisions, during which chromosome number are halved (Cobb and Handel, 1998; Hassold et al., 2000; Hunt and Hassold, 2002) (Fig. 2A). Meiosis I can be separated into distinct phases based on the cytological features and chromosome dynamics: prophase, metaphase, anaphase, telophase and cytokinesis

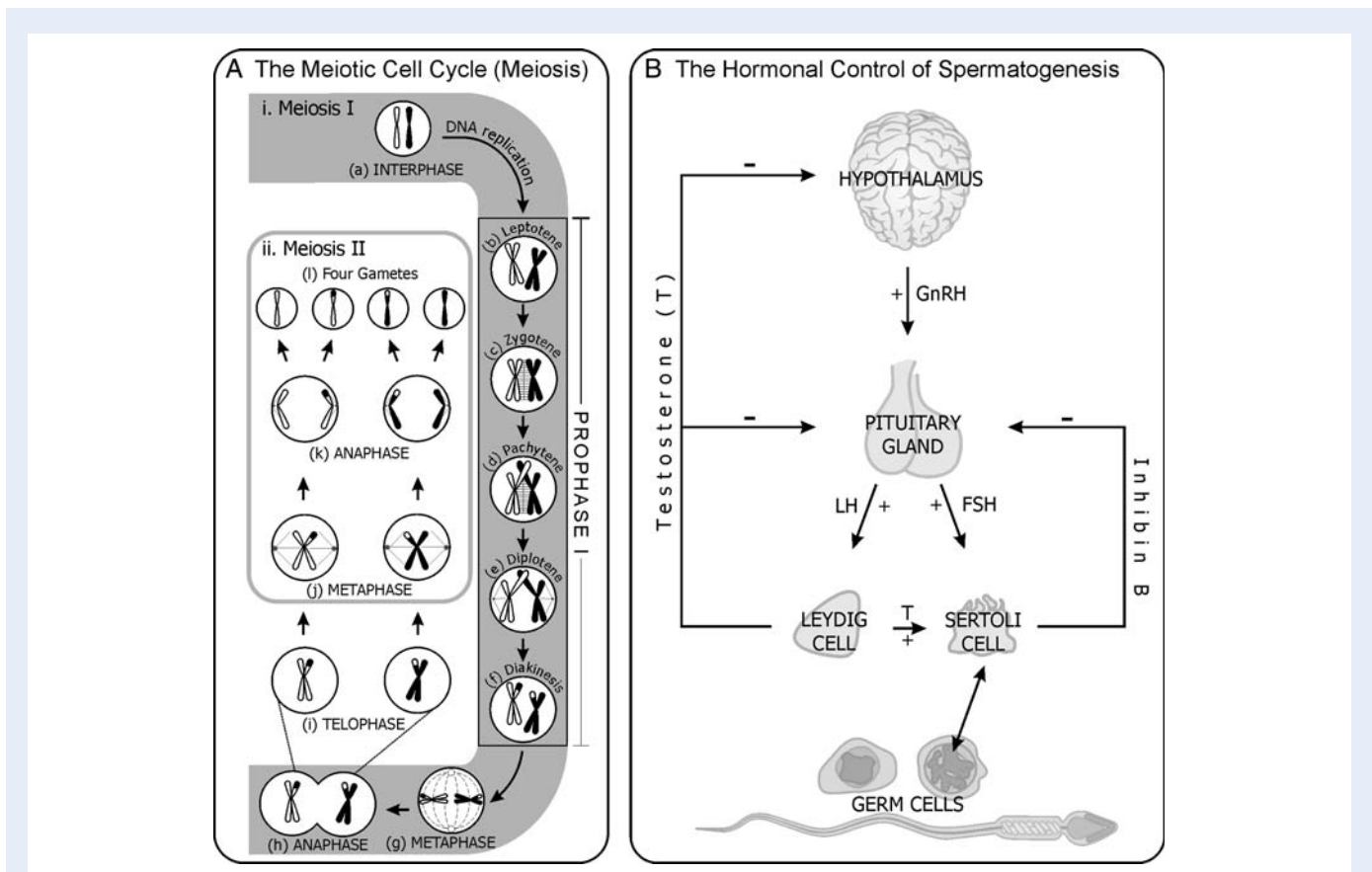


Figure 2: (A) The meiotic cell cycle: chromosomes replicate during interphase (a) of meiosis I (i). Following interphase, the chromosomes move into prophase I, beginning with the first phase, leptotene (b), where the chromosomes remain unpaired, but search each other out. The synaptonemal complex forms during zygotene (c) and homologous chromosomes begin to pair and the chromosomes become compact. Crossing-over occurs during pachytene (d) and the chromosomes are held together by sites of recombination well into diplotene (e). The chromosomes are completely separated during diakinesis (f) where they are pulled to separate poles of the cell. During metaphase (g) of meiosis I, chromosomes are pulled to separate poles by the spindle fibers. The cell divides at the end of anaphase (h), and contains intact/joined sister chromatids. Two separate cells are present at telophase (i). During metaphase (j) of meiosis II (ii), the sister chromatids are pulled to opposite poles of the cell and they separate during anaphase (k). At the end of meiosis II, four haploid gametes (l) have formed from each leptotene spermatocyte. (B) The hormonal control of spermatogenesis: a schematic representation of the hypothalamic–pituitary–testis axis in adulthood. Hypothalamic gonadotrophin-releasing hormone (GnRH) stimulates the pituitary to secrete the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). LH stimulates Leydig cells to produce testosterone (T). FSH and testosterone directly stimulate the Sertoli cells activity, which in turn regulates germ cell development. GnRH is under negative feedback control by testosterone. Pituitary secretion of LH and FSH is under feedback control by testosterone. Inhibin B is produced by the Sertoli cells and causes selective inhibition of FSH production.

(Fig. 2A). Prophase accounts for over 90% of meiosis, or ~3 weeks, and is divided into a further five periods: leptotene, zygotene, pachytene, diplotene and diakinesis, which are defined by the association between pairs of homologous chromosomes and the recombination events on each chromosome (Cobb and Handel, 1998; Russell *et al.*, 1990a, b, c). It is during the leptotene period that germ cells move from the basal to the luminal compartment of the seminiferous epithelium. Please see the meiosis results section for more details.

Following the completion of meiosis, round spermatids undergo a series of processes collectively known as spermiogenesis. Cell division no longer occurs, but round spermatids undergo extensive morphological changes to transform into spermatozoa (Fig. 3E–F). The molecular processes of spermiogenesis are not fully understood, however, several structural changes are well characterized including the formation of the acrosome, condensation of the nucleus, development of a flagellum (tail) and the reorganization/elimination of cytoplasm. The discrete types (morphologically) of spermatids are called 'steps' and haploid germ cell development can be broken down into 16 steps in the mouse. For a detailed description of spermiogenesis, readers are referred to (Kerr *et al.*, 2006a, b). Sperm are ultimately released from the seminiferous epithelium in a process known as spermiation (see below).

The hormonal control of spermatogenesis

The production of testosterone is an absolute requirement for the development of male secondary sexual characteristics, and its synthesis increases dramatically at puberty. Testosterone acts on the Sertoli and Leydig cells through the androgen receptor. Testosterone is required for the initiation, maintenance and restoration of spermatogenesis (O'Donnell *et al.*, 2006). Androgen synthesis and, by extension, sperm production are controlled by a feedback loop involving the testes, hypothalamus and pituitary gland (Fig. 2B). The pituitary controls testicular function by producing gonadotrophins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH). Pituitary production of these hormones, in turn, depends upon secretion of the gonadotrophin releasing hormone (GnRH) by the hypothalamus and elevated levels of GnRH initiate puberty. The control of gonadotrophin release involves a negative feedback of testosterone, from the Leydig cells, on LH, as well as the glycoprotein inhibin B, which is produced by Sertoli cells, on FSH (de Kretser and Robertson, 1989). An increase in testosterone acts on the pituitary gland to reduce the secretion of LH. There is also evidence that the action of FSH is important for the growth, activity and survival of the Leydig cells (Chen *et al.*, 1976; van Beurden *et al.*, 1976; Selin and Moger, 1977; Kerr and Sharpe, 1985; Vihko *et al.*, 1991). For a review of the hormonal regulation of male fertility, see (O'Donnell *et al.*, 2006).

