



From cyst to tubule: innovations in vertebrate spermatogenesis

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Although vertebrates share many common traits, their germline development and function exhibit significant divergence. In particular, this article focuses on their spermatogenesis. The fundamental elements that constitute vertebrate spermatogenesis and the evolutionary changes that occurred upon transition from water to land will be discussed. The life-long continuity of spermatogenesis is supported by the function of stem cells. Series of mitotic and meiotic germ cell divisions are 'incomplete' due to incomplete cytokinesis, forming syncytia interconnected via intercellular bridges (ICBs). Throughout this process, germ cells are supported by appropriate microenvironments established primarily by somatic Sertoli cells. In anamniotes (fish and amphibians) spermatogenesis progresses in cysts, in which developing germ cell syncytia are individually encapsulated by Sertoli cells. Accordingly, Sertoli cells undergo turnover with germ cells that they nourish. This mode of cystic spermatogenesis is also observed in nonvertebrates as insects. In amniotes (reptiles, birds, and mammals), however, Sertoli cells do not turn over but comprise a persistent structure of seminiferous tubules. Sertoli cells nourish different stages of germ cells simultaneously in distinct regions of their surface. This function of Sertoli cells is spatiotemporally orchestrated, and the seminiferous epithelial cycle and spermatogenic wave make the seminiferous tubules a high-throughput factory for sperm production. Furthermore, contrary to the organized differentiating cells, undifferentiated spermatogonia that comprise the stem cell compartment exhibit active motion over the basal layer of seminiferous tubules and the frequent breakdown of ICBs. Thus, amniote seminiferous tubules represent a typical facultative (or open) niche environment without a stem cell tethering anatomically defined niche. © 2015 The Authors. *WIREs Developmental Biology* published by Wiley Periodicals, Inc.

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INTRODUCTION

This article from the *WIREs Developmental Biology* review series describes the *male germline in vertebrates*. Vertebrates include fish, amphibians, reptiles, birds, and mammals, which share a number of properties such as vertebrae (a backbone), a closed circulatory

system with red blood, and a central nervous system containing the brain and spinal cord encased in bone. In contrast to the high similarity in the development and function of these common traits, a large diversity is found in the germline among vertebrate species.

This article will begin with a brief overview of both the common and divergent features of vertebrate germline development. Then, the main emphasis will be on the process of spermatogenesis in the adult testes, which highlights dramatic evolutionary innovations that occurred during the history of vertebrates. The transition of vertebrates to land was accompanied by important changes in their reproductive strategy. This

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is often described with respect to the egg and embryogenesis. The invention of watertight eggs and embryonic membranes, which eventually led to the evolution of placenta, characterize the group known as *amniotes* (including reptiles, birds, and mammals). However, the testicular anatomy and the process of spermatogenesis also clearly differentiate amniotes from *anamniotes* (fish and amphibians).

Figure 1 illustrates the common pathway of vertebrate germline development. Primordial germ cells (PGCs) are born outside of the gonads, which develop from a portion of the intermediate mesoderm. The divergent process of PGC establishment has drawn particular interest.¹ PGCs translocate into the gonads through active migration in the developing embryo in many species, while they reach the gonads via the blood stream in birds and some reptiles.^{1,2} PGCs determine their sex under a strong induction signal from the sexually differentiated somatic gonadal cells, although PGCs may show cell-autonomous sexual differences before reaching the gonads.³ When PGCs enter the

female pathway in embryonic ovaries, they initiate meiosis in earlier stages than in males. Male germ cells continue to mitotically proliferate for an extended period.

In the developing testis, the mitotic germ cells become the foundation for long-lasting spermatogenesis. In general, the entire process of spermatogenesis is established during the period of sexual maturation (puberty), wherein *stem cells* also appear to be established. The ontogeny of stem cells is interesting but still largely remains to be elucidated. In mice, like *Drosophila*, the first round of spermatogenesis bypasses the stage of self-renewing stem cells and originates directly from the progenitor population, called gonocytes.^{4,5} Spermatogenesis commonly comprises the phases of mitotic expansion, meiotic divisions, and the transformation of haploid spermatids to spermatozoa (spermiogenesis) before mature spermatozoa are released (spermiation).^{6–8} Here germ cell division usually occurs incompletely because of *incomplete cytokinesis*, through which the resultant daughter cells remain

