Sertoli cell structure and function in anamniote vertebrates

13

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I. Introduction

The "raison d'être" of Sertoli cells in the adult testis is to facilitate the survival and development of male germ cells so that spermatogenesis can provide spermatozoa in sufficient numbers to guarantee fertility. This function is conserved across vertebrates. However, taxonomic groups at the basis of the phylum—fish and amphibians collectively referred to as anamniote vertebrates—differ from the amniote vertebrates in having the cystic type of spermatogenesis. The main differences between cystic and noncystic spermatogenesis are summarized in Figure 13.1. In cystic spermatogenesis, Sertoli cell structure and function have a number of characteristics that are very interesting from a comparative point of view and provide new insights into Sertoli cell physiology. This chapter focuses on specific aspects of Sertoli cells in cystic spermatogenesis that are relevant for understanding important Sertoli cell functions in vertebrates in general.

Testicular anatomy in amphibians and fish (except for sharks, which do not have steroidogenic Leydig cells in the interstitial compartment) follows the general vertebrate pattern of two compartments, germinative and interstitial, that are separated from each other by a basement membrane and peritubular myoid cells [1]. The interstitial compartment contains steroid hormone-producing Leydig cells, blood vessels, macrophages and mast cells, neural and connective tissue elements that are continuous with the tunica albuginea, and the testis organ wall. The (intra)tubular compartment houses the germinal epithelium that holds two cell types, the somatic Sertoli cells and the germ cells. Via the pituitary gonadotropic hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), interstitial steroidogenic Leydig cells as well as Sertoli cells, expressing the receptors for FSH and LH, are integrated into endocrine regulatory circuits governing puberty and adult functioning of the testis. The survival and development of germ cells *in vivo* depends on their close and continuous interaction with Sertoli cells so that the Sertoli cell number limits the spermatogenic capacity of a testis [2]. This limitation renders

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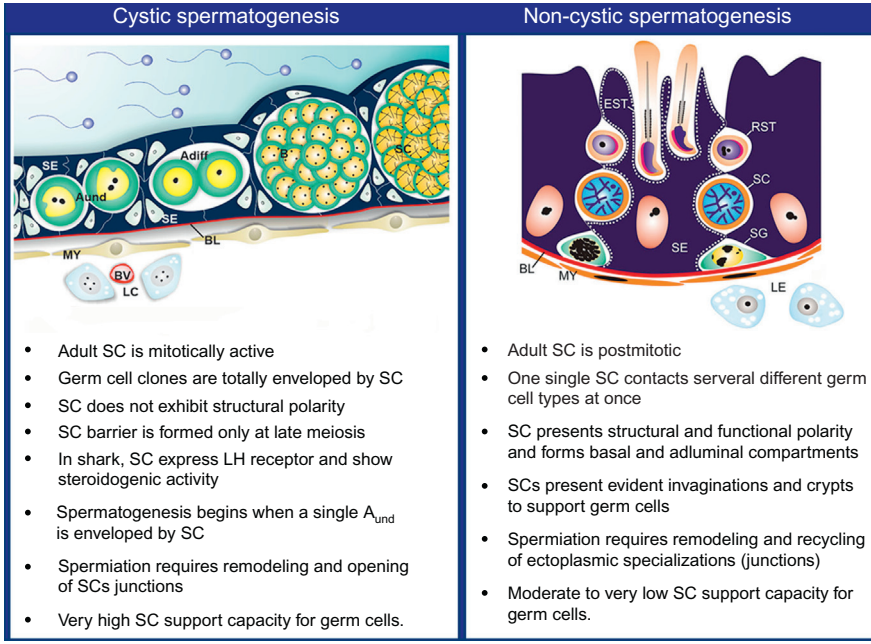

Figure 13.1 Schematic representation of the main differences between Sertoli cells in cystic and noncystic spermatogenesis. A_{diff}, type A differentiated spermatogonia; A_{und}, type A undifferentiated spermatogonia; B, type B spermatogonia; BL, basal lamina; BV, blood vessel; EST, elongated spermatid; LE or LC, Leydig cells; MY, peritubular myoid cells; RST, round spermatid; SC, spermatocytes; SE, Sertoli cell; SG, spermatogonia. Part of the figure was published previously [1].

determination of Sertoli cell number one of the most important aspects of testis physiology, and we discuss the occurrence and regulation of Sertoli cell proliferation in cystic spermatogenesis. Sertoli cell proliferation is accompanied by progressive morphological and functional differentiation that is discussed, along with information on the regulation of these processes.

II. Sertoli cell proliferation

A. Development of existing spermatogenic cysts

The manners of Sertoli cell proliferation differ between amniote and anamniote vertebrates, reflecting a major difference in the Sertoli cell/germ cell relationship.

In birds and mammals, Sertoli cells proliferate until puberty so that in the adult testis, a given number of resident, postmitotic Sertoli cells support successive waves of spermatogenesis. During these waves, a given Sertoli cell supports several different developmental stages of germ cells at a time. Typically, at the basolateral

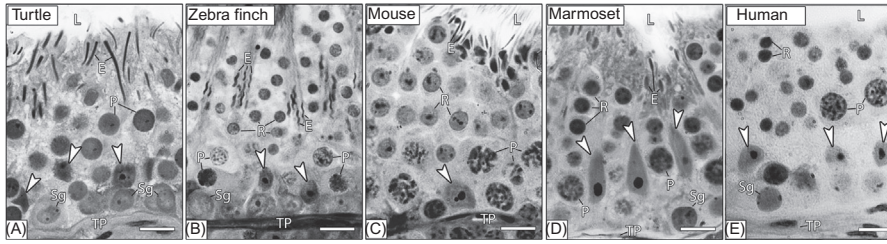

Figure 13.2 Seminiferous tubule cross sections of noncystic spermatogenesis in a turtle (A), bird, zebra finch (B), mouse (C), marmoset (D), and human (E), showing Sertoli cell nuclei (arrowhead), spermatogonia (Sg), pachytene spermatocytes (P), and round (R) and elongated (E) spermatids. Also shown are the tunica propria (TP) and lumen (L). In this mode of spermatogenesis, one single Sertoli cell contacts several different germ cell types at the same time, and the Sertoli cell barrier delineates two compartments in the seminiferous epithelium: the basal compartment in contact with the tunica propria, where spermatogonia and early spermatocytes are located; and the adluminal compartment containing spermatocytes and spermatids. Bars = 20 μm.

surface (basal compartment), mammalian Sertoli cells contact spermatogonia and early spermatocytes. Inside the blood−testis barrier, lateral parts contact spermatocytes and early (round) spermatids, while apical/adluminal parts contact late (elongating) spermatids (Figure 13.2 and Chapter 1).