Post-testicular sperm maturation

When sperm are released from the testis, they are transported via the efferent duct system into the epididymis. As this point, sperm are structurally complete, but functionally immature. In order to achieve functional competence they must undergo two additional processes, epididymal maturation and capacitation.

Sperm undergo epididymal maturation as they transit through the long and highly convoluted tubule that is the epididymis. Although the mouse epididymis can be divided into seven regions based on

epithelial morphology and the presence of tissue septae (Takano, 1980; Soranzo *et al.*, 1982; Abou-Haila and Fain-Maurel, 1984), it is most often considered in four sections; the initial segment, the caput, corpus and cauda epididymis (Fig. 1B). Epididymal maturation involves both the removal and addition of proteins onto and within sperm (Dacheux *et al.*, 2003; Sullivan *et al.*, 2005; Aitken *et al.*, 2007), a substantial remodelling of the membrane lipid composition and domains (Schlegel *et al.*, 1986; Martinez and Morros, 1996; Jones *et al.*, 2007) and protein post-translational modifications, including proteolysis, glycosylation and phosphorylation (Blobel *et al.*, 1990; Tulsiani *et al.*, 1995; Baker *et al.*, 2005; Morin *et al.*, 2005; Chandra *et al.*, 2008). While epididymal maturation remains a relatively poorly defined set of processes, it is apparent that these complex, regionalized and coordinated modifications are important in the ability of sperm to complete fertilization and their disruption may lead to compromised fertility (Baker *et al.*, 2003; Yoshinaga and Toshimori, 2003; Ecroyd *et al.*, 2004; Lin *et al.*, 2006).

Sperm functional competence is progressively obtained along the length of the epididymis. Key requirements for fertilization are largely complete by the time mouse sperm reach region 4 (the proximal corpus) (Baker *et al.*, 2003; Asquith *et al.*, 2004; Baker *et al.*, 2004; Ecroyd *et al.*, 2004; Lin *et al.*, 2006; Aitken *et al.*, 2007). Sperm acquire the ability to interact with the zona pellucida and an increased ability to achieve hyperactivated motility in region 4. The ability to manifest hyperactivated motility, however, continues to increase up to region 5b (the cauda epididymis) (Asquith *et al.*, 2004; Lin *et al.*, 2006). While sperm functional competence appears to be still developing within the cauda, these more distal sections of the epididymis are thought to function primarily in the storage of sperm and in keeping them in a suppressed state i.e. non-motile and non-capacitated (Jones, 1999).

Ejaculated sperm have the capacity for fertilization only after a period of residence in the female reproductive tract, during which time they undergo a process known as capacitation (Chang, 1951; Austin, 1952). During capacitation, sperm develop a forward progressive and then a hyperactivated form of motility and may switch periodically between the two forms (Suarez *et al.*, 1993; Ho and Suarez, 2001a, b; Suarez and Ho, 2003). Hyperactivated motility is characterized by exaggerated flagella bending and a very compact figure of eight path in low viscosity solutions, e.g. during *in vitro* capacitation. In high viscosity solutions, such as the secretions of the female reproductive tract, hyperactivated motility is thought to greatly facilitate progression and is required to dislodge sperm from the uterine epithelium and to penetrate the outer vestments of the oocyte (Carlson *et al.*, 2003; Suarez, 2008a, b). The ability to undergo the acrosome reaction and to bind to the oocyte are the end-points of sperm capacitation.

Many reviews describe the complexity of the signalling transduction mechanisms involved in capacitation (Baldi *et al.*, 2002; Visconti *et al.*, 2002). Specific mechanisms identified during capacitation include the removal of decapacitation factors (Bedford and Chang, 1962; Fraser, 1992); the loss of cholesterol to cholesterol acceptors (Visconti *et al.*, 1995a, b) and a subsequent increase in membrane fluidity (Wolf *et al.*, 1986; Baumber and Meyers, 2006; Jones *et al.*, 2007; Girouard *et al.*, 2008); redistribution or dissolution of lipid rafts (Sleight *et al.*, 2005; Nixon *et al.*, 2008); hyperpolarization of the plasma membrane and activation of adenylyl cyclase by HCO_3^- (Breitbart, 2002; Lefevre *et al.*, 2002; Demarco *et al.*, 2003; Beltran