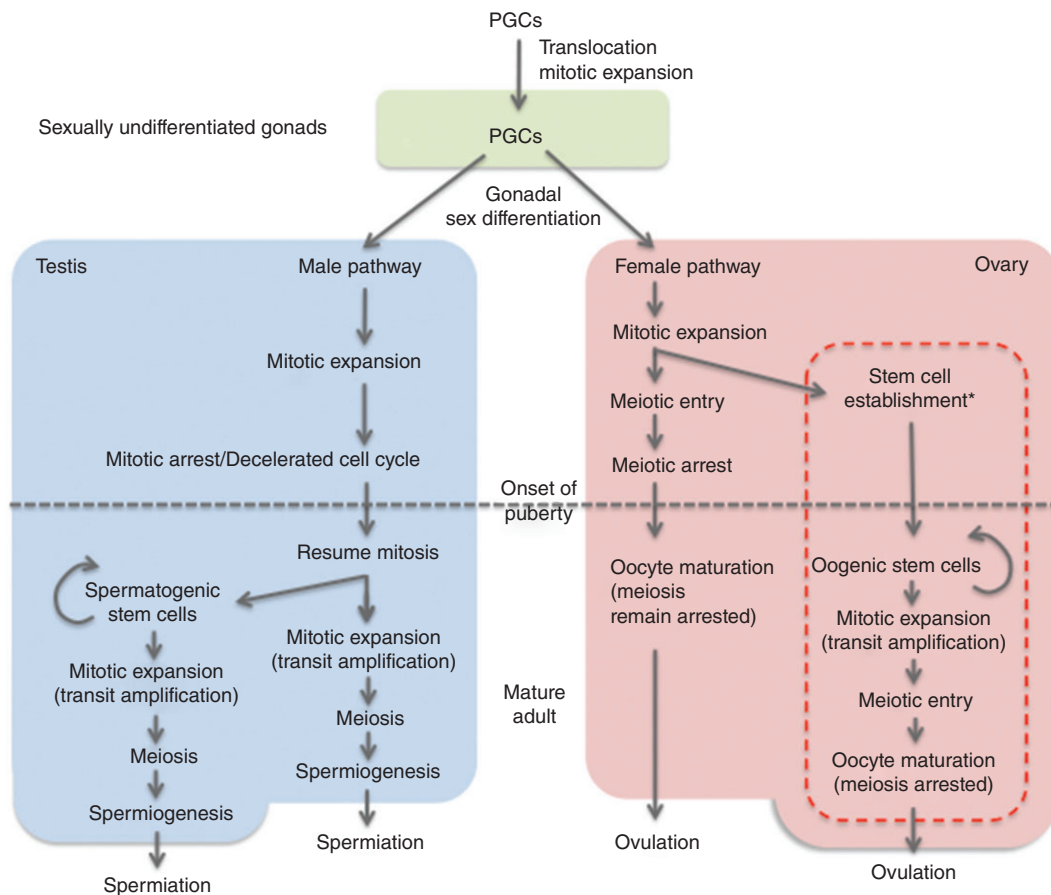


FIGURE 1 | General outline of vertebrate germline development, see text for details. Processes in the red-dotted line appear to be lost in mammals. *The process of stem cell establishment in females (in fish or amphibians) has not been clearly elucidated.

interconnected via intercellular bridges (ICBs) to form syncytia (Figure 2). This germline-specific trait is widely conserved across animals. The aforementioned process of spermatogenesis is thoroughly supported by testicular somatic cells. In particular, *Sertoli cells* are the primary supporting cells in the testis that make intimate contact with germ cells and nourish them. Thus, *stem cells*, *incomplete divisions (incomplete cytokinesis)*, and *Sertoli cells* are the fundamental elements that characterize vertebrate spermatogenesis.

Based on these common elements, vertebrate spermatogenesis exhibits significant divergence. The testicular architecture changed from the ancestral *cystic* form of anamniotes to an *acystic* form that occurs in *seminiferous tubules* of amniote testes. This is not a simple rearrangement of cells. Rather, this is a composite of several significant innovations including

functional changes of Sertoli cells and the development of the *seminiferous epithelial cycle* and the *wave of spermatogenesis*, which together ensure the high productivity and continuity of amniote spermatogenesis. In addition to that, a significant change also occurred in the regulation of sperm stem cells. In seminiferous tubules, the constraint that the stem cells receive from the tissue appears to be dramatically reduced, making vertebrate spermatogenesis a typical *open niche-supported stem cell system*.

In this article, descriptions will inevitably be made primarily on the basis of knowledge derived from a limited number of fish and mammalian species (especially mice) whose germ cell development has been investigated to a much larger extent than others. The statements herein are not necessarily representative for the entirety of these animal groups.

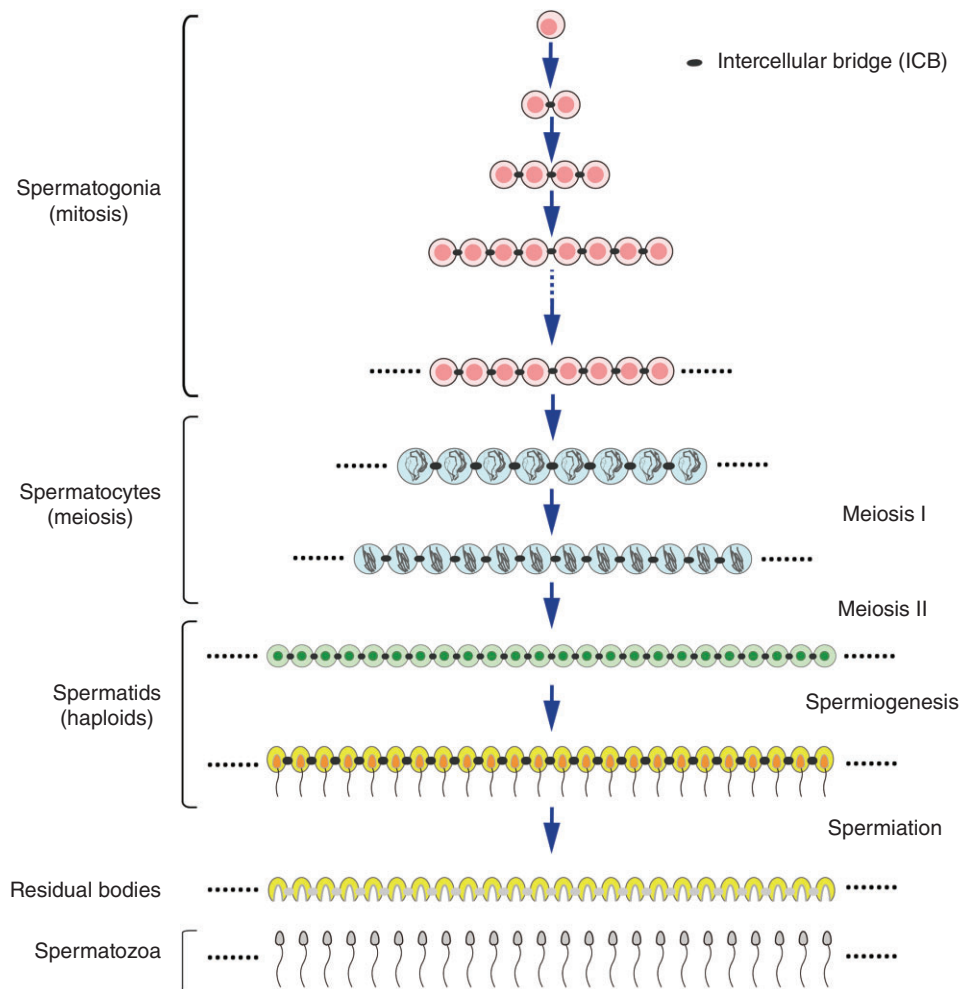


FIGURE 2 | Incomplete division in spermatogenesis. In general, spermatogenic differentiation accompanies incomplete mitotic and meiotic divisions, in which incomplete cytokinesis leaves the daughter cells interconnected through intercellular bridges. The number of premeiotic mitotic divisions varies between species. (Modified with permission from Ref 9. Copyright 1975 Saunders)