In anamniote vertebrates (fishes and amphibians), in contrast, the functional unit of the germinal epithelium is the spermatogenic cyst (Figure 13.3). This cyst consists initially of a single spermatogonium enveloped by the cytoplasmic extensions of Sertoli cells. When this initial cyst enters the spermatogenic process, both the cyst-forming Sertoli cells and germ cells proliferate and differentiate. A species-specific number of Sertoli cells is associated with cysts at specific stages of spermatogenesis, thereby establishing predictable Sertoli cell/germ cell ratios for a given stage of germ cell development [2−5]. Because Sertoli cells associated with growing cysts are positive in proliferation assays, at least part of the increase in Sertoli cell number per cyst is attributed to the division of these cells [4]. The major increase in cyst volume and Sertoli cell number per cyst occurs during the mitotic expansion of spermatogonia [2,4,5]. Therefore, unlike in amniote vertebrates, Sertoli cells are not postmitotic cells but proliferate in the anamniote testis. The situation is complicated somewhat by the fact that a Sertoli cell can contact more than one germ cell clone on the two sides of a cytoplasmic extension (Figure 13.4), although this ability is less complex than the typical situation of Sertoli cells in adult birds and mammals (Figure 13.2).

It seems that in all vertebrate species, testis function requires a functional Sertoli cell barrier and the interaction between the morphological and physiological barrier components to efficiently regulate the entry and exit of molecules [6]. This immunological barrier leads to an immune-privileged site that protects germ cells from immunological destruction [6]. The Sertoli cell barrier function is relatively well studied in mammals but still requires careful investigation in lower vertebrates. In fish, Sertoli cell proliferation stops when the germ cell clone completes meiosis and

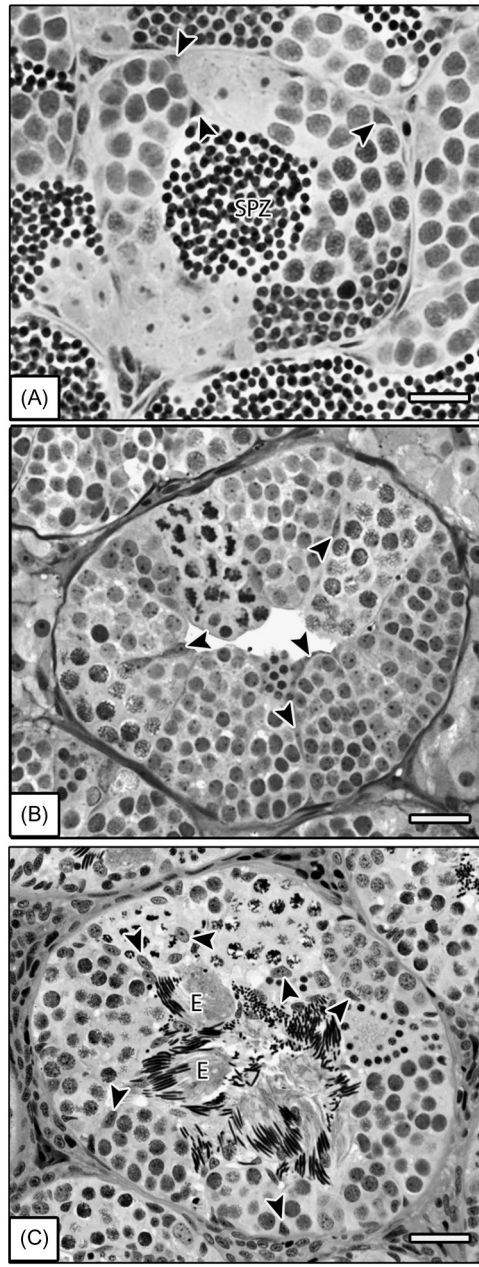

Figure 13.3 Seminiferous tubule cross sections showing cystic spermatogenesis in different phases of development and enveloped by Sertoli cells (arrowhead) in zebrafish (A), Nile tilapia (B), and amphibian bullfrogs (C). The zebrafish tubular lumen is usually filled with sperm (SPZ), while the elongate spermatids (E) cysts are open in bullfrogs but are still contacting SCs. Bars = 100 μm.

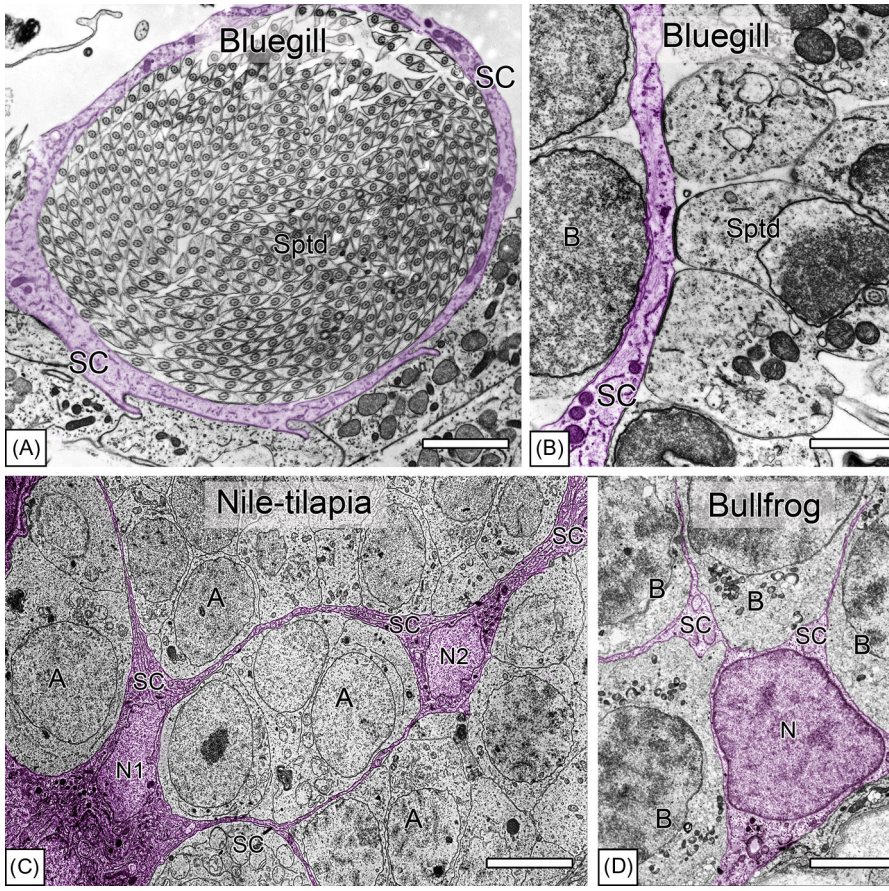

Figure 13.4 Seminiferous epithelium ultrastructure in fish and amphibians showing Sertoli cells (in purple) and germ cells. Strikingly, in the bluegill fish (A), a Sertoli cell is enveloping hundreds of spermatids (Sptd). This figure also shows that one Sertoli cell is able to contact different cysts, which can be observed in the bluegill (B), where Sertoli cell cytoplasm is facing type B spermatogonia (B) and Sptd. In the Nile tilapia (C), one Sertoli cell is apparently facing different type A spermatogonial (A) cysts. Sertoli cell cytoplasmic projections (D) are also observed between different germ cells in a bullfrog type B spermatogonial cyst. N, Sertoli cell nucleus. Bars: A = 2 μm; B = 5 μm; C = 5 μm; D = 3 μm.