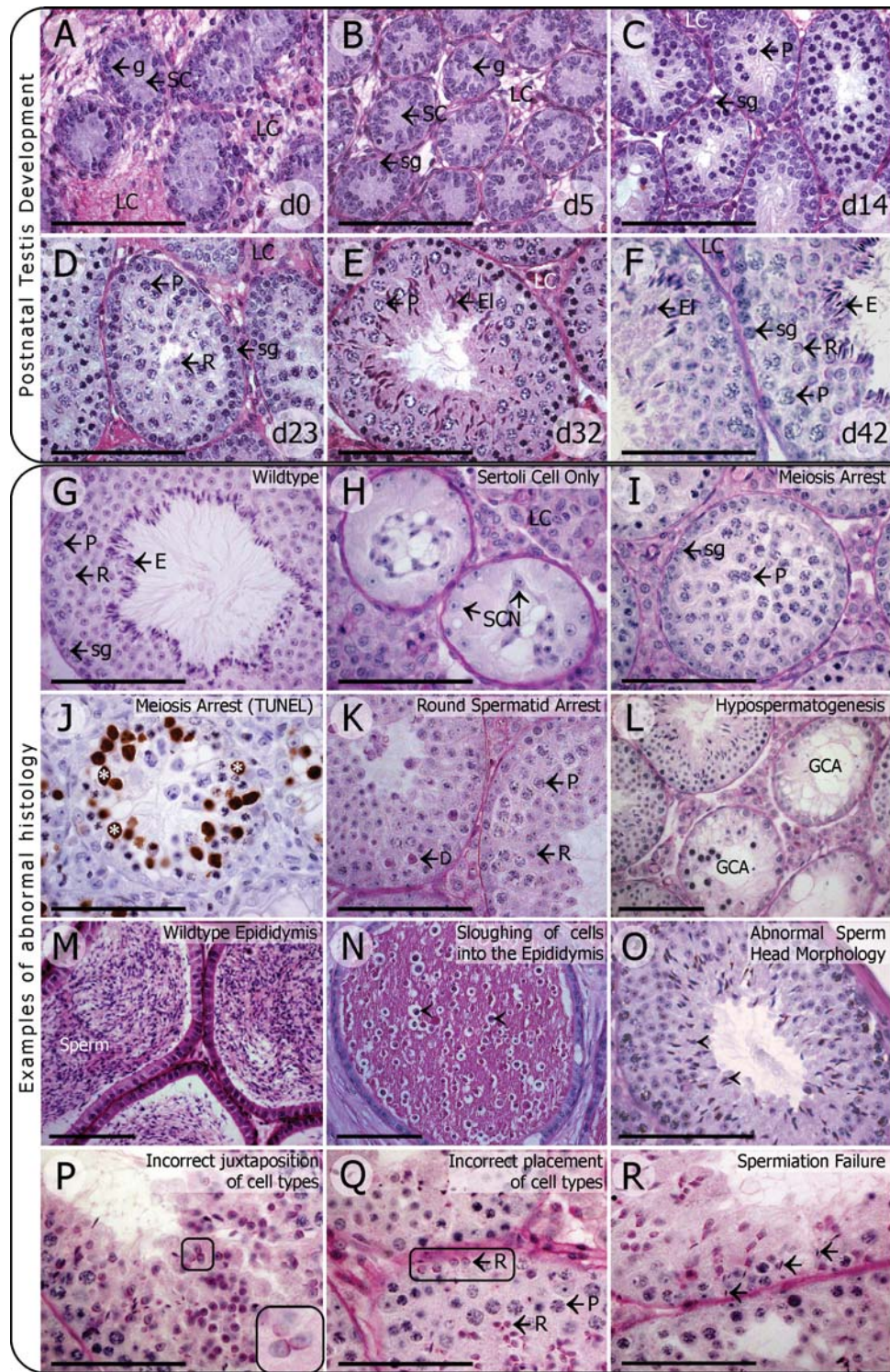


Figure 3: (A–F) Post-natal testis development: PAS stained sections of mouse testis at varying post-natal days, showing the most mature cell types present. Scale bar = 100 μ m. (A) Day 0. Centrally located gonocytes (g) surrounded by Sertoli cells (SC). (B) Day 5. Spermatogonia (sg) at the basement membrane surrounded by Sertoli cells. The occasional gonocyte may remain towards the centre of the tubule. (C) Day 14. Spermatogonia at the basement membrane and the beginning of meiosis with the primary spermatocytes (pachytene) (P) surrounded by Sertoli cells. (D) Day 23. Cells exiting meiosis are termed round spermatids (R), which begin to undergo the process of spermiogenesis towards the centre of the tubule. (E) Day 32. Elongating spermatids (El), undergoing the striking morphological changes to become a fully functional spermatozoon, line the centre of the tubule, anchored by the Sertoli cells. (F) Day 42 (Adult). All cell types are present in the testis forming defined cellular associations between sub-types of spermatogonia, meiotic and post-meiotic cells, called 'stages'. This tubule at the right contains spermatogonia, pachytene spermatocytes, round

et al., 2007); and increased outward K^+ permeability and the activation of voltage gated and ligand gated Ca^{2+} channels (Darszon *et al.*, 2006; Darszon *et al.*, 2007; Bedu-Addo *et al.*, 2008). Each of the hallmarks of sperm capacitation occur concurrently and while they have been shown to have distinct signalling components (Marquez and Suarez, 2004), they may also be linked through the activation of the cAMP–PKA signalling pathways.

Fertilization

The ultimate function of a sperm is fertilization (Primakoff and Myles, 2007; Vjugina and Evans, 2008; Sutovsky, 2009). A typical ovulated mammalian oocyte is enveloped in two layers, the cumulus cells and the thick extracellular matrix, the zona pellucida. In order to reach the plasma membrane of the oocyte, the sperm must penetrate both layers. This requires capacitated and hyperactivated sperm, and sperm surface and secreted proteins (Primakoff and Myles, 2002). Sperm ultimately bind to the zona pellucida, which in the mouse is made up of the three glycoproteins ZP1, ZP2 and ZP3 that act as a barrier to prevent cross-species fertilization (Yanagimachi, 1994). Binding to the zona is irreversible and once bound a calcium-dependent signal transduction pathway is initiated and results in the exocytosis of the sperm acrosome, a process known as the acrosome reaction (Abou-Haila and Tulsiani, 2000). The acrosome reaction exposes hydrolytic enzymes that help the sperm tunnel through the zona pellucida. Sperm then bind to the oocyte through an, as yet, incompletely understood series of receptors or receptor complexes involving CD9 and IZUMO (Inoue *et al.*, 2005) and fuse with the oocyte. For reviews on our current state of knowledge of the processes of fertilization, please see (Sutovsky, 2009).

Methods

We searched for English articles using PubMed, with the last computerized search taking place on 24 June 2009. Search terms encompassed all aspects of spermatogenesis, post-testicular sperm maturation and knockout mouse models resulting in male infertility, including, but not limited to, spermatogenesis, testis, sperm, spermatozoa and epididymis. The computerized search was also supplemented by a manual search of relevant genes and knockout mouse models. Additional information on mouse

genes and mouse models was extracted from reviews including (Matzuk and Lamb, 2008; Naz *et al.*, 2009). Given the volume of literature obtained, review articles are cited for more general or less controversial topics.

Results

Breeding experiments

If a mouse line is suspected of having a male infertility phenotype, the first thing to do is formally test it using controlled breeding experiments. The ability to produce copulatory plugs should be monitored and used as an indication of appropriate mounting behaviour. Those lines that continually fail to display mounting behaviour should be assessed for behavioural or endocrine profile and sex reversal as outlined elsewhere (Crews *et al.*, 2004; Wilhelm *et al.*, 2007). As both male and female fertility levels can change with age, it is best to use animals (including a comparison to control animals) within a defined and narrow age range. Use approximately equal numbers of females per male and be mindful of sample bias which can cause problems in lines where there are large animal-to-animal variations in fertility. The number of pups/copulatory plugs should be recorded i.e. sterile animals produce 0 pups/plugs, whereas subfertile males will produce ≥ 1 . Wild-type by wild-type matings should be used as a control in strains where subfertility is suspected. If an age-related decline in fertility is suspected, the number of pups/plugs should be plotted as a function of age (Gold *et al.*, 2009). Female age should be kept constant.

Histology

Mice should be killed and a testis and epididymis processed for histology. Tissues should be weighed and the testis capsule nicked with sharp scissors prior to immersion in fixative. The epididymis should be gently laid onto a piece of filter paper which assures that the epididymis is flat, making it possible to visualize all segments on a single histological slice (Fig. 1B). Both should be fixed in Bouin's fixative, although the epididymis may be fixed in paraformaldehyde if more convenient. Testes should not be fixed in paraformaldehyde as it does not penetrate the tissue quickly enough, resulting in inferior

spermatids and elongated spermatids (E) prior to spermiation (release from the testis). G-R. Examples of abnormal testis phenotypes: PAS stained sections of mouse testes showing abnormal phenotypes. All testes were taken from adult mice. Scale bar = 100 μm . (G) A Wild-type testis, showing all cell types in correct numbers. (H) A Sertoli cell only (SCO) testis, no germ cells are present, and only Sertoli cells and their nuclei (SCN) are observed. (I) A meiosis arrest testis, showing an arrest at the pachytene stage of meiosis I (P). (J) TUNEL staining of a meiosis arrest testis, showing an abundance of dying (apoptotic) cells stained in brown (*). (K) A round spermatid arrest testis, no cells past the round spermatid stage (R) are present. Dying cells can be seen (D). (L) A hypospermatogenic testis, wherein there are normal tubule cross-sections alongside abnormal cross-sections, in this case showing germ cell arrest (GCA). (M) An adult wild-type epididymis, displaying an abundance of sperm (S) in the caudal epididymal region. (N) Sloughing of testicular cells into the epididymis, no mature sperm can be seen in the caudal epididymal region, however, a large number of round cells that have prematurely left the testis are observed (arrowheads). (O) A testis containing elongating spermatids with abnormal sperm head morphology, sperm that are abnormally shaped (arrowheads) when compared with the classical falciform (hook) shaped sperm heads seen in the wild-type testis (G). (P) seminiferous epithelium containing incorrectly juxtaposed cell types, disorientation of round spermatids (circled, inset). The pink stained developing acrosomes should be facing the same direction. (Q) Seminiferous epithelium containing an incorrect placement of cell types, round spermatids are seen adjacent to the basement membrane (circled), whereas they should be seen approximately halfway towards the lumen. (R) Seminiferous epithelium containing retained elongated spermatids (arrows) are being drawn to the basement membrane (spermiation failure).

morphology. This is particularly critical in the assessment of meiosis. Tissues should be processed into paraffin wax (or resin) and stained with periodic acid Schiff (PAS) reagent and haematoxylin (Wolman, 1950). PAS stains glycoproteins and is the stain of choice for visualizing acrosome development and, thus, the particular step of spermiogenesis (Russell, 1990). Control animals should always be processed in parallel in order to account for strain-to-strain variations in the efficiency of spermatogenesis. Researchers should look carefully at testis histology and systematically detail the presence, or absence, of all germ types and their morphological integrity in comparison with wild-type mice. For definitive details on how to do this, please see (Russell, et al. 1990a, b, c).