FUNDAMENTAL ELEMENTS OF SPERMATOGENESIS

Stem Cells: The Persistence of Sperm Production

Over 60 years ago, Clermont and LeBlond analyzed rat spermatogenesis and arrived at the theory of stem cell renewal.¹⁰ The fact that this historic study was achieved in the study of mammalian spermatogenesis eloquently illustrates that vertebrate spermatogenesis harbors active and robust stem cell systems, supporting the continuity of sperm production.^{11–14}

In some fish species, particularly in medaka, mitotically active *stem cells* have been identified in both male and female gonads. These are found as small subpopulations of spermatogonia or oogonia, which are defined as mitotic stages of germ cells that have entered male or female programs that eventually produce sperm or eggs, respectively.¹⁵ A series of innovative intersexual transplantation experiments in trout, which have been corroborated in other fish species, indicated that both spermatogenic and oogenic stem cells in the testes and ovaries, respectively, retain sexual plasticity. Otherwise, they may remain in a sexually undifferentiated state.^{16,17}

In mammals, the female and male germline show much larger differences with regard to their stem cells (Figure 1). PGCs enter meiosis shortly after the female program has been initiated in the developing ovaries. Therefore, it remains unclear whether ‘oogonia’ can be defined unambiguously in mammals. The syncytial formation of early female germ cells in embryonic mammalian ovaries¹⁸ may result from the mitotic expansion of oogonia, or it may indicate the inherited interconnection of sexually undifferentiated PGCs (see the next section for the nature of the interconnection). Classically, it has been thought that all female mammalian germ cells enter meiosis in embryonic gonads and that no mitotic germ cells persist into the adult stage. Recent controversy surrounds the presence of mammalian female germline stem cells, while emerging data appear not to support this notion. On the other hand, mammalian males clearly harbor active stem cell populations that support life-long spermatogenesis.^{4,6,11}

Symmetric Incomplete Cell Division: The Formation of Syncytia

Like other animals, mitotic and meiotic divisions of vertebrate spermatogenesis often proceed incompletely due to the process of *incomplete cytokinesis* (Figure 2). This results in the interconnection of mitotic sister cells and meiosis-derived haploid cells

via cytoplasmic connections termed ICBs, the counterpart of the ring canals in *Drosophila*.^{6,19} Incomplete cytokinesis is typically observed in the transit-amplifying divisions of spermatogonia originated by the stem cells and in subsequent meiotic divisions. However, some other germ cell divisions are also found incomplete, including the first round of spermatogenesis derived directly from gonocytes, the progenitors.

Interconnected spermatogonia in a single syncytia are synchronized not only in their mitotic divisions but also in their progression of meiosis. Through meiosis, each cell (now called a spermatocyte) gives rise to four haploid spermatids, which remain connected. In the male program of spermatogenesis, both mitosis and meiosis occur *symmetrically*; that is, all the composite cells of a syncytia equally mature into sperm (Figure 2). This is in contrast to the female program, in which germ cells often develop *asymmetrically* into eggs. In mice, syncytia of early oocytes break down and a small number of surviving singly isolated oocytes enter the process of maturation.^{20,21} More dramatically, female meiotic divisions, which sometimes take place after fertilization, occur in a highly asymmetric manner and give rise to a large oocyte and tiny polar bodies.

The incomplete cytokinesis and resultant interconnection of cells derived from the same clonal origin may be fundamental for the germline, given its evolutionary conservation across sexes and species. To the best of my knowledge, this phenomenon is observed in all the animal species that have been examined. Using mice, it was shown that the interconnection between postmeiotic genetically haploid spermatids made these cells phenotypically diploid by means of sharing gene products from the haploid genomes of individual spermatids. These include the gene products from the X and Y chromosomes, which comprise a number of genes required for cell survival and spermatogenesis, respectively.²² However, other consequences of the interconnection of cells remain largely unknown, especially for earlier stages. Indeed, ICBs of early mouse spermatogonia break rather frequently²³ (see below). In accordance with this, in *Tex14* mutant mouse testes in which ICBs are not established and all germ cells are singly isolated, the development of spermatogonial stages does *not* appear to be severely affected. Rather, in this mutant, male germ cells initiate but do not complete meiosis for unknown reasons.²⁴ In female *Tex14* mutants, similarly, ICBs are not observed between oocytes in embryonic and neonatal ovaries. Although the number of maturing oocytes is reduced, however, apparent problems are not observed in their fertility.²⁵

Sertoli Cells: The Supporting Somatic Cells

Sertoli cells are the male counterpart of the female granulosa cells, which surround and nourish the oocytes in developing gonads. Sexually undifferentiated common progenitor cells are specified to become Sertoli cells by the cell-autonomous functions of sex-determination and sex-differentiation genes such as the *SRY* or *DMRT* genes. In addition to directing the sex of germ cells, Sertoli cells also act as the center of systemic sex differentiation.^{26–28} Further, in adult testes Sertoli cells are found as the primary cell type that nourish the germ cells undergoing spermatogenic differentiation.^{29,30}

In general, Sertoli cells form epithelia with prominent tight junctions and provide microenvironments appropriate for germ cell differentiation. Detailed below, anamniotes and amniotes have a marked difference in both the function of Sertoli cells and the testicular tissue architecture. Indeed, the grand design of fish gonads looks more similar to that of *Drosophila* than that of mammals. This narrates the magnitude of the innovations that occurred in the evolution of vertebrate testes.

