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Endocrine and paracrine regulation of zebrafish spermatogenesis: the Sertoli cell perspective

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Abstract

Spermatogonial stem cells (SSCs) either self-renew or differentiate into spermatogonia that further develop into spermatozoa. Self-renewal occurs when residing in a specific micro-environment (niche) while displacement from the niche would tip the signalling balance towards differentiation. Considering the cystic type of spermatogenesis in fish, the SSC candidates are single type A undifferentiated (A_{und}) spermatogonia, enveloped by mostly one niche-forming Sertoli cell. When going through a self-renewal cell cycle, the resulting new single type A_{und} spermatogonium would have to recruit another Sertoli cell to expand the niche space, while a differentiating germ cell cyle would result in a pair of spermatogonia that remain in contact with their cyst-forming Sertoli cells. In zebrafish, thyroid hormone stimulates the proliferation of Sertoli cells and of type A_{und} spermatogonia, involving Igf3, a new member of the Igf family. In cystic spermatogenesis, type A_{und} spermatogonia usually do not leave the niche, so that supposedly the signalling in the niche changes when switching from self-renewal to differentiation. Recombinant zebrafish (rz) Fsh down-regulated Sertoli cell anti-müllerian hormone (*amh*) mRNA levels, and rzAmh inhibited differentiation of type A_{und} spermatogonia as well as Fsh-stimulated steroidogenesis. Thus, for Fsh to efficiently stimulate testis functions, Amh bioactivity should be dampened. We also discovered that Fsh increased Sertoli cell Igf3 gene and protein expression; rzIgf3 stimulated spermatogonial proliferation and Fsh-stimulated spermatogenesis was significantly impaired by inhibiting Igf receptor signaling. We propose that in zebrafish, Fsh is the major regulator of testis functions and, supported by other endocrine systems (e.g. thyroid hormone), regulates Leydig cell steroidogenesis as well as Sertoli cell number and growth factor production to promote spermatogenesis.

Keywords: follicle-stimulating hormone, growth factors, Sertoli cells, sex steroids, spermatogenesis, spermatogonial stem cells, thyroid hormones, zebrafish.

Introduction

Sertoli cells (SCs) in the adult testis facilitate germ cell survival and development such that spermatogenesis can provide sufficient sperm cells to guarantee fertility. Different from amniote vertebrates, fish (and amphibians) show the cystic type of spermatogenesis. Still, the testicular anatomy in lower vertebrates follows the general vertebrate pattern: two compartments, germinative and interstitial, are separated from each other by a basement membrane and peritubular myoid cells (Schulz *et al*., 2010). The interstitium contains steroid hormone producing Leydig cells, blood vessels, immune cells and connective tissue that is continuous with the tunica albuginea. The (intra) tubular germinal epithelium holds - as usual - somatic SCs and germ cells. Mediated by the pituitary gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), Leydig and SCs, the former expressing receptors for both Fsh and Lh in fish, are integrated into endocrine regulatory circuits (Schulz *et al*., 2010). Since survival and development of germ cells depends on their continuous contact with SCs, SC numbers determine the spermatogenic capacity of a testis (Matta *et al*., 2002), rendering determination of the SC number an important aspect of testis physiology. Here, we will discuss SC proliferation as well as proliferation/differentiation of the associated germ cells in cystic spermatogenesis.

Sertoli cell proliferation

Development of existing spermatogenic cysts

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

In birds and mammals, SCs proliferate until puberty, resulting in the adult testis in a given number of resident, postmitotic SCs that support successive waves of spermatogenesis. During these waves, one SC supports germ cells in different (mostly mitotic) stages of development along the basolateral surface (basal compartment); inside the blood-testis-barrier, the lateral SC surface areas contact spermatocytes and early (round) spermatids, while apical/adluminal parts contact late (elongating) spermatids.