Where possible, testicular histology should be related to the equivalent human fertility analyses (McLachlan et al., 2007) (Table II). For example, are all germ cell types present (even if abnormally formed) and in qualitatively normal numbers, are there only Sertoli cells present, or does germ cell development arrest at a particular point? Phenotypic descriptors such as those outlined in Table II, which are commonly used in Assisted Reproductive Technology and semen laboratories, may facilitate the translation of mouse data into, and identification of, humans with similar mutations or perturbations.

The cycle of the seminiferous epithelium

Four or five generations of germ cells develop concurrently within the depth of the normal seminiferous epithelium, and because of the precisely regulated rate of transformation of one germ cell type into another, defined cellular associations between sub-types of spermatogonia, meiotic and post-meiotic cells can be recognized and predicted. Each association is called a 'stage' and is given a roman numeral description e.g. stage IX. Twelve stages occur in the mouse (Oakberg, 1956a, b; Gardner and Holyoke, 1964; Gardner, 1966; Russell et al., 1990a, b, c), 14 in the rat and 6 in humans (Clermont, 1963; Russell et al., 1990a, b, c). It is of note, that at first glance the

human testis histology appears chaotic in comparison with the regular and robust appearance of the mouse testis. This is due both to the relatively improved efficiency of rodent spermatogenesis, but also because in the human tracts of synchronized germ cell development are arranged in a spiral pattern running along the length of the seminiferous tubules, rather than as a wave (Clermont, 1963). At a histological level this means that all germ cells within a particular cross-section of a mouse tubule are at the same stage; whereas a cross-section of a human tubule contains several stages.

It takes ~34.5 days, for a single spermatogonium to differentiate into approximately 256 spermatozoa (Oakberg, 1956a, b). Importantly, from the mitotic divisions of spermatogonia through to spermiation, germ cells remain connected not only to their sister cells, but also preceding and succeeding generations via cytoplasmic bridges (Leblond and Clermont, 1952; Courot et al., 1970). Germ cells thus develop in a syncytium. When phenotyping a mouse testis at a light microscopic level, this is most easily evidenced through the apoptosis of strings of cells following an insult, or occasionally the collapse of several cells into multinucleate symplasts, as seen in the *Bclw* knockout mouse line (Print et al., 1998). At an electron microscopic level, it is possible to visualize cytoplasmic bridges and their presence at a light microscopic level can be marked using immunohistochemical markers such as TEX14 and HSF2 (Greenbaum et al., 2006).

Histological abnormalities

If an abnormality is present, the broad type of abnormality should be defined. A complete absence of germ cells is referred to as a Sertoli cell only epithelium (Fig. 3H). Relatively normal germ cell development up to a particular point, after which germ cells disappear, is called a germ cell arrest e.g. at meiosis (Fig. 3I) or round spermatids (Fig. 3K). Germ cells can disappear by sloughing, where they may be seen in the lumen of the epididymis (Fig. 3M versus 3N), and/or by apoptosis which can be marked using the TUNEL assay (Fig. 3J). Germ cell loss by necrosis is also possible, although rarely seen in genetically modified mouse models.

The presence of all germ cell types, but in reduced numbers, is referred to as hypospermatogenesis (Table II). This can manifest as either a global decrease in germ cell efficiency or the juxtaposition of relatively normal tubules next to those with germ cell arrest or loss of particular germ cell types (Fig. 3L). Such phenotypes may be reflective of the loss of a gene involved in multiple aspects of germ cell development or Sertoli cell function in the former instance, or germ cell colonization in the latter instance. If possible, an immunohistochemical analysis of native protein localization may be the most efficient way to narrow these possibilities. Gross abnormalities in sperm morphology can be seen in testis sections, e.g. abnormal sperm head morphology (Fig. 3O), but is more easily viewed using stained sperm smears or electron microscopy.

Occasionally, loss of a gene can result in a deregulation of the spermatogenic cycle and lead to an inappropriate juxtaposition of germ cell types (Fig. 3P), or their inappropriate placement within the depth of the epithelium (Fig. 3Q). In such instances, the staining of sections with PAS (or electron microscopy) is particularly helpful as it will allow a precise identification of germ cell sub-types. Similarly, several mouse models have resulted in a failure of sperm release (spermiation) (Fig. 3R). Generally, this is most apparent in stage IX tubules. In wild-type mouse spermatogenesis, sperm are rarely retained, meaning that

Table II. Types of male infertility in mice and men

Term	Histological feature
Sertoli cell only (syndrome)	No germ cells are present in the seminiferous tubule. Sertoli cells may be relatively mature as indicated by the presence of a tubule lumen, or may be immature as indicated by no lumen
Germ cell arrest	Spermatogenesis appears normal up to a particular phase of development after which germ cells are either sloughed off or die
Hypospermatogenesis	All germ cell types within spermatogenesis are present, but at least one appears at a reduced frequency within tubules or between tubules. Hypospermatogenesis includes the close juxtaposition of 'normal' tubules and tubules missing germ cell populations
Oligospermia	Mice produced reduced numbers of sperm
Azoospermia	Mice produce no sperm, i.e. the epididymis contains no sperm
Teratospermia	Sperm are produced but are abnormally shaped
Asthenospermia	Sperm display abnormal motility patterns

at stage IX the only germ cell types that are seen are type A spermatogonia, leptotene spermatocytes, pachytene spermatocytes and step 9 elongating spermatids (moving from the basement membrane towards the lumen). Retained spermatids will be seen as highly condensed nuclei at either the lumen interface or in the process of being drawn down towards the Sertoli cell nucleus prior to phagocytosis (Fig. 3R). Spermiation failure can be indicative of changes in hormonal stimuli and/or the dissolution of components of the ES.

The graphing of testis weights and an examination of testis histology during post-natal development can also indicate the onset of fertility defects, and thus, the cell or process of origin. For example, an analysis of several mouse models has revealed that the first wave of spermatogenesis proceeded further through spermatogenesis than subsequent waves (Print *et al.*, 1998; Chen *et al.*, 2005; Webster *et al.*, 2005).

When male mice are born, the seminiferous tubules should contain only gonocytes and immature Sertoli cells (Fig. 3A). The tubules have no lumen and should be surrounded by fetal Leydig cells, but no testicular macrophages which develop between days 14 and 28 (Li *et al.*, 1998). The gonocytes sit in the centre of the tubules and are surrounded by immature Sertoli cells (Fig. 3A). Between 1.5 and 5 days post-natal the gonocytes migrate to the basement membrane of the seminiferous tubule, after which they are termed spermatogonia (Nagano *et al.*, 2000) (Fig. 3B). In the mouse, spermatogonia enter spermatogenesis almost immediately (Table I). Delays in the establishment of spermatogenesis have been discovered in several mouse lines, including the POG insertional mutation (*FanCL* knockout) mouse line (Lu and Bishop, 2003), using a comparative histology approach.

Analysis of meiosis arrest phenotypes

Correct progression through meiosis is crucial for the production of viable gametes and a failure to accurately complete meiosis results in either the absence of sperm, or the production of abnormal gametes (aneuploidy) (Ashley, 2004; Hall *et al.*, 2006). A good way to identify when meiosis abnormalities are arising is the immunohistochemical analysis of meiotic chromosome spreads using markers that are present at different periods, particularly during prophase I (where most arrests seem to occur).