CYSTIC SPERMATOGENESIS IN ANAMNIOTES

In fish and amphibians, spermatogenesis proceeds inside a cyst of Sertoli cells (Figure 3). Each cyst surrounds a group of germ cells that comprise a single syncytium that synchronously divides (incompletely) and differentiates.^{8,31,32} Although the properties of spermatogenic stem cells of fish and amphibians are still largely unknown, those of medaka have been identified and found to be tightly linked to a somatic, cord-like structure.¹⁵ Generally, in fish species, the most primitive entities of spermatogonia are singly isolated and often called ‘type A spermatogonia,’ which presumptively contain both the stem cells and the youngest progenitors. Differentiating germ cells then undergo a series of incomplete divisions forming syncytia and are eventually spermiated. Intriguingly, germ cells are already encapsulated by Sertoli cells from the stage of ‘type A spermatogonia,’ and their specific link appears to persist until they differentiate to mature spermatozoa and are released⁸ (Figure 3).

Attuned to the progression of germ cell differentiation, Sertoli cells progressively change their gene expression and support the corresponding stages of germ cells, as schematically shown by different colors in Figure 3. In anamniotes, Sertoli cells develop tight junctions beyond the spermatid stage. This mode of *cystic spermatogenesis* is commonly observed over a range of animals other than anamniotes, including *Drosophila* and other insects, in which supporting somatic cells are designated as cyst cells.^{8,11,31,33} In the

testes of particular fish (those showing so-called tubular spermatogenesis) the developmental order of spermatogenic cysts is spatially recapitulated in the testes, similar to that found in the *Drosophila* testes. Such a spatial arrangement is often unclear in other fish species.^{8,32}

In cystic spermatogenesis, Sertoli cells turn over in coordination with the germ cells. This strongly suggests the presence of ‘Sertoli stem cells,’ although no direct evidence has been found to the best of our knowledge. The identity and regulation of both spermatogenic and (presumptive) Sertoli stem cells and their interplay in fish testes warrants future investigation. To address these important questions, findings from *Drosophila* gonads, in which germline and cyst stem cells appear to both be controlled by somatic hub cells,^{33,34} may be insightful. Given that the spermatogonium is already wrapped at its single-cell stage, their spermatogenic stem cells would be under strong constraints both anatomically and functionally from the gonadal somatic cells.

SEMINIFEROUS TUBULES IN AMNIOTES

Architecture of Seminiferous Tubules

Amniotes have an acyclic spermatogenic process that occurs in *seminiferous tubules*.³¹ Seminiferous tubules are long and convoluted tubular structures forming loops out of the rete testes, the common outlet of the sperm (Figure 4(a)). During active spermatogenesis, seminiferous tubules are roughly 0.2 mm in diameter regardless of species, but they demonstrate a high divergence in length among species.⁶ An average human testis is comprised of over 200 m of long seminiferous tubules in total. The tubules are rarely branched in mammals, but they are highly branched in birds forming a meshwork structure.⁷ The following description is based on findings obtained in mice, unless otherwise specified.

The seminiferous epithelium, the building block of seminiferous tubules, is primarily composed of Sertoli cells and germ cells (Figure 4(b)). Very tall and highly polarized Sertoli cells form an epithelium that harbors a basement membrane and a prominent network of tight junctions, the anatomical basis of the ‘blood–testis barrier.’^{6,29} The tight junctions separate the tubule into basal and adluminal compartments. The former is the gap between the junction and the basement membrane, which plasma components can freely reach, while the latter is ‘insulated’ from plasma and immune cells. Spermatogonia, the mitotic germ cells including the stem cells and differentiation-destined amplifying cells, are located in the basal compartment. Upon entering meiosis, they (now called

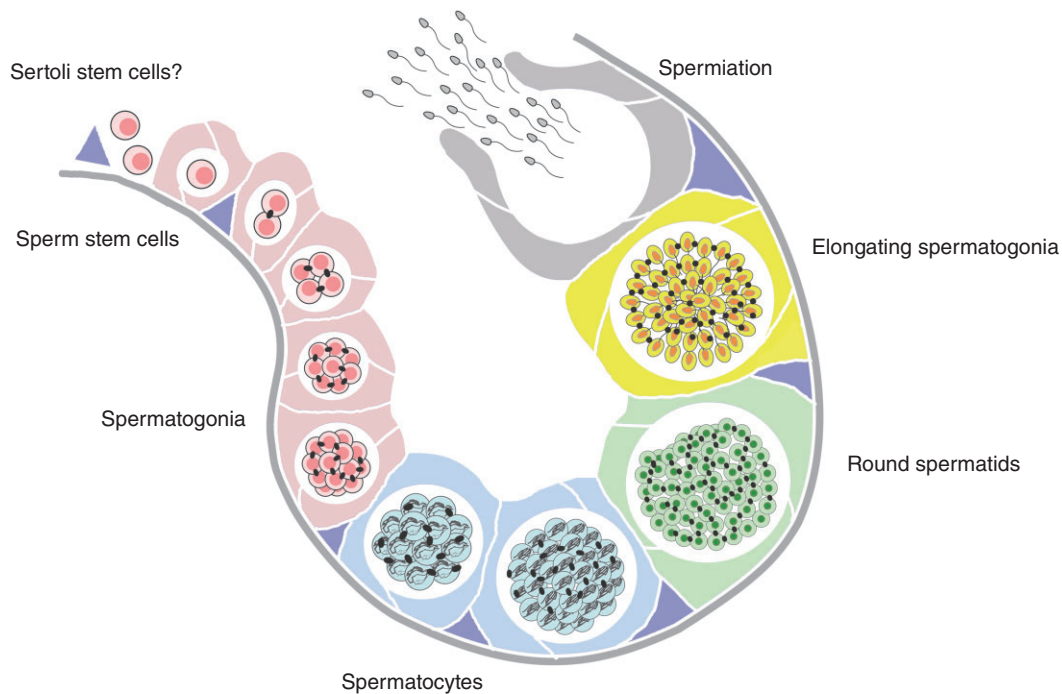


FIGURE 3 | Schematic drawing of cystic spermatogenesis observed in fish and amphibians. In amniote testes, a cyst of Sertoli cells surrounds each germ cell syncytium. Sertoli cells share their fate with the developing germ cell syncytium that they nourish, and eventually degrade when germ cells mature and spermiate. Such a turnover of both germ cells and Sertoli cells suggests the presence of self-renewing stem cells for both cell types. While germline stem cells are identified in some fish species, the Sertoli stem cells remain hypothetical. Tight junctions are established between Sertoli cells that cover haploid spermatids and more advanced germ cells. The spatial organization of cysts within the testis varies highly between species. They are aligned in the order of development in some fish, while others do not have such a polarized organization.