enters spermiogenesis, at which time tight junctions are also established among the Sertoli cells [2,4,5] (Figure 13.5). A similar trend has been observed in studies in bullfrogs in our laboratory. Because meiotic germ cells in fish are apparently not shielded from the vascular system, a tight Sertoli cell barrier seems not to be necessary for meiosis in fish. However, our knowledge of the barrier structure and function in fish is rather fragmentary. For instance, as shown for zebrafish

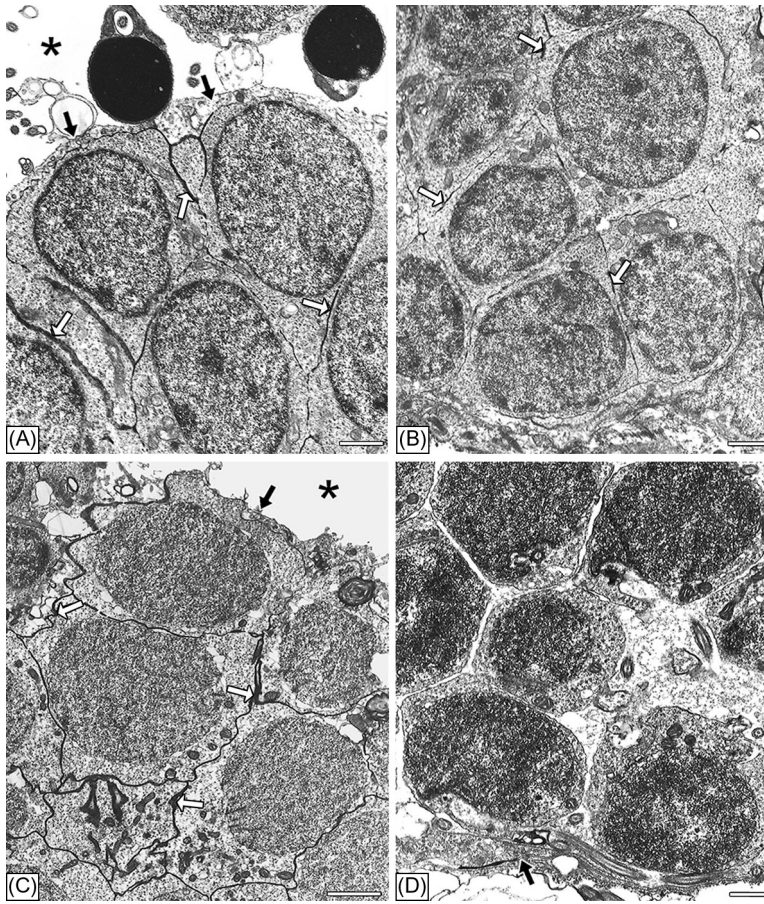

Figure 13.5 Ultrastructure of zebrafish testis using lanthanum as a tracer to investigate the Sertoli cell barrier. In cystic spermatogenesis, the barrier is not observed in cysts containing type B spermatogonia (A) or primary spermatocytes cysts at the initial stages of meiosis (leptotene/zygotene) (B and C) because lanthanum is present between the germ cells (white arrows in A–C). (D) Lanthanum is found at the level of tight junctions between Sertoli cells in spermatid cysts (black arrow) and are not present between the germ cells and in the lumen (asterisks in A and C). The black arrows in A and C indicate the Sertoli cell cytoplasm enveloping the cyst. Bars in A and B = 1 μm, C = 2 μm, D = 0.5 μm. The figure was published previously [5].

(Figure 13.5), even without a functional barrier, lanthanum, a tracer used to investigate the barrier efficacy, is never found in the tubular lumen. With respect to proliferation activity, however, Sertoli cells seem to behave in a similar way throughout vertebrates, given that Sertoli cells stop proliferating when tight junctions have been established. In this way, a predictable spermatid/Sertoli cell ratio is established. Interestingly, this ratio decreases during vertebrate evolution, being around

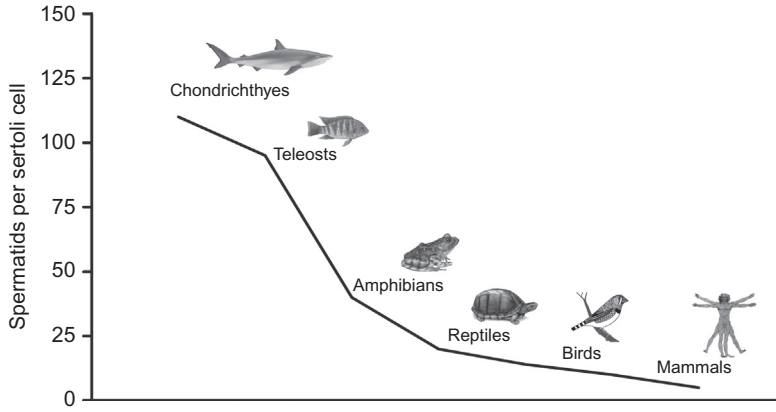

Figure 13.6 Number of spermatids per Sertoli cell, based on the available literature, for different vertebrate groups. This figure illustrates a progressive decrease in Sertoli cell efficiency during evolution.

100 in fish but decreasing to <10 in most mammalian species, reaching about 4 in humans (Figure 13.6).

After completion of spermiogenesis, the cyst opens to release sperm into the tubular lumen (spermiation). The seminiferous epithelium in anamniote vertebrates is composed of spermatogenic cysts in which different germ cell clones (i.e., descendants of different stem cells, usually in different stages of development) are taken care of by Sertoli cells enveloping the clone until spermiation. This finding seems to imply that an individual Sertoli cell is not a resident but rather a transient cell in the anamniote testis (see section III D).