Leptotene is the first period of meiotic prophase I and is similar to mitotic prophase. Leptotene begins with the condensation of the chromosomal axes into visible, thread-like chromosomes. During this period, the sister (or homologous) chromosomes are largely unpaired, but seek one another out. The initiation of synaptonemal complex (SC) formation begins during leptotene with the appearance of the central element, which is a single proteinaceous core that holds the homologues/sister chromatids of each chromosome together (synapsis) (Heyting, 1996). REC8 is frequently used as a marker of the axial elements and SCP3 as a marker of SC formation. SCP3 staining is present from the beginning of SC assembly and remains until the SC is disassembled in diplotene, however, the staining pattern observed is reflective of the extent of SC formation between the different periods of meiosis (Kuroda *et al.*, 2000). Mouse models with defects at the leptotene stage include: the platelet-activating factor acetylhydrolase 1b alpha 1 and alpha 2 double knockout mouse line (Yan *et al.*, 2003), and the *Msh5* (de Vries *et al.*, 1999) and *Spo11* knockout mouse lines (Romanienko and Camerini-Otero,

2000). For a review of the consequences of a failure in synapsis, readers are referred to (Burgoyne *et al.*, 2009).

During meiosis, the sister chromatids exist as chromatin loops which are connected to the axial element (referred to as the lateral element in mature SC) by a number of regularly spaced transverse filaments (TFs) (Roeder, 1997). The single TFs that span the distance between the lateral elements carry two symmetrically placed thickenings called pillars at fixed positions (Heyting, 1996). Recent analyses of TF protein knockout models in yeast (*ZIP1*), *Drosophila* (*C(3)G*) and mice (*SYCP1*) suggest that the TFs are involved in recombination (the exchange of genetic information between homologous chromosomes) (Sym *et al.*, 1993; Page and Hawley, 2001; de Vries *et al.*, 2005).

During the zygotene phase of meiosis, homologous chromosomes start to pair and the SC becomes more obvious. Mouse models with defects during zygotene include the *Dmcl* (Yoshida *et al.*, 1998) and the *Dnmt3L* knockout lines (Webster *et al.*, 2005).

An arrest during the pachytene phase of prophase I may indicate a defect in SC formation, crossing-over, mismatch-repair or recombination. Specifically, during the pachytene period of meiosis I, sister chromosomes become fully synapsed with the completion of the SC and chromosome compaction (Fig. 2A, d). The formation of the SC is accompanied by the appearance of electron dense structures known as recombination nodules (RNs), which are expected to be involved in homology searches and later mark sites of crossing-over (Heyting, 1996). Early RNs become apparent during the zygotene period. The late nodules are fewer and mark the sites of recombination, the chiasma (Carpenter, 1994). Meiotic recombination is initiated by programmed DNA double-strand break formation, which can be visualized using markers including *DMC1*, *RAD51* and γ H2AX (Masson and West, 2001). Repair occurs through the use of the unbroken homologous chromosome as a template and results in the formation of chiasma, which holds together the homologous chromosome and prevents mis-segregation (Petronczki *et al.*, 2003). At least one recombination must take place between each homologous chromosomal pair (Mather, 1936; Anderson *et al.*, 1999) and sites of crossover can be stained with *MLH1* (de Boer *et al.*, 2007). In reality, multiple recombination events occur in most chromosomes. Recently, several mouse models with decreased numbers of recombination sites per chromosome have been reported (e.g. the *Tex15* and *Ews* knockout lines) (Li *et al.*, 2007; Yang *et al.*, 2008).

At the diplotene stage, the chromosomes begin to separate as the SC disintegrates, but are held together by the sites of recombination (Fig. 2A). The chromosome pairs remain in contact with one another, disentangle but do not yet move to separate poles. At this point prophase has ended.

At metaphase the association between homologous chromosomes is resolved and the chromosomes migrate to opposite poles with sister chromatids still attached. At anaphase, the chromosomes segregate/separate. The decision to separate is governed by the spindle checkpoint which restrains cells from entering anaphase until all chromosomes form proper attachment to a functional bipolar spindle (Hoyt, 2001). Telophase sees the cytoplasm begin to divide and give rise to two daughter cells. The second division of meiosis closely resembles mitosis, with the separation and segregation of sister chromatids to create haploid gametes. As a consequence, each spermatocyte divides to produce four spermatids, each with

IN chromosome content. Defects at metaphase can be informatively analyzed using electron microscopy or staining sections with a spindle marker e.g. β -tubulin, and SCP3 plus a chromatin stain such as DAPI. Examples of mouse models with an arrest at metaphase include the *Siah1a* (Dickins et al., 2002), *Cks2* (Spruck et al., 2003) and *Mlh1* knockout mice (Eaker et al., 2002).

In addition to the pairing of autosomes during pachytene of meiosis I, the regions of the sex chromosomes not involved in pairing form the XY body (also known as a sex body) and become transcriptionally silenced (Khalil and Wahlestedt, 2008; Zamudio et al., 2008). This is referred to as meiotic sex chromosome inactivation (MSCI). The full list and exact order of events leading up to XY body formation is unclear, though several epigenetic modifications of histone are known to occur. An early event in sex chromosome inactivation is the phosphorylation of H2AX. Knocking out *H2ax* function results in the sex chromosomes being transcriptionally active during pachytene, in a developmental arrest and ultimately germ cell death (Fernandez-Capetillo et al., 2003). Antibodies directed against H2AX are good markers for XY bodies on chromosome spreads (Chicheportiche et al., 2007). Therefore, based on published information, if a failure of MSCI is suspected, it may be informative to undertake a comparative analysis of histone modifications using immunocytochemistry or of X–Y gene expression using microarrays on purified germ cell populations.

Defects in spermiogenesis

Sperm head shaping: acrosome formation and nuclear condensation

The shape of a sperm head is species-specific and in the mouse is falconiform (sickle-shaped) and formed through the coordinated action of a number of processes both intrinsic to the germ cell and extrinsic via the Sertoli cell including: the formation of the acrosome; the condensation and elongation of the nucleus; and Sertoli cell lipid metabolism. There are still many aspects of these processes that remain to be discovered, but a body of literature is beginning to form a picture wherein the structure of the sperm head is far more complicated than its small size indicates. Abnormalities in sperm head shaping result in teratozoospermia, including the specific disorder globozoospermia, whereby the sperm acrosome is missing.

Acrosome formation

Simplistically, the acrosome is a bag of enzymes which sits at the anterior pole of the sperm head (Olson et al., 2003; Yoshinaga and Toshimori, 2003) (Fig. 1C). The acrosome contains the enzymes necessary for the sperm to penetrate the surrounding layers of the oocyte. The formation of the acrosome begins with the production of proacrosomal granules from the Golgi apparatus (reviewed in (Kerr et al., 2006a, b)). The granules translocate to what will become the anterior pole of the sperm head and fuse to become the acrosomal vesicle. Later in spermiogenesis, the acrosome also elongates (see below).

The acrosome appears to become attached to the nuclear membrane via the perinuclear theca (Toshimori and Ito, 2003). In the mature sperm head, the perinuclear theca can be visualized as a thin layer that sits between the nuclear and acrosomal membranes. The perinuclear theca contains both a cytoskeletal component and soluble factors, which are thought, among other things, to be involved

in oocyte activation (oocyte activation factor) (Kimura et al., 1998) and the activation of zygote transcription post-fertilization (STAT4) (Herrada and Wolgemuth, 1997). The attachment of the acrosome appears to involve proteins including SUBH2BV (Aul and Oko, 2001). Abnormalities in proacrosomal granule fusion, as in the *Zbbp1* mouse (Lin et al., 2007), or acrosome attachment to the underlying perinuclear theca and nucleus, as in the *Hrb* (Kang-Decker et al., 2001), *Gopc* (Yao et al., 2002) and *Pick1* knockouts (Xiao et al., 2009), result in globozoospermia (Dam et al., 2007). Defects in acrosome formation can be detected using a combination of PAS staining of testis section, and transmission electron microscopy on testis and sperm samples.

