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Spermatogenesis in fish

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ABSTRACT

Spermatogenesis is a developmental process during which a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome. We characterise morphologically the different germ cell stages with particular attention for the spermatogonial generations, including the stem cells and their specific capacity to colonise a recipient's testis after transplantation. We propose a nomenclature for fish germ cells to improve the comparability among different teleost fish but also to higher vertebrates. Survival and development of germ cells depends on their continuous and close contact to Sertoli cells, and we review their multiple roles in the cystic mode of spermatogenesis seen in fish. We then discuss gene expression patterns associated with testis maturation. The endocrine system of vertebrates has evolved as master control system over spermatogenesis. In fish, both pituitary gonadotropins LH and FSH stimulate gonadal sex steroid hormone production directly by activating Leydig cells. Information is reviewed on the effects of progestin, androgens, and estrogens on global testicular gene expression patterns (microarray analysis), and on the molecular mechanisms by which steroids regulate specific candidate genes (identified by subtractive hybridization approaches) during early stages of testis maturation. Moreover, progestin and androgen effects on spermiation and milt hydration are discussed. Sex steroids mainly act via receptors expressed by Sertoli cells. One type of response is that Sertoli cells change growth factor expression, which subsequently modulates germ cell proliferation/differentiation via mechanisms yet to be characterised. Finally, we review data on germ cell autonomous processes, mainly derived from loss-of-function mutant fish lines, before identifying a number of focus areas for future research activities.

1. Introduction

The cellular basis of sexual reproduction in animals are haploid gametes, one sex producing a relatively small number of large gametes, eggs that are rich in reserves, the other sex producing a much larger number of small gametes, spermatozoa, which evolved as highly specialized, motile genome vectors. Although the development of eggs and sperm show common principles, many aspects of gametogenesis differ between sexes. With regard to oocyte development, the reader is referred to Lubzens et al. (2010). The present chapter will first deal with morphological aspects of male germ cell development, will then turn to gene expression patterns accompanying spermatogenesis, before discussing extrinsic (hormones and growth factors) and intrinsic (cell autonomous) regulation of germ cell development.

Fish are the most diverse and numerous group of vertebrates. Our knowledge on spermatogenesis in fish, however, is limited to a few species used in basic research and/or in aquaculture biotechnology (e.g. catfish, cod, eel, guppy, medaka, salmon, tilapia, trout, and zebrafish). While trying to distil general principles from the available information, we will also indicate knowledge caveats that require more research efforts, and be cautious with too detailed speculations that might melt soon in the light of new data.

An important aim of the morphological section is to characterise teleost spermatogenic cells. Considering the existing literature, we feel that in particular the identification and naming of the different spermatogonial generations needs clarification and standardization; also the different meiotic and post-meiotic stages will be discussed. We hope that this exercise will contribute to forming a basis necessary for comparative and applied research on spermatogenesis in fish, and allowing intra- and inter-species comparisons among fish, but also with higher vertebrates, where

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generally accepted classification systems for spermatogenic cells exist (Hess and França, 2007).

Attention will then be directed towards the transcriptomic characterization of germ cells, based on the recent publication of large-scale EST sequences, and using the rainbow trout as example (Govoroun et al., 2006; Mazurais et al., 2005).

In the following section, we will review the regulation of spermatogenesis via pituitary hormones (FSH, LH, GH), sex steroids, and locally produced growth factors. The reader is referred to Kah et al. and Levavi et al. (both this volume) as regards the brain and pituitary parts of the BPG axis.

The subsequent section will summarize the information in fish on processes operating in a germ cell-autonomous manner at specific stages of spermatogenesis, as opposed to the endocrine regulation of spermatogenesis via signalling molecules derived from sources outside germ cells.

Finally, we will discuss points that we consider as major knowledge caveats requiring significant research attention in the future. Here, we will encounter key-points of spermatogenic development, such as the equilibrium between stem cell self-renewal and differentiation. Some of these key-events are located at the cross-roads of basic and applied research (e.g. aquaculture biotechnology and ecotoxicology), therefore justifying particular attention.