B. Generation of new spermatogenic cysts—Sertoli cell progenitors

In addition to the Sertoli cell proliferation that accompanies the development of spermatogenic cysts, another mode of Sertoli cell proliferation takes place in the fish testis in the context of the production of new spermatogenic cysts. Because germ cells cannot survive unless they are in close contact with Sertoli cells, we hypothesize that the first new Sertoli cells are produced, thereby creating a niche into which a newly formed, single type A_{und} (type A undifferentiated) spermatogonium, a spermatogonial stem cell (SSC) candidate can be recruited. This assumption explains the observation made in the seasonally reproducing Atlantic salmon, for example, that at the beginning of the testis growth phase, groups of Sertoli cells appear that are not (yet) in contact with germ cells or several Sertoli cells are grouped around a single germ cell (Figure 13.7). In this setting, it seems possible that similar to recent observations in mice [7], Sertoli cells produce a paracrine factor, glial cell-lined derived neurotrophic factor (GDNF) that increases the number of SSCs in the vicinity of these Sertoli cells, for example, by stimulating SSC

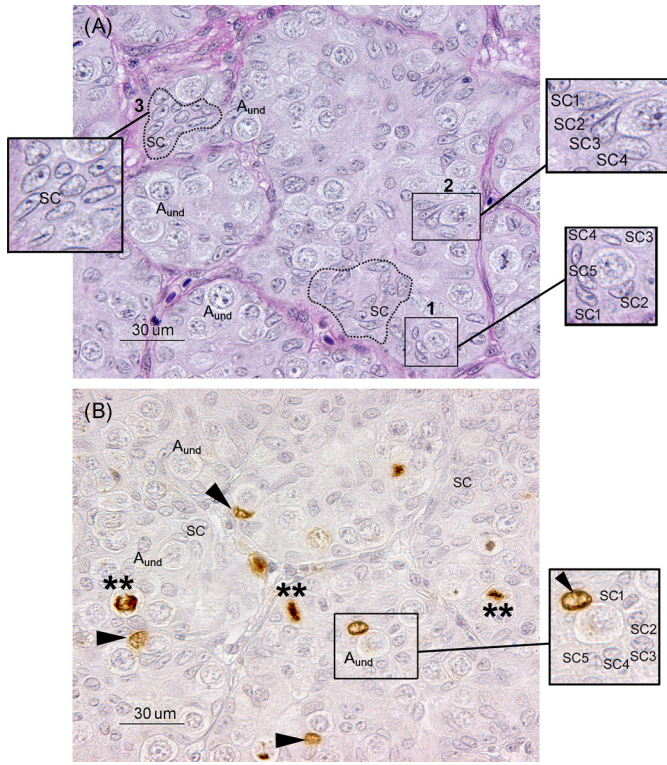

Figure 13.7 Sertoli cell groups in Atlantic salmon testis at the beginning of the seasonal testis growth phase. (A) **Squares** (**1,2**) show several Sertoli cells, indicated by numbered Sertoli cell nuclei that are grouped around a single type A undifferentiated spermatogonium (**A_{und}**). Areas delineated by **dashed lines** (e.g., **3**) show Sertoli cells apparently not (yet) in contact with germ cells (possibly containing Sertoli cell progenitor cells). Staining: Hematoxylin and eosin. (B) Immunocytochemical detection of a proliferation marker [phosphorylated histone H3 (pH3)] on another section of the same testis shown in A. pH3-positive Sertoli cell nuclei (**arrowheads**) indicate proliferation of Sertoli cells at the beginning of testis growth. The square highlights several Sertoli cells (**SC1−SC5**) that are already associated with a single spermatogonium type A_{und}. Sertoli cells continue proliferating, potentially providing niche space for new, single type A_{und} spermatogonia (cyst formation). Double asterisks indicate pH3-positive single type A_{und} spermatogonia.

self-renewal divisions or by attracting SSCs from other areas. Interestingly, in rainbow trout, Gdnf and its receptor have been detected in spermatogonia type A_{und}, suggesting that this factor can also function in an autocrine loop [8]. Studies in adult zebrafish have revealed a regulatory link (via thyroid hormone and a growth factor of the Igf family; see below) between the proliferation of Sertoli cells and of single type A_{und} spermatogonia [9]. Therefore, our current model of cyst formation assumes that Sertoli cells are first generated to provide new niche space that can then be occupied by single type A_{und} spermatogonia.

New cysts are produced at different stages of the life cycle, and the intensity of cyst production varies with the reproductive strategy of a species, in particular in seasonally reproducing species. Regardless of the reproductive strategy, cyst generation takes place in all species between completion of male sex differentiation and puberty. This slow growth populates the prepubertal testis that often grows not more than allometrically with cysts containing mainly type A_{und} spermatogonia. Because many fish species display lifelong growth, a certain level of (allometric) testis growth associated with the generation of new cysts and Sertoli cells continues after puberty.

In seasonally reproducing species, large (e.g., 50-fold) changes in testis weight are recorded [10,11]. These changes reflect the production of new spermatogenic cysts associated with a relatively slow growth of the testis at the beginning of a seasonal cycle. This growth becomes rapid when the cysts go through spermatogenesis, and germ cells and the associated Sertoli cells proliferate and differentiate until the cysts open to release spermatozoa into the tubular lumen. At a certain point in time, the generation of new cysts ceases and existing cysts continue to develop and complete spermatogenesis, such that the fully mature testis in these species typically shows spermatogenic tubuli with lumina filled with spermatozoa and a small number of quiescent cysts with a single type A_{und} spermatogonium, representing the start-up reservoir for the next season's spermatogenic wave.

The testis weight built up during spermatogenic activity declines in a stepwise way. The first step occurs during completion of spermiogenesis, when Sertoli cells remove residual bodies while the production of new cysts stops. The second step of testis weight decrease is due to the use of sperm during the spawning season. Testis weight finally returns to low levels when residual spermatozoa are phagocytized by Sertoli cells after completion of the spawning season in preparation for the start of the next seasonal growth period. Thus, in seasonally reproducing species, Sertoli cell proliferation for the formation of new cysts is activated at the beginning of the seasonal testicular growth phase and is turned off to initiate the completion of the ongoing spermatogenic wave. In contrast, many species living in (sub)tropical zones display continuous spermatogenesis after puberty; prominent examples used as experimental models are medaka, tilapia, and zebrafish. In these species, Sertoli cell proliferation in the context of the formation of new cysts is observed continuously after puberty.

We hypothesize that the new Sertoli cells required for the generation of new spermatogenic cysts are derived from a Sertoli cell precursor population that potentially has stem cell characteristics. There is no formal proof for this hypothesis yet, but circumstantial evidence suggests the presence of an undifferentiated population of somatic cells in the testis. One line of evidence is the long-term capacity to produce new Sertoli cells during successive reproductive cycles. Another line of evidence is the fully functional sex reversal in adults, for example, in female goldfish with androgen-induced female-to-male sex change [12], in estrogen-induced male-to-female sex change in medaka [13], or in the natural sexual plasticity in sequentially hermaphroditic species [14]. These observations suggest that an undifferentiated somatic precursor cell population can produce cells that have a male (Sertoli) or

female (granulosa) cell fate, depending on the environment in which they are situated. Clearly, germ cell-supporting somatic cells (or their precursors) in fish show an astonishing developmental plasticity in terms of sexual fate and proliferation capacity.