Head elongation

The mechanisms responsible for the elongation of the sperm head are not fully understood, but are believed to involve the coordination actions of DNA condensation (see below), the perinuclear theca, the acroplaxome and the manchette. Simplistically, it appears that the DNA of the head condenses and then is progressively elongated and reshaped, and extends the acrosome in the process. In the mouse, head elongation occurs in the latter half of spermiogenesis (from step 9) and gives rise to elongating spermatids (step 9–12) and elongated spermatids (step 13–16) (Russell et al., 1991).

Our current state of knowledge of the role of the acroplaxome and the manchette in nuclear elongation can be found in (Kierszenbaum, 2001; Kierszenbaum, 2002; Kierszenbaum et al., 2003; Toshimori and Ito, 2003). Abnormalities of the manchette result in teratozoospermia, such as that seen in the *Azh* (Meistrich et al., 1990; Russell et al., 1991) and *Krt9* mutant mouse lines (Rivkin et al., 2005). Defects in manchette formation can be most easily discerned using electron microscopy and staining for α tubulin or using phalloidin.

Nuclear condensation

The reshaping of the head (and acrosome) and nuclear condensation occur in parallel. During spermiogenesis, the size of the spermatid head decreases to ~5% of the size of a somatic cell nucleus (Brewer et al., 1999). This compaction occurs through dramatic changes in the way the DNA is packed and falls under the broad banner of epigenetic changes in that it results in changes in chromatid structure that affect transcription. The initial stage of chromatin reorganization is the remodelling of the chromatin from a nucleosomal form to a 'thread-like filamentous' form. The chromatin filaments thicken and become coarse forming a dense chromatin mass which eventually further condenses to form a homogeneous chromatin mass (Okamoto and Clermont, 1999). Nuclear condensation occurs by the removal of the major nuclear proteins, the histones, and their replacement initially with transition proteins, then subsequently protamines. Protamines are small, basic proteins. Mice and humans have two protamines (PRM1 and PRM2), whereas most other mammals have only one. Targeted disruption of either mouse *Prm1* or *Prm2*, in a heterozygous state resulted in sterility (Cho et al., 2001). Similarly, data from humans primarily suggest that the ratio of PRM1 to PRM2 is strongly associated with male infertility (Torregrosa et al., 2006).

While the incorporation of the transition proteins and protamines is the most overt change occurring during the re-packing of the spermatid DNA, there are other more subtle changes in histone composition and their post-translational modifications, including the

hyperacetylation of histone H4. For more details please see (Doe-necke *et al.*, 1997; Govin *et al.*, 2004; Zamudio *et al.*, 2008). If defects are suspected in a mouse line, it may be informative to assess sperm for the retention of transition proteins similar to that of the *Camk4* knockout line (Wu *et al.*, 2000).

Sertoli cell involvement

Recent data from both the human and the mouse suggest that aspects of sperm head formation are influenced by the Sertoli cell. It appears that lipid metabolism via the endoplasmic reticulum (ER) is critically important for acrosome formation and attachment. The ablation of the ER protein β -glucosidase 2 (GBA2) in mice results in the accumulation of lipid in the ER of Sertoli cells and a resultant globozoospermia phenotype (Yildiz *et al.*, 2006). A proposed mechanism of action for this pathway is presented in (Roy *et al.*, 2006).

Of relevance to sperm shaping, elongating and elongated spermatids interact with the Sertoli cells via a unique cytoskeletal structure called the ES which is thought to play a role in the positioning and the elongation of the spermatids (Beardsley and O'Donnell, 2003; Mruk and Cheng, 2004; Yan *et al.*, 2007; Wong *et al.*, 2008). Mature sperm are released from the seminiferous epithelium by a series of events collectively known as spermiation. Just before spermiation, the apical ES is partially replaced by another modified adherens junction, the apical tubulobulbar complex (TBC) which is located on the concave surface of the spermatid head (Russell, 1979a, b). These specialized structures are also thought to be critical in removing excess cytoplasm from germ cells prior to spermiation, i.e. the volume of the spermatid is reduced to ~25% of its initial volume (Sprando and Russell, 1987). This occurs via three processes: (i) the loss of water during elongation (Sprando and Russell, 1987); (ii) the elimination of cytoplasm by TBCs (Russell and Clermont, 1976; Russell, 1979a,b; Russell, 1980); and (iii) the formation of the residual body, which is engulfed and destroyed by the Sertoli cells. Residual bodies contain excess cytoplasm, packed RNA and organelles (Fawcett and Phillips, 1969). Within normal mouse spermatogenesis, residual bodies are most clearly visible at stage IX, where they can be seen as basophilic vesicles being drawn down towards the basement membrane by Sertoli cells.

If a defect in attachment between Sertoli and germ cells is suspected, for example, mice showing either the abnormal sperm retention (spermiation failure) or premature sloughing of germ cells, or producing sperm containing excessive cytoplasm, the structure of the ES and associated structures may be analyzed using electron microscopy or using ES markers e.g. Espin. It is of note that in several species, including humans and rats, spermiation failure is also an indication of hormonal perturbations and as such a comparison of testosterone and LH levels may be of value (Saito *et al.*, 2000; Beardsley and O'Donnell, 2003; D'Souza *et al.*, 2009). Mouse models with abnormal spermiation include the *Sox8* knockout (O'Bryan *et al.*, 2008) and the repro32 point mutant mouse (Geyer *et al.*, 2009).

Sperm tail development and structure

Defects in sperm tail development have been reported in many mouse models. For a detailed description of sperm tail formation and the function of the accessory structures, readers are referred to (Oko, 1998; Eddy *et al.*, 2003; Kerr *et al.*, 2006a,b). Briefly the axoneme,

which is the 'drive shaft' of the tail, is a microtubular structure. It is composed of a 9+2 microtubular structure which is common to motile cilia/flagella throughout the body and many species (Satir and Christensen, 2007). Unlike cilia in other tissues, however, the sperm tail contains several accessory structures: the mitochondrial sheath (midpiece), the outer dense fiber (ODF) (mid- and principal pieces); and the fibrous sheath (the principal piece) (Fig. 1C). Both the ODF and fibrous sheath provide structural rigidity to the tail and protect against shearing forces e.g. during ejaculation and motility (Baltz *et al.*, 1990). The ODF is also suspected of having an active role in the regulation of tail motility (O'Bryan *et al.*, 1998; Nakamura *et al.*, 1999) and the fibrous sheath is integrally involved in the generation of ATP by glycolysis for microtubule sliding and motility of the axoneme as evidenced by the *Gapd2* knockout mouse line (Eddy *et al.*, 2003; Miki *et al.*, 2004). The fibrous sheath is also a scaffold for signal transduction molecules/cascades which regulate sperm motility and/or capacitation, as evidenced by the *Pka* and *Rho* knockout lines (Moos *et al.*, 1998; Nakamura *et al.*, 1999; Eddy *et al.*, 2003).

External to the ODF, and in the midpiece of the sperm tail, is the mitochondrial sheath. It provides at least a proportion of the ATP required for microtubule sliding within the axoneme and, thus, sperm motility. For a review of the origin and importance of ATP for sperm motility in different species, please see the review by (Ford, 2006). At the distal end of the mitochondrial sheath is the annulus (Fig. 1C).

As evidenced in many of the references listed above, a mouse line with defects in sperm tail development or motility produce a phenotype equivalent to teratospermia and or asthenospermia in humans (Table II). Methods to analyze such defects could include an electron microscopic assessment of axoneme and/or accessory structures. See below for methods on how to analyze sperm motility. An assessment of the axoneme structure is particularly informative if the line is suspected of having primary cilia dyskinesia as in the *Tek2* (Tanaka *et al.*, 2004), *Dnah5* (Olbrich *et al.*, 2002) and *Mdhc7* knockout mice (Neesen *et al.*, 2001) and mice homozygous for the nml054 mutation (PCDPI loss of function) (Lee *et al.*, 2008) mouse lines, in which case an assessment of cilia function in other tissues would be relevant (Badano *et al.*, 2006; Zariwala *et al.*, 2007). For a recent review on mouse models with abnormal sperm tail formation please see (Escalier, 2006).