In anamniote vertebrates (fishes and amphibians), the functional unit of the germinal epithelium is the spermatogenic cyst. It consists initially of a single spermatogonium enveloped by the

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cytoplasmic extensions of usually one SC (see below for exceptions), thus forming a cyst. Upon entering the spermatogenic process, both the cyst-forming SCs and germ cells proliferate and differentiate. As a consequence, a species-specific number of SCs is associated with a cyst at a specific stage of spermatogenesis, thereby establishing predictable SC/germ cell ratios, for a given stage of germ cell development (Billard, 1969; Matta *et al*., 2002; Schulz *et al*., 2005; Leal *et al*., 2009). Since SCs associated with growing cysts are positive in proliferation assays, at least part of the increase in SC number per cyst is attributed to SC proliferation (Schulz *et al*., 2005). The major increase in cyst volume and SC number per cyst occurs during the mitotic expansion of spermatogonia (Matta *et al*., 2002; Schulz *et al*., 2005; Leal *et al*., 2009), so that mitotically active SCs are found regularly in the anamniote testis. The situation is complicated somewhat by the fact that a SC can contact two germ cell clones at the two sides of a cytoplasmic extension, a situation still being much less complex than in birds and mammals.

In fish, SC proliferation stops (very few of them still proliferate when associated with spermatids) when the germ cell clone completes meiosis and enters spermiogenesis, at which time also tight junctions are established among the SCs (Matta *et al*., 2002; Schulz *et al*., 2005; Leal *et al*., 2009). After completion of spermiogenesis, the cyst opens to release sperm into the tubular lumen (spermiation). Hence, the seminiferous epithelium in anamniote vertebrates is composed of spermatogenic cysts, in which different germ cell clones (i.e. descendants of different stem cells and usually in different stages of development) are taken care of by mitotically active SCs enveloping the clone until spermiation. This also seems to imply that an individual SC is not a resident but rather a transient cell in the anamniote testis.

Generation of new spermatogenic cysts; Sertoli cell progenitors

Next to the SC proliferation accompanying the development of an existing spermatogenic cyst, a second mode of SC proliferation is observed in fish in association with the production of new spermatogenic cysts. Since germ cells cannot survive unless being in close contact to SCs, we assume that first, new SCs are produced, thereby creating a niche into which a newly formed, single type A_{und} spermatogonium, a SSC candidate, can be recruited. This assumption would explain an observation made in the seasonally reproducing Atlantic salmon, in which at the beginning of the testis growth phase groups of SCs appear that are not (yet) in contact with germ cells, or where several SCs are grouped around a single type A_{und} spermatogonium. In this setting, it seems possible that similar to recent observations in mice (Dovere *et al*., 2013), SCs produce a paracrine factor (GDNF) that increases the number of SSCs in the vicinity of these SCs, for example by stimulating SSC self-renewal divisions. Interestingly, in rainbow trout, Gdnf and its receptor have been detected in type A_{und} spermatogonia, suggesting that this factor can also function in an autocrine loop (Nakajima *et al*., 2014). Recent studies in adult zebrafish revealed that thyroid hormone and a growth factor of the Igf family (see below) link proliferation of SCs and of single type A_{und} spermatogonia (Morais *et al*., 2013). Therefore, we assume that first SCs are generated to provide new niche space that then can be occupied by single type A_{und} spermatogonia.

Cyst production occurs at different stages of the life cycle and the intensity can vary with the reproductive strategy of a species, being particularly evident in seasonally reproducing species. Irrespective of the reproductive strategy, cyst generation takes place in the period between completion of male sex differentiation and puberty, and populates the often not more than allometrically growing prepubertal testis with cysts containing mainly type A_{und} spermatogonia. Many fish species display life-long growth and a certain level of (allometric) testis growth by generating new cysts and SCs continues after puberty.

In seasonally reproducing species, large (e.g. 50-fold), seasonally recurrent changes in testis weight are recorded (Billard and Breton, 1978; Almeida *et al*., 2008). 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, which switches to a rapid growth period when cysts go through spermatogenesis. At a certain point in time, the generation of new cysts ceases, existing cysts continue to develop and complete spermatogenesis. Fully mature testes in these species typically show tubular lumina filled with spermatozoa while a few quiescent cysts with a single type A_{und} spermatogonium represent the startup reservoir for next season's spermatogenic wave.

Hence, in seasonally reproducing species, SC proliferation for the formation of new cysts is activated at the beginning of the seasonal testicular growth phase, and ceases to initiate the completion of the ongoing spermatogenic wave. On the other hand, many species living in (sub)tropical zones display continuous spermatogenesis after puberty, prominent examples as experimental models being medaka, tilapia and zebrafish. In these species, SC proliferation in the context of formation of new cysts is observed continuously after puberty.