C. Intratesticular sites of Sertoli cell proliferation

Where does Sertoli cell proliferation in the context of cyst production take place in the testis? Depending on the species, cysts with a single type A_{und} spermatogonium, among which are SSCs, are distributed apparently at random throughout the germinal epithelium or are restricted to the periphery of the testis near the tunica albuginea [15]. In the first case, the cysts show little mobility during spermatogenesis. In the latter case, the cysts move away from the tunica as the germ cells divide and differentiate toward the region of the spermatic duct located centrally in the testis, where spermiation occurs and the cysts open to release spermatozoa. This arrangement is typical for teleost orders that are considered more evolved (atheriniformes, cyprinodontiformes, and beloniformes), while the former arrangement is found in less evolved taxonomic groups, such as in characiformes, cypriniformes, and salmoniformes [16]. Intermediate situations exist in perciformes (e.g., tilapia, *Oreochromis niloticus* [17]) or some pleuronectiformes (e.g. sole, *Solea senegalensis* [18]), where cysts with single type A_{und} spermatogonia have a preferred, but not exclusive, location close to the tunica albuginea. Close inspection of the Atlantic cod *Gadus morhua* revealed that new cysts are formed in a germinative zone in the periphery of the testicular parenchyma, which is divided into several lobes arranged around an efferent duct [11]. This structure results in a zonation of the lobes because early stages of development take place in the periphery, while advanced stages occur close to the efferent duct. The setting is based on appositional growth that is fuelled by the cyst-generating activity of the germinative zone rather than through movement of developing cysts. Hence, positional cues for the formation of new cysts exist in certain species, such that progenitor germ and Sertoli cells are likely to be found close to the tunica in the periphery of the testis. In the bluehad wrasse *Thalassoma bifasciatum*, it is possible that cellular elements from the tunica can differentiate into Sertoli cell progenitors [19].

However, in species in which the cysts with single type A_{und} spermatogonia (i.e., the potential site for the production of new cysts) are distributed throughout the testis, such as zebrafish, the intratubular location of these cysts is not random. As in rodents [20–22] and other mammals, such as horses [23] and peccaries [24], single type A_{und} spermatogonia in zebrafish preferentially locate to areas of the seminiferous tubules near blood vessels and the interstitium [25].

D. Regulation of Sertoli cell proliferation

The two modes of Sertoli cell proliferation, one in the context of the production of new spermatogenic cysts (mode 1) and the other in the context of the growth and development of existing cysts (mode 2), are regulated differently (Figure 13.8). This conclusion is based on the observation that these modes can take place

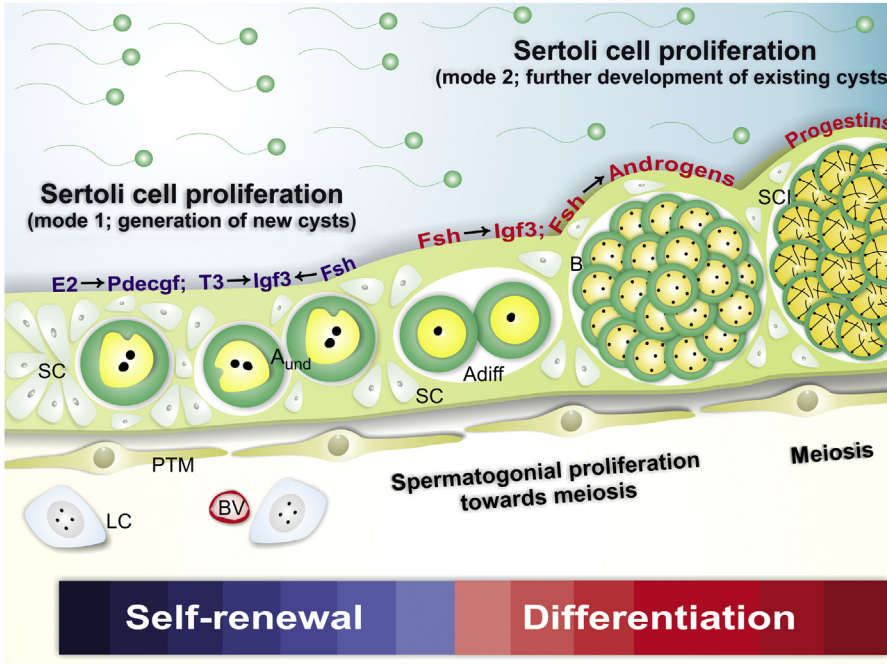

Figure 13.8 Schematic representation of Sertoli cell proliferation in relation to endocrine and paracrine regulation of fish spermatogenesis. Generation of new cysts occurs by **mode 1** of Sertoli cell proliferation, while **mode 2** accompanies the development of existing cysts. Proliferation in **mode 1** can occur when Sertoli cells are not (yet) in contact with germ cells or when multiple Sertoli cells contact a single type A undifferentiated spermatogonium ($\mathbf{A_{und}}$). In zebrafish, cysts with single type A_{und} spermatogonia are preferentially found close to the interstitial area (Leydig cells [LC]) and blood vessels [25] (BVs). We hypothesize that mode 1 of Sertoli cell proliferation increases niche space into which single type A_{und} spermatogonia can be recruited for the generation of new cysts. **Mode 1** is stimulated by thyroid hormone, in part by increases in the production and release of Sertoli cell-derived insulin-like growth factor 3 [9] (**Igf3**). Because *igf3* gene expression is strongly stimulated by follicle-stimulating hormone, this gonadotropin may also be relevant to mode 1, as is the case for estrogens via stimulation of Sertoli cell production of Pdecgf [26]. **Mode 2** of Sertoli cell proliferation accompanies the development of existing cysts from the expansion of the spermatogonial population until the end of meiosis/beginning of spermiogenesis and stops when tight junctions are formed between Sertoli cells in teleost testes [2,4,5]. During spermatogonial proliferation toward meiosis, FSH stimulates Sertoli cell Igf3 and LC androgen production, which both promote cyst development. In the meiotic phase, progestins might be involved in **mode 2**. Sertoli cell (**SC**), type A undifferentiated spermatogonia ($\mathbf{A_{und}}$), type A differentiated spermatogonia ($\mathbf{A_{diff}}$), type B spermatogonia (**B**), primary spermatocyte (**SCI**), Leydig cell (**LC**), peritubular myoid cell (**PTM**), and blood vessel (**BV**).

independently of each other. In seasonally reproducing species, such as the Atlantic salmon, Sertoli cell proliferation for cyst formation (mode 1) starts earlier than the second mode of Sertoli cell proliferation. The latter mode commences once cysts that have started to differentiate reach a size that requires increasing the Sertoli cell number to accommodate the growing germ cell clones. When mode 2 of Sertoli cell proliferation starts, mode 1 is still ongoing. Later in the season, when mode 1 of Sertoli cell proliferation stops at around the time that the testis reaches its maximum weight, mode 2 of Sertoli cell proliferation continues in the context of completing the development of existing cysts.