2. Testis structure, spermatogenesis, and germ cell morphology

2.1. General structure of the teleost testis

In all vertebrates, from fish to mammals, the testis is composed of two main compartments, the intertubular (or interstitial) and the tubular compartment. The intertubular compartment contains steroidogenic Leydig cells, blood/lymphatic vessels, macrophages and mast cells, neural and connective tissue cells, the latter being continuous with the tunica albuginea (Koulish et al., 2002), i.e. the testis organ wall. The tubular compartment is delineated by a basement membrane and peritubular myoid cells and houses the germinal epithelium. This epithelium contains only two cell types, the somatic Sertoli cells and the germ cells, which are found at different stages of development. In vivo, germ cells can only survive in close and continuous interaction with Sertoli cells, so that Sertoli cell number determines the spermatogenic capacity of a testis and is an important target of signalling systems regulating spermatogenesis (Matta et al. 2002).

In amniote vertebrates (reptiles, birds, mammals) it is considered that Sertoli cells proliferate until puberty when only spermatogonia and a few early spermatocytes are present in the germinal epithelium. Hence, the adult amniote testis contains a fixed number of "immortal" Sertoli cells supporting successive waves of spermatogenesis. During these waves, a given Sertoli cell supports at the same time different developmental stages of germ cells (i.e. cells belonging to different clones; see below). For example, the Sertoli cell basis contacts spermatogonia, whereas lateral parts contact spermatocytes and early spermatids, and adluminal parts late spermatids (Fig. 1A).

In anamniote vertebrates (fishes and amphibians), on the other hand, we find the cystic type of spermatogenesis (Callard 1996). There are two main differences compared to the testis of higher vertebrates. First, within the spermatogenic tubules, cytoplasmic extensions of Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium (Fig. 1B). Second, the cyst-forming Sertoli cells retain their capacity to proliferate also in adult fish (Schulz et al., 2005). Hence, the basic functional unit of the spermatogenic epithelium in fish is a spermatogenic cyst formed by a dynamic group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone. Different clones being in different stages of development generate the typical histological picture of fish testes, where the tubular compartment contains differently sized groups of germ cells in different stages of spermatogenesis (Fig. 1B, inset). Comparing cystic and non-cystic spermatogenesis, cystic Sertoli cells might be more efficient in supporting germ cell development, e.g. by concentrating specific growth factors required for each developmental phase, resulting in a low percentage of germ cell apoptosis, and consequently high spermatogenic yield. The non-cystic Sertoli cells, on the contrary, are more diversified or regionalized in context with the complex requirements for nursing different germ cell clones at the same time.

Two types of spatial arrangements have been described in the literature for the teleost testes based on the distribution of spermatogonia in the germinal compartment (Grier, 1981). In the first type (restricted spermatogonial distribution), the distal regions of the germinal compartment, near the tunica albuginea, are occupied by Sertoli cells surrounding early, undifferentiated spermatogonia (see below for a more detailed description of spermatogonial generations). As the cells divide and enter in meiosis, the cysts migrate towards the region of the spermatic ducts located centrally in the testis, where spermiation occurs, i.e. the cysts open to release spermatozoa. This type of arrangement is found in the higher teleosts, such as in the order Atheriniformes, Cyprinodontiformes and Beloniformes (Parenti and Grier, 2004). In the second type (unrestricted spermatogonial distribution), spermatogonia are spread along the germinal compartment throughout the testis. The cysts do not migrate or get displaced during their development (Grier, 1981). The unrestricted distribution of spermatogonia is considered a more primitive pattern found in less evolved taxonomic groups, such as in the order Cypriniformes, Characiformes, and Salmoniformes (Parenti and Grier, 2004). However, also intermediate forms appear to exist between restricted and unrestricted spermatogonial distribution, such as found in Perciformes, tilapia *Oreochromis niloticus* (Vilela et al., 2003); Pleuronectiformes, *Solea senegalensis* (García-López et al., 2005) or Gadiformes, *Gadus morhua* (Almeida et al., 2008). In these species, undifferentiated spermatogonia show a preferred, but not exclusive, location close to the tunica albuginea. In cod, spermatogonia are derived from a germinative zone in the periphery of the testicular parenchyma, which produces new spermatogonial cysts (Almeida et al., 2008). This results in a zonation of the testis: early stages of development reside in the periphery and advanced stages are found close to the collecting duct, a situation arising through appositional growth that is fuelled by the cyst-generating activity of the germinative zone rather than through movement of developing cysts.