We assume that when new SCs are required to generate new cysts, they are derived from a precursor that may have stem cell characteristics. There is no proof for this assumption, but the presence of such an undifferentiated somatic cell population may explain the long-term capacity to produce new SCs during successive reproductive seasons, the fully functional, experimentally

inducible (Shibata and Hamagushi, 1988; Kobayashi *et al*., 1991) sex reversal in adults in several species, or the natural sexual plasticity seen in sequentially hermaphroditic species (Kobayashi *et al*., 2009), even suggesting that a somatic cell precursor is sufficiently undifferentiated to assume either a male (Sertoli) or a female (granulosa) identity.

Regulation of Sertoli cell proliferation

The two modes of SC proliferation, one in context with the production of new spermatogenic cysts (mode 1), the other in context with the growth and development of existing cysts (mode 2) seem to be regulated differently, since these modes can be observed independently of each other. In seasonally reproducing species, such as the Atlantic salmon, SC proliferation for cyst formation (mode 1) starts earlier than the second mode of SC proliferation. The latter commences once cysts have started to differentiate, thereby reaching a size that requires an increase of SC number to accommodate the growing germ cell clone. When mode 2 of SC proliferation starts mode 1 is still ongoing. Later in the season, mode 1 of SC proliferation stops while mode 2 continues with already existing cysts completing their development.

The production of new cysts also requires the production of single type A_{und} spermatogonia derived from self-renewal divisions of SSCs. SSCs are among the single type A_{und} spermatogonia in all vertebrates; however, in mice, also paired or aligned undifferentiated spermatogonia can generate stem cells (Yoshida, 2012). In the cystic type of spermatogenesis, SSCs do not contact the basement membrane and are enveloped by usually one SC, which are thought to contribute importantly to the niche characteristics. In order to survive, a newly formed, single type A_{und} spermatogonium has to recruit its own SC support to form a new spermatogenic cyst.

Thyroid hormones exert well-known (inhibitory) effects on SC proliferation in mammals (Cooke *et al*., 2005). Recent studies in zebrafish showed, however, that in contrast to findings in rodents, tri-iodothyronine (T3) increased the mitotic index of SCs, in particular of SCs 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 suggested that T3 increased the available niche space by stimulating mode 1 of SC proliferation, resulting in the production of new spermatogenic cysts containing a single type A_{und} spermatogonium (Morais *et al*., 2013). T3-stimulated proliferation involved the Igf signaling system, since the T3 effect on proliferation was abrogated fully (A_{und}) or in part (SC), by an Igf receptor inhibitor. Interestingly, fish gonads express the *igf3* gene, a new Igf family member (Wang *et al*., 2008) that may have arisen as an *igf1* paralog in a neo-functionalization event in context with the teleost-specific whole genome duplication. Thyroid hormone receptors are expressed by SCs in zebrafish (Morais *et al*., 2013), as is Igf3 protein/*igf3* mRNA, and exposure to T3 increased testicular *igf3* mRNA levels. Since T3-stimulated proliferation of spermatogonia and (part of) SC proliferation can be blocked by an Igf receptor inhibitor, we can conclude that T3-induced effects are mediated by SC-derived Igf3.

The *igf3* gene provides a link to the next aspect of regulation of SC proliferation, the role of gonadotropins, considering that Fsh is a more potent stimulator of *igf3* gene expression than T3 in the zebrafish testis (Morais *et al*., 2013).

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 directly stimulate steroidogenesis, while Sertoli cell functions are predominantly regulated by Fsh (Garcia-Lopez *et al*., 2010). Consequently, an important difference to the situation in higher vertebrates is that Fsh in fish is also a potent steroidogenic hormone, next to regulating SC activities. Most information on circulating Fsh levels in male fish is available from salmonid species (Prat *et al*., 1996; Gomez *et al*., 1999; Campbell *et al*., 2003), showing annual or once in a lifetime reproductive cycles. Transiently elevated Fsh blood levels were associated with the period of spermatogonial proliferation at the beginning of the testis growth phase. Fsh blood levels re-increased when spermiation becomes increasingly prominent, but decreased before the actual spawning season commenced, at which time prominent increases in plasma Lh levels occurred.

In mammals, FSH is an important regulator of SC proliferation and adult SC functioning. Many of these FSH functions were uncovered by analyzing experimentally induced or spontaneous FSHR loss-of-function mutations in animal models or patients (Huhtaniemi and Themmen, 2005). What do we know about the possible roles of Fsh as a direct regulator of SC activities in fish?