The production of new cysts also requires the production of single type A_{und} spermatogonia derived from self-renewal divisions of SSCs. The self-renewal of SSCs is observed when they divide in a specific niche environment, while displacement from the niche would result in the tipping of the signaling balance toward differentiating cell division (see Chapter 4). SSCs are among the single type A_{und} spermatogonia that are present in all vertebrates; however, in mice, paired or aligned undifferentiated spermatogonia can also retain stem cell potency [27]. In the cystic type of spermatogenesis, SSCs are not in contact with the basement membrane and are enveloped by Sertoli cells, which are thought to contribute importantly to the niche characteristics. To survive, a newly formed, single type A_{und} spermatogonium must recruit its own Sertoli cell support to form a new spermatogenic cyst. As shown in Figure 13.7, Sertoli cells proliferate excessively at the beginning of the testicular growth phase in the seasonally reproducing Atlantic salmon, which transiently leads to a situation in which a single type A_{und} spermatogonium is either surrounded by several Sertoli cells that nevertheless keep proliferating, or in which Sertoli cells form groups that are not in contact with germ cells (yet).

What is the available information regarding the regulation of mode 1 of Sertoli cell proliferation? Studies in different fish species have shown that a low dose of estrogen stimulates SSC self-renewal (eel [26]; huchen [28]; medaka [29]). In eel, this effect involved estrogen receptor-dependent expression of a Sertoli cell-derived growth factor [26]. Although it has not been studied directly, we believe that this stimulatory effect of low estrogen doses is associated with the production of new spermatogenic cysts which must involve mode 1 of Sertoli cell proliferation.

Thyroid hormones exert well-known (inhibitory) effects on Sertoli cell proliferation in mammals [30]. Studies in zebrafish showed, however, that in contrast to findings in rodents, triiodothyronine (T3) increased the mitotic index of Sertoli cells, in particular of Sertoli cells not associated with germ cells (yet) or associated with type A spermatogonia. Moreover, proliferation of type A_{und} spermatogonia was stimulated as well. Jointly, these observations suggest that T3 increases the available niche space by stimulating mode 1 of Sertoli cell proliferation, resulting in the production of new spermatogenic cysts containing a single type A_{und} spermatogonium [9]. T3-stimulated proliferation involves the Igf signaling system because the T3 effect on proliferation is abrogated fully (A_{und}) or partially (Sertoli cell) by an Igf receptor inhibitor. Interestingly, fish gonads express the *igf3* gene, a new Igf family member [31] that may have arisen from the *igf1* gene in a neofunctionalization event in the context of the teleost-specific whole genome duplication.

Thyroid hormone receptors are expressed by Sertoli cells in zebrafish [9], as is Igf3 protein/mRNA (see below), and exposure to T3 increased testicular *igf3* mRNA levels so that T3-stimulated proliferation of spermatogonia and (part of) Sertoli cell proliferation are mediated by Sertoli cell-derived Igf3.

A point not studied yet in this concept is that, as mentioned above, we believe that mode 1 of Sertoli cell proliferation is fueled by a Sertoli cell progenitor or stem cell population. Therefore, we must postulate a niche for Sertoli cell progenitor cells as well. Signaling molecules that are potentially relevant to the SSC niche and are derived from other somatic elements (e.g., myoid, Leydig, or endothelial cells) but also from Sertoli cell progenitors themselves might be relevant to a potential Sertoli cell niche in the fish testis.

The *igf3* gene provides a link to the next aspect of regulation of Sertoli cell proliferation, the role of gonadotropins, given that FSH is a more potent stimulator of *igf3* gene expression than T3 in the zebrafish testis [32]; stimulatory effects of FSH on *igf3* mRNA have also been described for other species (e.g., rainbow trout [33]).

With regard to the biological activity of piscine gonadotropins, cellular localization data show that Leydig cells express the receptors for FSH and LH, which both stimulate steroidogenesis directly, while Sertoli cell functions are predominantly regulated by FSH [1]. Consequently, an important difference for the situation in higher vertebrates is that FSH in fish is also a potent steroidogenic hormone, while also regulating Sertoli cell activities. Most information on circulating FSH levels in male fish is available from salmonid species [34–36]; these species have annual reproductive cycles or their reproduction occurs only once in a lifetime. Transiently elevated FSH blood levels typically are associated with the period of spermatogonial proliferation at the beginning of the testis growth phase. FSH blood levels increase again when spermiation becomes increasingly prominent, but they decrease before the actual spawning season commences, at which time prominent increases in plasma LH levels occur.

In mammals, FSH is an important regulator of Sertoli cell proliferation and adult Sertoli cell function. Many of these functions were uncovered by studies that analyzed experimentally induced or spontaneous FSHR loss-of-function mutations in animal models or humans [37].

What do we know about the possible roles of FSH as a direct regulator of Sertoli cell activities in fish? With respect to Sertoli cell proliferation, it is tempting to speculate that elevated FSH plasma levels at the start of spermatogonial proliferation are important. In support of this concept, we found that incubating adult zebrafish testis tissue with recombinant zebrafish FSH in the presence of trilostane (which inhibits the production of biologically active steroid hormones) stimulated the proliferation of Sertoli cells and of type A_{und} and type A_{diff} spermatogonia (Nóbrega, Morais, França, Schulz, Bogerd; unpublished results), suggesting that not only can new cysts be formed but also that the further differentiation of existing cysts is stimulated. Experiments in prepubertal male sea bass that were injected with recombinant FSH point in the same direction. Next to plasma androgen levels, testis growth was stimulated, along with Sertoli cell and germ cell proliferation; postmeiotic stages of development were reached 23 days after injection [38]. In

adult African catfish, surgical removal of one testis induced increased androgen release and *fshr* mRNA levels in the contralateral, remaining testis, which were associated with increased proliferation activity of Sertoli cells and spermatogonia type A_{und}, that is compatible with activated FSH signaling [39].

As mentioned above, FSH is a potent steroidogenic hormone in fish and induces the testicular release of 11-ketotestosterone (11KT), the main androgen in fish [40,41]. In a tissue culture system with prepubertal eel testis (which is rich in type A spermatogonia), incubation with 11KT induced full spermatogenesis, such that all stages of germ cell development were present [42]. This stimulatory effect of 11KT has been observed in other species as well (e.g., Japanese huchen [28] and zebrafish [43]). Although the effects of FSH/androgen on mode 2 of Sertoli cell proliferation, which accompanies the growth of differentiating cysts, have not been studied directly yet, we predict that FSH/androgen stimulation of germ cell proliferation toward advanced spermatogonial generations will also stimulate mode 2 of Sertoli cell proliferation.

A progestin is relevant to spermatogenesis in fish. There are two peaks of circulating 17α,20β-dihydroxy-4-pregnen-3-one (DHP) during the reproductive cycle of salmonid fish—a prominent peak in the spawning season and a small peak during the progression of spermatogonial proliferation and entry into meiosis [44−46]. Studies in Japanese eel showed that DHP stimulated spermatogonial DNA synthesis and was required to initiate meiosis [47]. A strong, stimulatory effect of DHP on spermatogenesis was also reported in adult zebrafish [48]. In this study, spermatogenesis was first interrupted by estrogen-induced androgen insufficiency and was then restarted by exposure to DHP, which induced proliferation of type A and B spermatogonia and entry into meiosis.