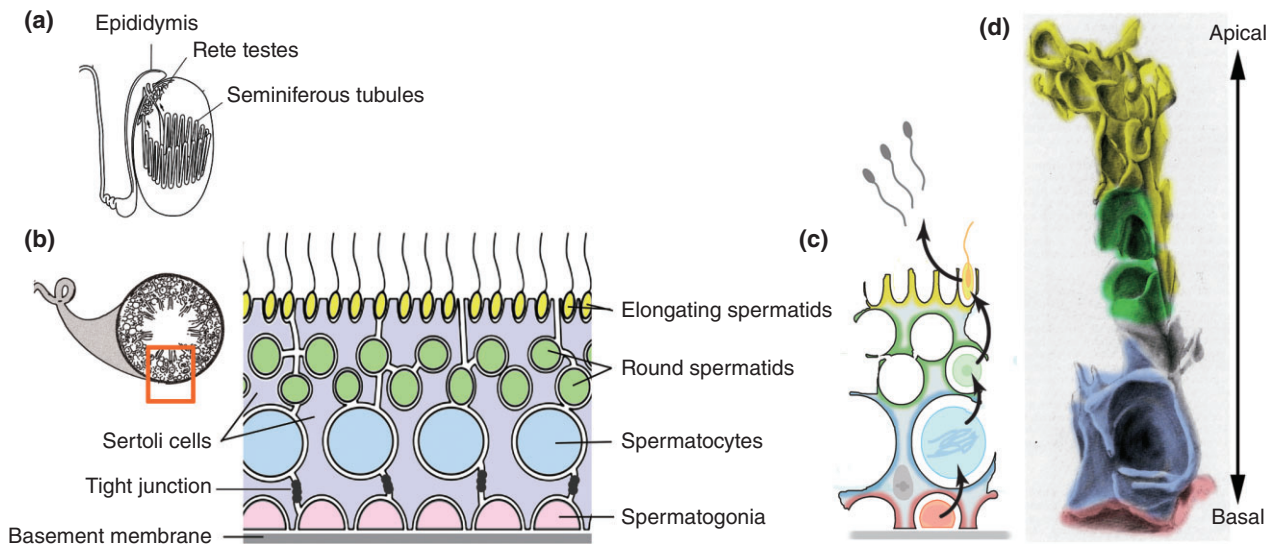


FIGURE 4 | Spermatogenesis in seminiferous tubules in amniotes. (a) In amniote testis, seminiferous tubules loop out of the rete testes connected to the epididymis. (Reprinted with permission from Ref 35. Copyright 2006 University of Tokyo Press) (b) In seminiferous tubules, different steps of germ cells are found among Sertoli cells, which are quiescent in their cell cycle and comprise single-layered epithelium inside the tubules. (c) Each Sertoli cell simultaneously nourishes different (typically four) steps of germ cells in different areas of their plasma membrane (illustrated by colors; from basal to the apical side). Germ cells turn over as they mature on the surface of Sertoli cells, which in contrast never turn over during adulthood. (d) A diagram of a single rat Sertoli cell, showing its columnar shape (approximately 90 μm in height) and numerous processes of cytoplasmic sheets that form crypts for different stages of germ cells, as indicated by the same colors as in (c). (Modified with permission from Ref 36. Copyright 1983 John Wiley & Sons Ltd.)

spermatocytes) translocate to the adluminal compartment. This is followed by two rounds of meiotic divisions to form haploid spermatids, which are eventually released into the lumen after maturing into spermatozoa (Figure 4(b)). Intriguingly, Sertoli cells that have already terminated their cell cycle before puberty expand their plasma membrane to an extreme degree and simultaneously 'hold' germ cells of all four stages (spermatogonia, spermatocytes, round spermatids and elongating spermatids) at different areas of their plasma membrane (Figure 4(c) and (d)).^{6,29} Thus, a single Sertoli cell provides various microenvironments appropriate for the different stages of germ cell differentiation, allowing the germ cells to progressively differentiate from the basal to the apical side.

Tight junctions between Sertoli cells are important for providing the meiotic and haploid cells with a specially insulated microenvironment (adluminal compartment). Interestingly, junctions remain functional while spermatocytes translocate from the basal to the adluminal compartments. This mystery has been recently elucidated; it occurs by the transient formation of an intermediate compartment in which the young spermatocytes are sandwiched by upper and lower junctions similar to an 'airlock'.³⁷

The Seminiferous Epithelial Cycle

An undifferentiated population of spermatogonia located in the basal compartment harbors the stem cell function. These cells supply the differentiating cell types (beginning with A₁ spermatogonia in mice) that subsequently differentiate in close association with Sertoli cells (Figure 5(a)). Of note, differentiation starts in a periodic manner, showing a species-specific interval (e.g., 8.6 days in mice and 16 days in humans).⁶ Regardless of species, it takes four times longer than this interval period for germ cells to complete spermatogenesis and leave the seminiferous epithelium. This explains why four layers of germ cells at different stages (differentiating spermatogonia, spermatocytes, and round or elongating spermatids) are always observed in addition to the undifferentiated population of spermatogonia.