As regards SC proliferation, it is tempting to speculate that the elevated Fsh plasma levels in salmonid fish at the start of spermatogonial proliferation are important. Experiments with prepubertal male sea bass injected with recombinant Fsh support this notion: plasma androgen levels and testis growth was stimulated, along with SC and germ cell proliferation and postmeiotic stages of development were reached 23 days after injection (Mazón *et al*., 2014). In adult African catfish, surgical removal of one testis increased androgen release and elevated *fshr* transcript levels in the remaining testis, associated with an increased proliferation activity of SCs and type A_{und} spermatogonia, observations that are all compatible with activated Fsh signaling (Schulz *et al*., 2012).

As mentioned above, Fsh is a potent steroidogenic

hormone in fish, and induces the testicular release of 11-ketotestostrone (11-KT), the main androgen in fish (Idler *et al*., 1961; Borg, 1994). In a tissue culture system with prepubertal eel testis (rich in type A spermatogonia), 11-KT induced full spermatogenesis (Miura *et al*., 1991). This stimulatory effect of 11-KT has been observed also in other species, e.g. Japanese huchen (Amer *et al*., 2001) and zebrafish (Skaar *et al*., 2011). Although Fsh/androgen effects on mode 2 of SC proliferation, accompanying the growth of differentiating cysts, have not been studied directly yet, we predict that germ cell development towards advanced spermatogonial generations will also stimulate mode 2 of SC proliferation, considering the recorded increases in SC numbers per cyst (Schulz *et al*., 2005; Leal *et al*., 2009).

Next to androgens, also a progestine is relevant for 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 (Dépêche and Sire, 1982; Scott and Sumpter, 1989; Vizziano *et al*., 1996). In Japanese eel, DHP stimulated spermatogonial DNA synthesis and moreover was required for initiating meiosis (Chen *et al*., 2013). A strong, stimulatory effect of DHP on spermatogenesis has also been reported in adult zebrafish (Miura *et al*., 2006). In this study, spermatogenesis was first interrupted by an estrogen-induced androgen insufficiency *in vivo*, and was then restarted by exposure to DHP in tissue culture, which induced proliferation of type A and B spermatogonia and entry into meiosis.

Considering an individual spermatogenic cyst, the SC number increases steadily during the mitotic phase, levels off during meiosis to reach the maximum value upon completion of meiosis/start of spermiogenesis, when also tight junctions are established between neighboring SCs (Matta *et al*., 2002; Leal *et al*., 2005). Switching from the level of a single cyst to the tissue level in seasonally reproducing species, mode 1 of SC proliferation for the production of new spermatogenic cysts stops at a certain moment, after which mode 2 still continues, until all cysts have entered spermiogenesis. At this point, testis weight usually reached maximum values, before decreasing due to the loss of germ cell mass in the context of spermiogenesis. When then spermiation occurs, 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 awaiting the start of the subsequent spermatogenic wave (Schulz *et al*., 2010). Recent studies in Atlantic salmon showed that the stop of the production of new cysts was associated with a decrease in the expression of *fshb* mRNA in the pituitary (Melo *et al*., 2014). Since Fsh plasma levels decrease in salmonids at this stage (Gomez *et al*., 1999), one possible explanation for the stop of the production of new cysts is a reduced Fsh stimulation.

Sertoli cell functions

After discussing different aspects of SC proliferation, we review SC functions, starting with their role as paracrine communicator translating endocrine into local signals.

Paracrine relay station

With respect to germ cell signaling towards SCs, experimental evidence has yet to be published in fish. In rat, the density of undifferentiated spermatogonia modulates growth factor production by SCs (Johnston *et al*., 2011), and the changing germ cell composition during the epithelial cycle is associated with changes in FSH receptor expression (Kliesch *et al*., 1992). However, as there is no direct experimental evidence available yet that germ cells modulate SC functions in fish.

When discussing SC proliferation, we have introduced the major endocrine input to SCs, via Fsh and sex steroids. In some selected cases, (part of) the molecular mechanisms involved have been elucidated and this will be summarized below.