In an individual spermatogenic cyst, the Sertoli cell number increases steadily during the mitotic phase and levels off during meiosis to reach its maximum value upon completion of meiosis and the start of spermiogenesis, when tight junctions are also established between neighboring Sertoli cells [2,5]. We have explained that estrogens, thyroid hormones, and FSH can stimulate mode 1 of Sertoli cell proliferation, while FSH, androgens, and progestins can stimulate mode 2 of Sertoli cell proliferation (Figure 13.8), which stops when spermiogenesis starts. Leaving the level of a single cyst towards the tissue level in seasonally reproducing species, mode 1 of Sertoli cell proliferation (i.e., the production of new spermatogenic cysts) stops at a certain moment, after which mode 2 continues until all cysts have entered spermiogenesis. When spermiation follows, these species typically show spermatogenic tubules filled with spermatozoa and a limited number of quiescent cysts with single type A_{und} spermatogonia scattered along the basement membrane and awaiting the start of the subsequent spermatogenic wave [1]. Studies in Atlantic salmon showed that the cessation of the production of new cysts was associated with a decrease in the expression of *fshbeta* mRNA in the pituitary gland [49]. Because FSH plasma levels decrease in salmonids at this stage [35], one possible explanation for the cessation of the production of new cysts is reduced FSH stimulation. However, it is also possible that FSH receptor expression decreases in Sertoli cells in association with type A_{und} spermatogonia in testes that have reached their seasonal

maximum weight, which might bear some similarity to the stage-dependent (i.e., induced by the specific germ cell composition) changes occurring in FSH receptor mRNA levels in adult rat testis [50].

III. Sertoli cell functions

After discussing different aspects of Sertoli cell proliferation, we now review Sertoli cell functions, starting with the function of these cells as paracrine communicators that translate endocrine signals into locally acting signals, and potentially also respond to signals from the germ cells.

A. Paracrine relay station

With respect to germ cell signaling to Sertoli cells, experimental evidence has yet to be published in fish. However, ongoing ultrastructural work in our laboratory showed that a single Sertoli cell can be in contact with different germ cell clones in quite different stages of spermatogenesis (Figure 13.5). We believe that Sertoli cells in this situation receive signals from the two different germ cell clones and respond appropriately. In rats, the density of undifferentiated spermatogonia modulates growth factor production by Sertoli cells [51], and the changing germ cell composition during the epithelial cycle is associated with changes in FSH receptor expression [50]. In zebrafish, the transforming growth factor (Tgf) β family member anti-Müllerian hormone (Amh) is produced by Sertoli cells in contact with type A spermatogonia. However, *amh* gene expression is downregulated when Sertoli cells contact type B spermatogonia or at later stages of germ cell development [43]. As mentioned above, there is no direct experimental evidence available yet to show that germ cells modulate Sertoli cell functions in fish, so we turn to the opposite direction of information flow, Sertoli cell signaling to germ cells.

When we discussed the regulation of Sertoli cell proliferation, we introduced the concept of the major endocrine input received by Sertoli cells through FSH and via the steroidogenic activity of (in fish) FSH and LH. All three types of sex steroids modulate Sertoli cell functions. In some selected cases, (part of) the molecular mechanisms involved have been elucidated, and these findings are summarized below.

With respect to the early stages of spermatogenesis, we referred to the stimulatory effects of low estrogen levels and thyroid hormone on the production of single type A_{und} spermatogonia. For thyroid hormone, this stimulation also involved Igf3. The effect of low estrogen levels is mediated by a nuclear estrogen receptor located in Sertoli cells that triggers the expression of platelet-derived endothelial cell growth factor (Pdecgf); recombinant Pdecgf mimics estrogen's effects and was designated in eel as an SSC renewal factor [26].

Although thyroid hormone is stimulatory, FSH has a much stronger effect on upregulating *igf3* mRNA in zebrafish Sertoli cells (Nobrega, Morais, de Waal, França, Schulz, Bogerd, unpublished results). These ongoing studies also show that zebrafish Igf3 stimulates the mitotic indices of A_{und} and A_{diff} spermatogonia

without modulating androgen production. Still, FSH does have clear steroidogenic potency, and sex steroids have clear effects on Sertoli cell functions. As in other vertebrates, the androgen receptor is expressed by Sertoli cells but not by germ cells, and in zebrafish in particular, by Sertoli cells in contact with type A spermatogonia [52]. In general, the stimulatory effect of androgens on spermatogenesis and Sertoli cell proliferation has already been discussed, while little is known yet about the molecular mechanisms involved in fish. One exception is a finding in eel that androgen-stimulated Inhba (activin) production is involved in the stimulation of spermatogonial proliferation toward meiosis [53].

FSH-driven androgen production also provides a cross-link to thyroid hormone effects. In zebrafish, thyroid hormone receptors are not only expressed by Sertoli cells but also by Leydig cells, and thyroid hormone potentiates the stimulatory effect of FSH on androgen production [9]. In this way, thyroid hormone in the presence of low FSH levels stimulates the production of new spermatogenic cysts (as discussed earlier), while in combination with FSH, differentiation-inducing signals prevail in the context of the upregulation of Igf3 production and the potentiation of steroidogenic effects.

Also relevant to FSH is the Tgf β family member Amh. Fish do not have Müllerian ducts, and Amh probably has evolutionarily older functions in teleosts. Amh inhibited the onset of gonadotropin- or androgen-stimulated spermatogenesis in prepubertal Japanese eel [54]. Later work in adult zebrafish showed that Amh inhibited FSH-stimulated androgen production by downregulating the expression of steroidogenesis-related genes, resulting in reduced androgen release. Amh also inhibited androgen-supported spermatogenesis by blocking the differentiation of type A spermatogonia, resulting in an accumulation of type A_{und} spermatogonia [43]. Finally, FSH downregulated *amh* mRNA levels in adult zebrafish testis. Taken together, these observations place FSH in a central position with respect to the switch to differentiation—FSH downregulates Amh expression in Sertoli cells, which would otherwise inhibit differentiation of type A_{und} spermatogonia and compromise steroidogenesis. At the same time, FSH directly stimulates Leydig cell androgen production, which is potentiated by thyroid hormone. FSH also stimulates the expression of Igf3, which promotes proliferation of Sertoli cells and of type A spermatogonia. Thus, the biological activities of FSH coordinate signaling systems to increase the number of spermatogenic cysts and then support their progress through the mitotic phase of spermatogenesis.

Other research, again based on the eel model, revealed a signaling system regulated by the fish-specific progestin that becomes relevant when germ cells approach meiosis. DHP, which is typically also found in male fish, induced trypsin expression [55] and taurine biosynthesis [56] in Sertoli cells. Trypsin then stimulated germ cells to express a solute carrier gene (*slc6a6*) that functions as a taurine transporter so that elevated taurine levels in germ cells could trigger germ cell entry into meiosis [57].