Hormone analysis

As in humans, maintaining balanced levels of key reproductive hormones (e.g. testosterone, FSH, inhibin, activin A and LH) is critical for normal fertility. During prenatal development in the mouse, FSH is a critical regulator of Sertoli cell proliferation. FSH also regulates Sertoli cell function during later development. As such, aberrant FSH stimulation in the peri-natal period can result in decreased (or increased) adult Sertoli cell numbers and as a consequence can affect sperm output (Rich and de Kretser, 1977; O'Donnell *et al.*, 2006). Inhibin is involved in regulating FSH via a negative feedback loop. Inhibin is principally secreted by the Sertoli cells and, as such, alterations in the levels of this hormone are indicative of Sertoli cell number, function and spermatogenic failure (Rich and de Kretser, 1977; Kennedy *et al.*, 2005).

Similarly, serum levels of LH and testosterone can provide insights into causes of infertility. LH is produced solely by the Leydig cells,

and while it is not absolutely required for the differentiation and function of foetal Leydig cells, post-natal steroidogenesis relies on LH signalling. Thus, a reduction in LH levels will have an effect on the efficiency of spermatogenesis by reducing the amount of testosterone in the testis. During development, reduced LH may impede the initiation of spermatogenesis and in the adult may compromise daily germ cell production or epididymal function; for example, see (Ma et al., 2004). Similarly, a change in the LH to testosterone ratio may be indicative of altered receptor function or steroid synthesis. As indicated in the hormonal control of spermatogenesis section, even an absence of LH (or signalling via its receptor) is unlikely to result in a frank absence of germ cell types (Ma et al., 2004). It is of note, however, that the measurement of testosterone in the mouse is notoriously unreliable and large numbers of animals are often required to form a consensus on any variation from normal levels. Examples of mouse models with infertility as a consequence of perturbed endocrine signalling include: a variety of androgen receptor inactivation models (reviewed in (Wang et al., 2009)); the LH and its receptor (Ma et al., 2004; Huhtaniemi et al., 2006); and FSH and its receptor (Abel et al., 2000; Abel et al., 2008).

Spermatogenic efficiency

Occasionally genetic modifications result in qualitatively normal, but quantitatively abnormal spermatogenesis. This is termed 'hypospermatogenesis' and the mice usually have reduced testis weights. The equivalent phenotype in humans would result in an oligospermic semen sample (Table II). If germ cells are 'disappearing' late in haploid germ cell development, however, the difference in testis weights between wild-type and mutant mice may not be statistically significant. Such mice may be sterile, subfertile or have normal fertility. In order to quantify the absolute loss of sperm production, daily sperm production can be calculated (Robb et al., 1978; Cotton et al., 2006). This is an easy technique and does not require the use of specialized equipment. It relies upon the resistance of elongated spermatid heads to detergent solubilization. If mice have a phenotype resulting in inappropriate spermatid head condensation or spermiation failure, this technique may not, however, indicate the true magnitude of the abnormality. In the latter instance, the calculation of epididymal sperm content may be more indicative of the germ cell loss.

If a precise identification of where germ cells are being lost is required, then a stereological approach will be more appropriate. This technique relies upon an automated random sampling (thus avoiding bias) of testis sections and the precise identification of germ cell types. By comparing absolute germ cell numbers and ratios, e.g. Sertoli cells to particular germ cell numbers, it is possible to identify where germ cells are being lost (Robertson et al., 1999; Wreford et al., 2001; Myers et al., 2004).

Analyzing post-testicular sperm maturation and fertilizing ability

The presence of relatively normal testis histology, sperm morphology and count, but sterility or subfertility in a mouse line can be indicative of a post-testicular maturation defect. This type of infertility falls into two main categories: those caused by defects in epididymal formation and fluid resorption; and those that affect sperm function once they enter the female reproductive tract.

The latter are crudely equivalent to defects in epididymal sperm maturation and capacitation and can be further subdivided by the ability / inability of sperm to manifest the hallmarks of capacitation including motility and subsequently hyperactivated motility, the ability to undergo increased global tyrosine phosphorylation, the ability to undergo the acrosome reaction and the ability to penetrate the outer vestments of the oocytes and fuse with oocyte. As with all of the analyses described in this review, the precise biochemical pathway perturbed in each type of infertility may require an analysis of specific biochemical pathways and will depend upon what is known about the function of the gene involved.

A note of caution for researchers who have not worked with sperm before: mouse sperm are extremely fragile cells. They are easily damaged by shearing forces, e.g. pipetting or high G forces, UV light, and their function is critically dependent upon media composition, e.g. salt concentration, albumin quality and osmolarity. To ensure cell viability is not affected, researchers should use wide bore pipette tips and should not exceed 400 g when centrifuging. It is essential that during collection of sperm from the cauda epididymis the capillary, or all of the blood from the capillary running the length of the epididymis, is removed. Components of blood will dramatically inhibit sperm capacitation *in vitro*.

Defects in epididymal development and fluid resorption

At the early stages of epididymal maturation, the efferent ducts and initial segment of the epididymis reabsorbs the vast majority of the fluid that arrives via testicular secretions (Clulow et al., 1998; Dacheux et al., 2003; Da Silva et al., 2006). The result of fluid resorption is to dramatically increase the sperm concentration in the epididymal tubule and is mediated at least in part by aquaporins, sex steroids and cAMP-mediated signal cascades. Defects in these processes, as evidenced by the G-protein coupled receptor *Gpr64* knockout, can lead to tubule blockages and infertility (Davies et al., 2004). Another example of a defective fluid regulation leading to male infertility, but this time in an age-dependent manner, is the estrogen receptor alpha knockout mouse (Hess et al., 1997).

Many, or even the majority, of the mechanisms of epididymal development are unknown, however, a few mouse models have clearly shown that a disruption to epididymal regionalization can result in male infertility. For example, *Ros1* deficient males show abnormal epididymal epithelial differentiation, aberrant regionalization and infertility *in vivo* (Sonnenberg-Riethmacher et al., 1996; Yeung et al., 1999). Sperm from these mice could, however, fertilize normally *in vitro*, suggesting in addition to a physical abnormality in epididymal structure, the loss of ROS1, a receptor tyrosine kinase, resulted in a loss of, or defect in, at least one protein with a critical role in capacitation (Nixon et al., 2006).

Defects in post-testicular maturation and infertility

As outlined in previous sections, epididymal sperm maturation encompasses a wide, and still poorly understood, series of events that are thought to have key roles in male fertility, but their importance does not become apparent until sometime later in the female reproductive tract during capacitation. Many of the processes occurring during capacitation can be recapitulated in chemically defined solutions, e.g. Biggers, Whitten and Whittingham medium (Biggers et al., 1971) and modified tyrodes (mT6) medium (Fleming et al.,

1986) thus facilitating a dissection of the signalling cascades within sperm. For the sake of simplicity, herein we describe only the analysis of mice with defects in motility, signal transduction mechanisms as evidenced in the ability to manifest changes in global tyrosine phosphorylation and the ability bind and fuse with an oocyte. For the analysis of mice with defects in early fertilization events readers are referred to the following reviews (Kurokawa *et al.*, 2004; Whitaker, 2006; Sutovsky, 2009).