As can be seen in Figure 5(a), these layers exhibit routine associations with particular cell types. As these cells progressively differentiate, the initial associations are observed again after one interval period (8.6 or 16 days) when individual cells have shifted to the next layer.^{38,39} Thus, the observed germ cell combination changes periodically. This is called the 'seminiferous epithelial cycle' and was first discovered in rat testes.⁴⁰ The seminiferous epithelial cycle is divided into stages I through XII in mice.^{6,41} Along with the cycle, Sertoli cells periodically change their morphology, function,

and underlying gene expression profiles.^{29,42} Here, Sertoli cells are similar to a university that persists for decades or centuries. The university welcomes freshmen (spermatogonia) every year (cycle), educates them as they become sophomores (spermatocytes), juniors (round spermatids), and then seniors (elongated spermatids). After four years (cycles), the university eventually graduates the students (as spermatozoa). In addition, it has an annual cycle and delivers different lectures every season according to the students' grade levels, like the periodic change of Sertoli cells during a cycle of seminiferous epithelium.

Spermatogenic Wave

Perhaps more amazingly, in addition to the local synchronization of the seminiferous epithelial cycle in a particular region of the tubules, the temporal order of stages is spatially recapitulated on the seminiferous epithelium. In rodents, synchronicity is observed all around the circumference and the phase of the cycle shifts along the axis of the tubule. As a result, the entire process of spermatogenesis travels along the tubule length. This is designated as the 'spermatogenic wave',⁶ which is sometimes explained by an analogy of the 'wave' in a football stadium (Figure 5(b)). The wave pattern differs between species. In some primates including humans and birds such as quail, the pattern of the wave is complex and not fully determined. Although classically described to be spiral, it remains controversial.^{7,43-45} In any case, this wave ensures the constant production of fresh sperm in a particular portion of the seminiferous tubules. Thus, the seminiferous epithelial cycle and the spermatogenic wave make the seminiferous tubules a high-throughput factory for sperm production.

Despite its interesting properties, little is known about how the cycle and the wave are generated and maintained. Studies using rats and mice suggest that retinoic acid (RA) plays a crucial role in these processes by triggering the differentiation of spermatogonia and adjusting the Sertoli cells' cycle. For readers interested in this issue, please refer to the referenced literature.^{38,39,46}

SPERM STEM CELLS AND THEIR REGULATION IN SEMINIFEROUS TUBULES

Stem Cells Become Freed in Seminiferous Tubules

As described, the relationship between germ cells and Sertoli cells experienced a significant change between anamniotes and amniotes. Seminiferous tubules are a

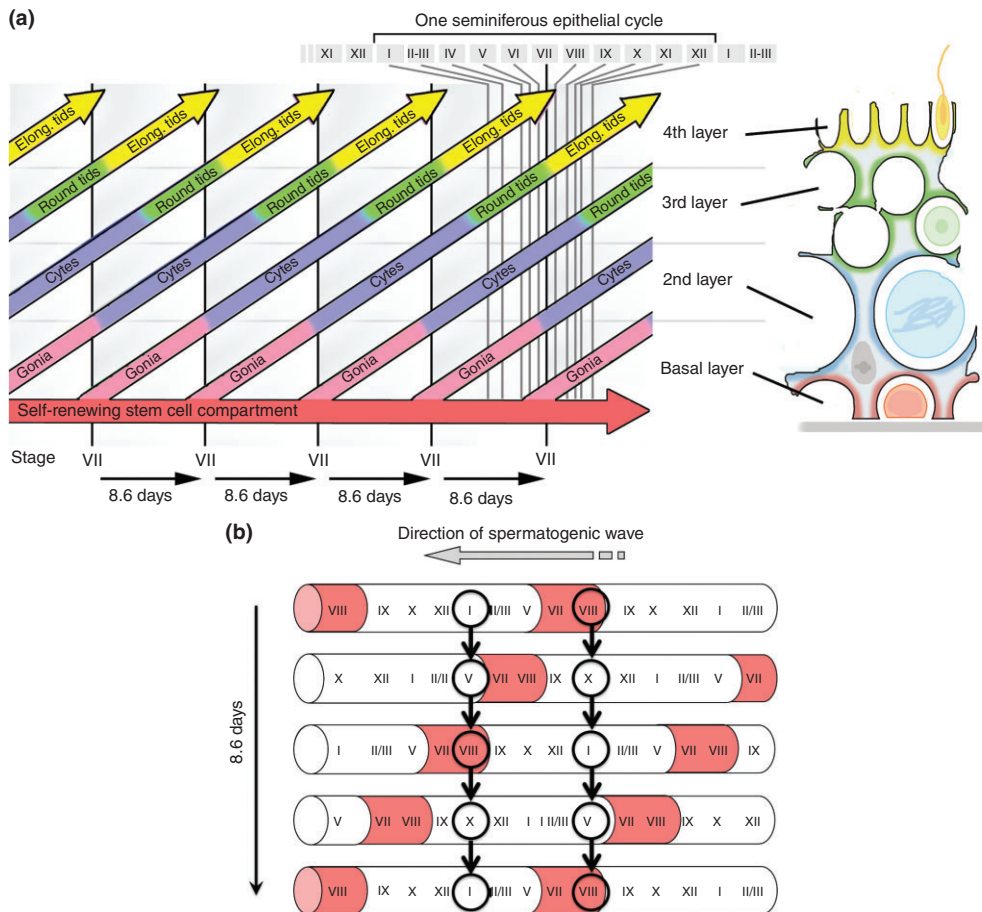


FIGURE 5 | Seminiferous epithelial cycle and spermatogenic wave. (a) A schematic explanation of the seminiferous epithelial cycle in mice. The self-renewing pool gives rise to the first step of differentiating cells periodically with a particular interval (8.6 days in mice), which corresponds to the length of one seminiferous epithelial cycle. The differentiating germ cells subsequently differentiate into sperm after periods of four cycles, which is how four different steps of germ cells are simultaneously nourished by a single Sertoli cell. A cycle is divided into different stages on the basis of the combination of germ cell types. Duration of a cycle and the number of stages in a cycle is species-specific. See text for details. (b) A diagram showing the spermatogenic wave in mice. Roman numerals indicate the local stages of a seminiferous epithelial cycle. The areas where spermiation occurs (in particular those in stages VII and VIII in mice) are colored. Over time, the stages of the seminiferous epithelial cycle progress periodically, as indicated for two positions by circles. Because the temporal order of the stages is recapitulated by the spatial order along the tubules, the area of spermiation progressively shifts along the tubules like a wave (leftward in this scheme). The pattern of the wave is simple in mice but varies among species. See text for details.