B. Spermiation

In most fish species, spermatogenic cysts open after completion of spermiogenesis, so that the lumen of the opening cyst becomes continuous with the lumen of the

spermatogenic tubule. The close contact between germ cells and Sertoli cells is broken and spermatozoa are released. This process is called "spermiation." In mammals, spermiation takes place at a specific stage of the cycle of the germinal epithelium that is known to be particularly dependent on androgens, and androgen signaling is required for the disintegration of the ectoplasmic specializations that connect late spermatids and Sertoli cells [58,59].

Experimental data on cellular and molecular mechanisms operating in Sertoli cells during spermiation in fish are limited. Data in Atlantic salmon indicate that spermiation is associated with elevated plasma androgen levels; the mitotic, meiotic, and most of the spermiogenic phases take place in the presence of plasma androgen levels (15−20 ng/ml) that are clearly higher than the levels found in immature fish (<2 ng/ml). However, the transition to large-scale spermiation, which leads to the typical histological picture of mature testes, in which the tubular lumen is filled with (and serves as a storage place for) spermatozoa, is associated with a second step of plasma androgen level increase to >40 ng/ml [49]. However, a direct role of androgens in spermiation has not been demonstrated yet. In fish, the highest levels of circulating progestins are observed in fully mature males in seasonally reproducing species. Progestins like DHP or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) are believed to induce or advance spermiation in salmonid and cyprinid fishes [60], increasing testicular fluid production [61,62], and stimulating spermatozoa motility [63,64] by altering the pH and fluidity of the seminal fluid [65].

In a number of taxonomic groups of fish (e.g., opheliidae [66], scorpaenidae [67], bleniidae [68], and soleidae [18]), spermiation takes place shortly after the completion of meiosis and spermatogenesis is referred to as "semicystic" [66]. In bullfrogs, cysts also open early (Figure 13.3), but spermatids remain in close contact with Sertoli cells until the completion of spermiogenesis; in this species, opening of the cyst and spermiation are separate events. In fishes with semicystic spermatogenesis, round spermatids complete spermiogenesis while in a free state in the tubular lumen. The Sertoli cells that formed the cysts are transformed into a single-layered epithelium lining the tubular lumen, and these Sertoli cells may still provide support to spermatids. Spermiogenesis in fish is less complex than in tetrapod vertebrates, as suggested by the absence of an acrosome in teleost spermatozoa and by its short duration (e.g., 2 days in zebrafish [5]). Direct, individual contact between Sertoli cells and spermatids during spermiogenesis may not be available to all members of spermatid clones in teleosts in general because the clone size usually amounts to hundreds of cells while only the peripheral layer(s) seem to have easily access to direct Sertoli cell contact.

C. *Phagocytosis of apoptotic germ cells and removal of residual sperm*

Fish Sertoli cells are astonishing with regard to their efficiency as phagocytotic cells, which is part of the normal physiological function of Sertoli cells. Electron microscopy studies have demonstrated Sertoli cell phagocytic activity and shown

vesicles with hydrolytic enzymes, lysosomes, or phagosomes with degenerating spermatids and spermatozoa. In addition, residual bodies were observed in the cytoplasm of Sertoli cells. Acid phosphatase in Sertoli cell lysosomes is a marker of phagocytic activity in fish and rodents [69,70]. Altogether, these morphological features indicate the capability of Sertoli cells to remove residual bodies during spermiogenesis, apoptotic germ cells (in fish only 30−40% loss compared to the expected number [3,5,17]), and residual sperm after spermiation. These characteristics have been reported for the Sertoli cells of several teleost species, especially after spermiation and at the end of a spawning season [71−73].

In many fishes, the tubular lumen is an important sperm storage site. At the end of a spawning season, residual spermatozoa can remain in the tubular lumen. These spermatozoa are phagocytized by the Sertoli cells that form the epithelial lining of spermatogenic tubules, an aspect of Sertoli cell physiology that is absent in higher vertebrates in which derivatives of mesonephric tissue form efferent duct and sperm storage tissues. Interestingly, the clearing of residual sperm is completed before the next wave of spermatogenesis can start. The timing of these processes differs locally because neighboring tubules can have rather different activities. For example, phagocytosis of residual sperm was ongoing in one tubule in which spermatogonia seemed to be quiescent, while spermatogonial proliferation resumed in an adjacent tubule in which residual sperm were not visible [11,74]. This finding suggests that local signaling, potentially originating from Sertoli cells still occupied with phagocytosis, can suppress the resumption of spermatogonial proliferation in the vicinity. No information is available about the endocrine regulation (if it does exist) of the phagocytotic activity of Sertoli cells in fish. In rats, phagocytosis mediated by acid phosphatase in Sertoli cells is not hormone dependent [69]. In humans, however, testicular acid phosphatase gene expression is upregulated by androgens and downregulated by estrogens [75].

D. Fate after completion of cyst development

The lifelong generation of new Sertoli cells along with the production and differentiation of spermatogenic cysts supports the hypothesis that Sertoli cells are at least partially lost when a cyst has completed its development, in particular because seasonally reproducing species show large increases and then similarly large decreases in testis weight after the spawning season before embarking on the next season's growth phase.

So far, clear evidence for Sertoli cell apoptosis is only available for sharks. In the shark testis, developing cysts migrate during development through the testis from the dorsal to the ventral surface, where cysts open to release sperm into an efferent duct system while the cyst-forming Sertoli cells become apoptotic [76]. There are no results available in teleosts or amphibians yet. However, it is conceivable that during spermiation, some Sertoli cells are lost, perhaps especially those Sertoli cells that had a mainly adluminal position and did not contact another germ cell clone. Other Sertoli cells—perhaps preferentially those that had extended contact areas with the basement membrane—can become integrated into the epithelial lining of the spermatogenic tubule, while Sertoli cells that do contact another germ cell clone may continue to support this clone [19].

IV. Concluding remarks

From a comparative point of view, there are, in our opinion, two main differences worth pinpointing between anamniote and amniote Sertoli cells. One difference is the astonishing plasticity of the numbers and stages of differentiation of Sertoli cells in the adult anamniote testis. The other difference is the considerably higher germ cell-supporting capacity. With respect to the first point, it will be exciting to investigate further the presence, characteristics, and regulation of Sertoli cell precursor cells and to search for formal proof for the presence of a true somatic stem cell population in the anamniote testis. The potential to provide additional Sertoli cells via mode 1 and the tailored increase in Sertoli cell number during the progress of cystic spermatogenesis via mode 2 of Sertoli cell proliferation may be part of the "secret" of the high capacity of anamniote Sertoli cells to support germ cells. However, several other structural and functional aspects of Sertoli cell/germ cell interaction probably contribute to this high efficiency and await clarification and comparative analysis in higher vertebrates. For both issues, we wish to stress the equal importance of developing technical approaches on the molecular, physiological, and morphological levels.

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