Some tropical species do not display apparent seasonal variations in spermatogenic activity. However, in many species with habitats at higher latitudes, reproduction is a seasonal or cyclic event related to environmental cues (Billard and Breton, 1978; Nash, 1998). Active spermatogenesis may take place in summer (trout, carp, pike), in spring (tench, bream, whiting, sea bream), or may begin in autumn and finish in spring (killifish, stickleback, roach) (Billard and Breton, 1978; Billard, 1986). For species with a clear-cut seasonal reproduction such as salmonids, pike, or Atlantic cod, cysts with rapidly proliferating spermatogonia can be observed only at the beginning of testicular recrudescence. In these species, tubules are filled after completion of spermatogenesis with only two types of germ cells, large numbers of spermatozoa and some scattered, quiescent type A spermatogonia (Billard, 1986). In other cyclic species, germ cells remaining in the regressed testis may be spermatogonia and spermatocytes (tench), or even all germ cell types, such as in goldfish, where the seasonal regression is of a quantitative rather than of a qualitative nature (Billard, 1986).

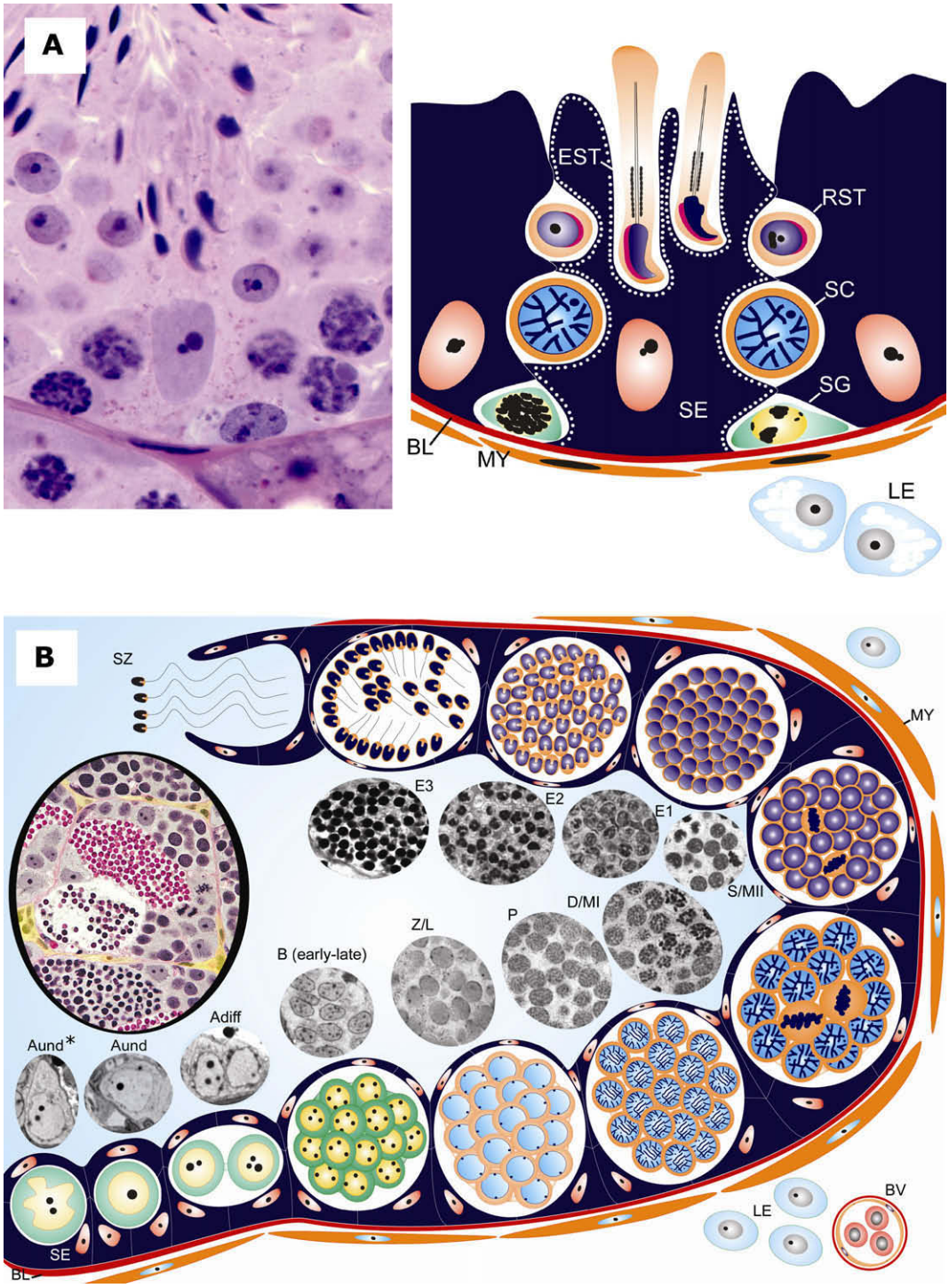

Fig. 1. Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between cystic (B) and non-cystic (A) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. (A) spermatogonia (SG); spermatocyte (SC); round spermatid (RST); and elongated spermatid (EST). (B) Type A undifferentiated spermatogonia (A_{und}^{*}) (stem cell?); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); spermatogonia type B [B (early–late)]; leptotenic/zygotenic primary spermatocytes (L/Z); pachytenic primary spermatocytes (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ).

2.2. Sertoli cells