Sperm motility

Upon entering the female reproductive tract (or physiological media), sperm begin to manifest a model form of motility. Subsequently, they undergo a series of biochemical events and start to show hyperactivated motility. Obviously, an inability to produce any motility (asthenospermia), or sluggish motility, will result in infertility and has been reported in several mouse models, including models of primary cilia dyskinesia (see above). The inability to manifest hyperactivated motility is more difficult to detect, but equally important. Hyperactivation is critically regulated by Ca^{2+} flow (Suarez *et al.*, 1993; Ho and Suarez, 2001a,b; Ho *et al.*, 2002) and is triggered by the release of Ca^{2+} from intracellular stores (Marquez and Suarez, 2007; Marquez *et al.*, 2007) leading to Ca^{2+} influx via the activation of Catsper ion channels as evidenced by each of the Catsper 1 to 4 knockout models (Ren *et al.*, 2001). This subsequently leads to cyclic changes in intracellular Ca^{2+} , which correlate with flagellar activity (Harper *et al.*, 2004). Many of the upstream signalling cascades leading to hyperactivation remain to be determined, but appear to be linked to PKA signalling. Another example of a mouse model with a failure of hyperactivation is the *Pmca4* knockout mice (Okunade *et al.*, 2004; Schuh *et al.*, 2004). As evidenced in the above references, the dynamic imaging of Ca^{2+} is highly informative in the analysis of hyperactivated mutants. This does however, require specialized equipment and a high level of training. A preliminary analysis of sperm motion characteristics over time, however, can be informative before undertaking more complex analyses.

To analyze motility, sperm are typically diluted in a capacitation permissive medium to between 10^6 and 10^7 per mL, with dilution being important for removing the effect of decapacitation factors. In an initial assessment of sperm motility it should be noted whether sperm show any form of motility. Do they move forward, and after 60 min (and increasing up to 120 min) are there increased amounts of hyperactivated motility? Hyperactivated motility can be easily recognized as a frenetic form of motility characterized by exaggerated asymmetric flagellar beating which often results in a compact figure of eight motility pattern. Sperm motility is best viewed in a droplet on a microscope slide or in a 80–100 μm deep well chamber, rather than under a cover slip, as the weight of the cover slip can affect motility.

A more precise definition of motility problems can be obtained using a computer-assisted sperm analyzer (CASA). Using software specifically written for mouse sperm, this instrument can quantify multiple aspects of motility, e.g. percentage total and progressive motility, velocities, motion tracts (as an indication of modal versus hyperactivated motility), beat frequencies and the magnitude of head displacement. An analysis of parameters over a time course, e.g. 0–2 h, may be helpful in pinpointing defects in sperm motility. Motility parameters as measured by CASA should also be verified visually to ensure the

instrument parameters reflect what is observed. This is particularly critical when measuring mouse sperm hyperactivation as the standard settings on several CASAs lack sensitivity (Miki *et al.*, 2004; O'Bryan *et al.*, 2008).

Defects in signal transduction mechanisms

Another hallmark of sperm capacitation is a pronounced increase in the tyrosine phosphorylation of many proteins. This occurs predominantly in the sperm tail, but to a lesser extent in the sperm head. Assessing the time-dependent increase in global tyrosine phosphorylation can be a useful prelude to a more precise analysis of a particular signal transduction cascade. In order to do this, sperm should be incubated in capacitation permissive conditions and samples taken at regular intervals. Total sperm proteins should be size fractionated by SDS–PAGE, alongside wild-type control sperm, and probed for tyrosine phosphorylated proteins on a western blot using antibodies such as 4G10 (Visconti *et al.*, 1995a, b). Normal capacitation is exemplified by a progressive increase in the number and intensity of phosphorylated proteins. Conveniently, a germ cell specific hexokinase (molecular weight of 116 kD) is abundant and constitutively phosphorylated in sperm and can act as an internal loading control. At a cytological level on individual sperm, tyrosine phosphorylation appears first in the principal piece and then along the midpiece as capacitation proceeds (Aitken *et al.*, 1995, 2006; Jha *et al.*, 2008). Examples of mouse lines with abnormal capacitation include the following: the FGFR dominant negative mouse (Cotton *et al.*, 2006), the *Rkip1* knockout (Moffitt *et al.*, 2007) and the soluble adenylyl cyclase (sAC) mouse (Hess *et al.*, 2005).

Zona pellucida binding and the acrosome reaction

The ability of sperm to bind the zona pellucida and undergo the acrosome reaction is a critically important end-point of epididymal maturation and capacitation. In order for this to occur *in vivo*, sperm must be motile and reach the oocyte, and they must express the sperm receptor on the apical surface of the head and have 'primed' the signalling cascades to permit the zona pellucida induce the acrosome reaction. Within the laboratory this can be partially recapitulated using IVF techniques. IVF can, however, by-pass several aspects of sperm function that are important for *in vivo* fertility, e.g. the hyperactivated motility. In order to test the ability of a genetically modified mouse to undergo the acrosome reaction and to present the appropriate array of sperm–oocyte binding proteins, IVF can be extremely valuable (Harrison, 1996). An absence of these processes results in sperm that fail to bind and effectively 'bounce off' the zona, such as in the calmegin knockout line (Ikawa *et al.*, 2001) and the fertilin beta knockout line (Cho *et al.*, 1998). A failure of zona binding is also a common manifestation of human infertility (Liu and Baker, 2000).

The acrosome reaction is an endocytotic event, stimulated by binding the zona pellucida protein ZP3, that results in the release of proteases and digestion of the zona pellucida. A failure of the acrosome reaction results in sperm binding to the zona pellucida, but a failure to reach the perivitelline space. The source of an acrosome defect may be during spermiogenesis, inappropriate epididymal maturation or capacitation and may be hinted at by the sites of expression. In order to analyze a mouse line with a suspected acrosome defect, the presence of an appropriately formed acrosome should be assessed

using a combination of PAS stained testis section, electron microscopy and fluorescent-PNA labelling of epididymal sperm. If an acrosome is present, it may be informative to assess the ability of sperm to undergo the acrosome reaction in response to stimuli such as progesterone (Nixon *et al.*, 2006). There are a few cases of a specific failure of the acrosome reaction in mouse sperm, without other defects, however; they include the slowly progressive hyh mouse which has a point mutation in the α SNAP protein (Batiz *et al.*, 2009) and the PLCdelta4^{-/-} mouse line (Fukami *et al.*, 2001). Importantly, the human ortholog of α SNAP is a known participant in the human acrosome reaction (Tomes *et al.*, 2005).

Oocyte binding and fusion

The last major event for a sperm is its fusion with an oocyte. A failure at this stage results in sperm entering the perivitelline space and either failing to bind to the oocyte or failing to trigger uptake by the oocyte. The Izumo knockout animal and the CD9 knockout animal have provided tantalizing insight into this key molecular event (Miyado *et al.*, 2000) (Ikawa *et al.*, 2008). The Izumo phenotype was characterized through IVF experiments and through assessment of sperm fusion to zona pellucida-free oocytes.

Conclusion

As evidenced in many publications, the manifestation of male infertility in genetically modified mice is often unexpected. Unfortunately, such mice are often poorly analyzed. The identification of infertile lines is likely to increase as a consequence of international efforts to inactivate every gene in the mouse genome (Nord *et al.*, 2006; Collins *et al.*, 2007). This and the fact that the processes inherent in developing a functional male gamete can be viewed as a dynamic recapitulation of many processes occurring in somatic tissues, and in disease, we view a case of poor analysis as a lost opportunity. Spermatogenesis, for example, involves the continual division of stem cells, cell divisions by both mitosis and meiosis and dramatic morphological processes. It is critically dependent on all of transcription, translation and post-translational modifications. It is also exquisitely sensitive to environmental pollutants and changes in the epigenetic state. We hope that herein we have provided a path by which the non-expert researcher can tackle the challenges of 'what is wrong with the fertility of my mouse' and that in doing so, their research may shed light not only on the causes of the high incidence of human infertility, but also other disease processes.

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