Considering early stages of spermatogenesis, we have mentioned the stimulatory effects of thyroid hormone on the production of single type A_{und} spermatogonia, which also involved Igf3 (Morais *et al*., 2013). However, Fsh has a much stronger effect on up-regulating *igf3* mRNA in zebrafish SCs than thyroid hormone (Nóbrega, Morais, de Waal, França, Schulz, Bogerd, 2014, Utrecht University, The Netherlands; IB/Unesp, Botucatu, SP, Brazil; ICB/UFMG, Belo Horizonte, MG, Brazil; 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 of SC functions. As in other vertebrates, the androgen receptor is expressed by SC but not by germ cells, in zebrafish in particular by SCs contacting type A spermatogonia (de Waal *et al*., 2008). The stimulatory effect of androgens on spermatogenesis and SC proliferation has already been mentioned, while little is known yet on the molecular mechanisms involved in fish. One exception is a finding in eel, showing that androgen-stimulated activin production is involved in the stimulation of spermatogonial proliferation towards meiosis (Miura *et al*., 1995), while androgens down-regulated the inhibitory factor anti-Müllerian hormone (Amh; Miura *et al*., 2002; see below).

Fsh-driven androgen production also provides a cross-link to thyroid hormone effects. In zebrafish, thyroid hormone receptors are not only expressed by SCs

but also by Leydig cells, and thyroid hormone potentiates the stimulatory effect of Fsh on androgen production (Morais *et al*., 2013). In this way, thyroid hormone in the presence of low Fsh levels stimulates the production of new spermatogenic cysts, while in combination with elevated Fsh levels, signals inducing differentiation would prevail in context with the up-regulation of Igf3 production and the potentiation of the steroidogenic effects of Fsh.

Also relevant in context with Fsh is the Tgfbeta family member Amh since, depending on the species, either Fsh (Skaar *et al*., 2011) or androgen (Miura *et al*., 2002) down-regulated testicular *amh* transcript levels. Fish do not have Müllerian ducts, and it is therefore likely that Amh has evolutionary older functions in teleosts. Amh inhibited the onset of gonadotropin- or androgen-stimulated spermatogenesis in prepubertal Japanese eel (Miura *et al*., 2002). In adult zebrafish, Amh inhibited Fsh-stimulated androgen production by down-regulating the expression of steroidogenesis-related genes. Moreover, Amh inhibited androgen-supported spermatogenesis by blocking the differentiation of type A spermatogonia (Skaar *et al*., 2011). Taken together, these observations place Fsh in a central position with respect to the switch to differentiation: Fsh down-regulates Amh expression in SCs, which would otherwise inhibit differentiation of type A spermatogonia and compromise steroidogenesis. At the same time, Fsh directly stimulates Leydig cell androgen production, which is potentiated by thyroid hormone. Back to SCs, Fsh also stimulates expression of Igf3, which promotes proliferation of SCs and of type A spermatogonia. Hence, the biological activities of Fsh coordinate signaling systems to increase the number of spermatogenic cysts and then to support their progress through the mitotic phase of spermatogenesis.

Recent work, again based on the eel model, unraveled a signaling system regulated by the fish-specific progestin that becomes relevant when approaching meiosis. DHP, typically found also in male fish, induced trypsin expression (Miura *et al*., 2009) and taurine biosynthesis (Higuchi *et al*., 2012) in SCs. Trypsin then stimulated germ cells to express the solute carrier gene *slc6a6* that functions as taurine transporter, allowing taurine to trigger germ cell entry into meiosis (Higuchi *et al*., 2013).

Fate after completion of cyst development

Considering the ongoing production of new cysts and SCs allows speculations about the at least partial loss of SCs after a cyst opened to release spermatozoa into the tubular lumen. In particular when considering seasonally reproducing species that show large increases and then similarly large decreases in testis weight after the spawning season, the loss of SCs seems very likely. So far, clear evidence for SC apoptosis is only available in the shark testis, where spermatogenic cysts migrate dorso-ventrally through the testis during spermatogenesis. Cysts then open to release sperm into a ventral efferent duct system while the cyst-forming SCs become apoptotic (McClusky, 2013). However, it is conceivable that also in teleost fish some SCs are lost during spermiation, perhaps in particular those SCs that had a mainly adluminal position and did not contact another germ cell clone. Other SCs can become integrated in the epithelial lining of the spermatogenic tubule, maybe preferentially those SCs that showed extended contact areas with the basement membrane, while SCs that did contact another germ cell clone may continue to support this clone (Koulish *et al*., 2002).

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