uniform-looking tissue composed of epithelial sheets of postmitotic Sertoli cells. Unlike the medaka testes, no specific structure reminiscent of the stem cell tethering hub in the *Drosophila* testes has been found in amniote seminiferous tubules.^{11,47} In *Drosophila* testes, singly isolated cells (germline stem cells and differentiation-destined gonialblasts) and cysts of two, four, eight, and more cells are spatially arranged in order. A similar polarity is observed in the testes of some fish (those with so-called ‘tubular’ spermatogenesis).⁸ However, in the basal compartment of mouse seminiferous tubules, singly isolated spermatogonia (termed ‘A_s’ or ‘A_{single}’ in rodents) are irregularly intermingled with variable lengths of syncytia.^{2,3,48–51} Significantly, direct live

imaging has shown that A_s spermatogonia and short syncytia of ‘undifferentiated spermatogonia’ actively migrate between immotile Sertoli cells^{23,52} (Figure 6 (a)). On transition into the scheduled differentiation, in harmony with the seminiferous epithelial cycle, they become evenly distributed over the tubules and less motile.^{52,53} This process is similar to high school students who choose the universities to enter and become freshmen. Once admitted, they would stay in the same universities (Sertoli cells) until graduation (unless withdrawal through apoptotic death).⁵⁴

Thus, seminiferous tubules provide the stem cells with a typical *open niche environment*.^{55,56} In stark contrast to the advanced germ cell types whose

differentiation is exquisitely synchronized with its neighbors to form the ‘cycle’ and the ‘wave’ (i.e., differentiating spermatogonia, spermatocytes, and round or elongating spermatids), undifferentiated populations of spermatogonia appear to receive minimal constraint from the tissue. It is interesting to point out that the stem cells appear to be much less constrained and behave more freely, than their differentiating progeny. This may sound quite different from the general notion that the stem cells are tightly tethered to a discrete area while differentiating cells appear to become free from the constraint by the stem cell niche. We will close this article with recent knowledge and discussions related to mouse sperm stem cells.

Sperm Stem Cell Behavior in Mice

The identity of mouse sperm stem cells has long been a focus of discussion.^{12–14,57} Classically, it was postulated that A_s cells are the self-renewing stem cells, while A_{pr} and longer syncytia are committed for

differentiation^{58–60} (Figure 6(c)). This hypothesis, consistent with the behavior of germline stem cells in *Drosophila* gonads, has become prevalent and is known as ‘the A_s model.’

It has been experimentally established that ‘undifferentiated spermatogonia’ (including A_s and short syncytia of up to approximately 16-cell long chains) have major responsibility for the stem cell function supporting long-lasting steady-state spermatogenesis. Among them, the GFR α 1-positive (GFR α 1+) subpopulation appears to be the most important. However, a differentiation-destined GFR α 1-negative (GFR α 1–) subset of undifferentiated spermatogonia also retains stem cell potential, which becomes apparent in regeneration following tissue damage or in colony formation following transplantation.^{51,61,62} Morphologically, the GFR α 1+ population is comprised of A_s , A_{pr} (A_{paired} , two-cell syncytia), and fewer A_{al} ($A_{aligned}$, mostly comprised of four cells, some containing eight cells as well as few ‘odd numbers’ of three, five, and six cells) spermatogonia.^{2,3,49–51,63}

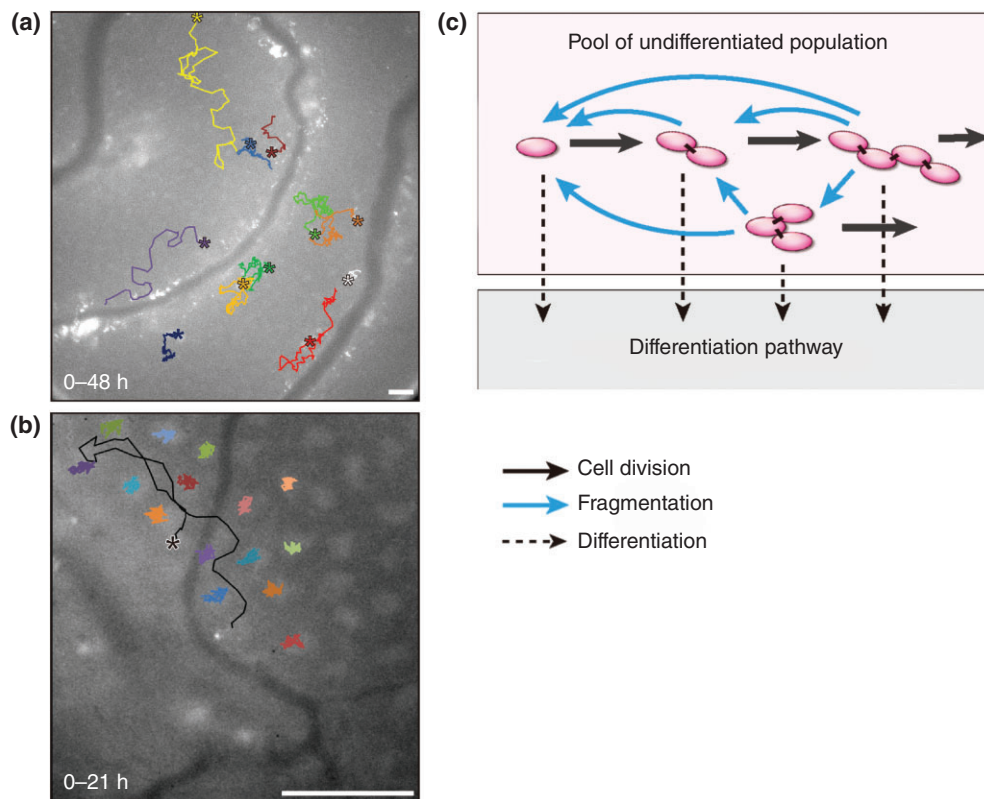


FIGURE 6 | Behavior of undifferentiated populations of spermatogonia in mouse seminiferous tubules. (a) Seemingly random migration of GFR α 1-positive (GFR α 1+) spermatogonia observed by intravital live imaging: the trajectories of 11 spermatogonia for 48 h are shown. Blood vessels running between the tubules are observed in black. (b) The GFR α 1+ spermatogonia weave their way (black trajectory for 21 h) between immotile Sertoli cells (colored trajectories). Bars indicate 50 μ m. (a, b: Reprinted from Ref 23.) (c) Continual interconversion between singly isolated and syncytial GFR α 1+ spermatogonia in adult mice is schematically shown on the basis of live imaging observations.

Recently, some genes have been reported to be expressed in a subset of GFR α 1+ spermatogonia, with particular enrichment to A_s cells, including *ErbB3*, *Id4*, *Pax7* and *Bmi1*.^{64–67} Accordingly, the authors of these studies hypothesized that stem cell activity may be limited to a subset of GFR α 1+ A_s cells that show characteristic gene expression. Although these genes do not appear to be expressed in the same cells, these scenarios extend the classic idea of the A_s model (Figure 6(c)). On the other hand, recent intravital live imaging demonstrated that, in most cases, GFR α 1+ A_s spermatogonium divide incompletely and give rise to an A_{pr}.²³ It was also demonstrated that GFR α 1+ A_{pr} and A_{al} frequently fragment through the breakdown of ICBs and replenish the lost A_s cells.²³ Combined with the clonal fate analysis of pulse-labeled GFR α 1+ spermatogonia and biophysical modeling studies, it is further suggested that the entire population of GFR α 1+ spermatogonia comprises a single stem cell pool. Within this pool, cells continually interconvert between morphologically distinct states of A_s and syncytia via symmetrical incomplete divisions and fragmentation²³ (Figure 6(c)). Thus, the identity and dynamics of the mouse sperm stem cells in seminiferous tubules is currently surrounded by some controversy. However, I believe that researchers will eventually reach a full understanding of this long-held question using functional experimentation that has become available in the last few decades.¹²

Regardless of the identity of the stem cells, early stages of mouse spermatogonia flexibly interconvert between the single and syncytial states while moving around the open environment of seminiferous tubules. Although it remains to be elucidated whether such freedom is also given to the sperm stem cells of fish, the testicular morphology and, more importantly, the presumptive necessity of coordination with ‘Sertoli stem cells’ should constrain their behavior.

Unresolved Problems Related to Stem Cell Regulation

However, stem cells do not appear to be completely free in mouse seminiferous tubules. Although irregularly scattered in mouse seminiferous tubules, the average density (number per unit tubule length) of the presumptive stem cells (GFR α 1+ cells) has been found to be constant.^{23,50} In a closed niche-supported system, the physical area of the niche determines the size of the stem cell pool.^{33,55} What mechanism determines the *stem cell pool size* in seminiferous tubules? This is one of the biggest problems that should be addressed to understand the functionality of seminiferous tubules.

In support of understanding the control of the stem cell pool by the seminiferous tubules, a clue has been provided by the observation that an increase in the number of Sertoli cells causes an increase of transplantable stem cells,⁶⁸ showing the significance of Sertoli cells. However, the answer appears to be more complex given that the number of GFR α 1+ spermatogonia is much smaller than that of Sertoli cells, and these spermatogonia are in constant motion changing their contact with the Sertoli cells.²³ Of particular note, while migrating over the basal compartment, undifferentiated spermatogonia show a biased localization to areas adjacent to the blood vessels (in particular arterioles and venules, which accompany the interstitial cells) running between the tubules.^{23,52,69,70} Characterization of this area of the basal compartment may also provide important information to address this issue.

PERSPECTIVES

Many amniotes undergo external fertilization in water, producing large numbers of eggs and sperms in a well-timed manner. Thus, sexual difference in their gamete production is relatively small. Amniotes have attained watertight eggs, in which embryonic membranes compartmentalize the developing embryo, nutrient, and the waste matter, enabling the transition from water to land. This increased the amount of maternal resources invested to each single egg and largely decreased the number of ovulated eggs and offspring. Moreover, brooding of eggs (birds) and pregnancy (mammals) as well as nursing (including lactation in mammals) significantly limit the window of females for copulation. Making a stark contrast to this, amniote males came to regularly produce a huge number of sperm, greatly enlarging the sexual difference. Even the seasonally breeding mammals and birds exhibit continuous spermatogenesis in their breeding season. A speculation may be that the continual production of sperm, which makes amniote males always ready for mating, has enabled the females to concentrate on the small number of eggs and kids. The continual mass-production of sperm in seminiferous tubules may be a given treasure of amniotes, including ourselves.

The cyst-to-tubule transformation that vertebrate testes have undergone during evolution is not simply a morphological change. Sertoli cell function has diverged significantly. Like operating software, the processes of ‘cycle’ and ‘wave’ make tubules similar to high-throughput factories. Furthermore, germ cells, including stem cells, had to develop new modes of

action. Each of these events appears to have been required simultaneously to achieve functional seminiferous tubules. How could such an innovation have

occurred? Further investigations in amphibians and reptiles, as well as comparative studies at the molecular level, will shed light on this important issue.

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