The main Sertoli cell functions are to support germ cell survival, development, and physiological functioning. Moreover, Sertoli cells secrete fluid that generates the tubular lumen, and they phagocytise apoptotic germ cells, residual bodies discarded by spermatids during spermiogenesis, and residual sperm. Therefore, the development of spermatogenic cells strictly depends on their interaction with the somatic elements of the testis, amongst which Sertoli cells play a crucial role in animals in general. In invertebrates like in fruitfly, for example, Sertoli cells (also called cyst cells) are derived from somatic stem cells (hub cells). When cyst cells contact differ-

entiating germ cells, they form cysts similar to those in the testes of anamniote vertebrates (Decotto and Spradling, 2005). Sertoli cells are the first somatic cell type to differentiate in the vertebrate testis and this cell type plays a crucial role in directing testis differentiation and development (DiNapoli and Capel, 2008). Also, as already mentioned Sertoli cell number per testis quantitatively limits (spermatogenic ceiling) sperm production in sexually mature animals throughout the vertebrate phylum (Hess and França, 2007; Nóbrega et al., 2009).

In the cystic mode of spermatogenesis in fish, germ cell number and volume increases greatly per cyst during the spermatogenic process. Therefore, it is not surprising that the number of Sertoli cells per cyst also increases, which occurs in a predictable manner. A specific number of Sertoli cells is observed per cyst in a given species and for a given stage of germ cell development (Billard, 1969b; Matta et al., 2002). At least part of the increase in Sertoli cell number per cyst is based on Sertoli cell proliferation (Schulz et al., 2005). The major increase in cyst volume occurs during the period of rapid mitotic expansion of the spermatogonia, which may explain why proliferating Sertoli cells are mainly found associated with spermatogonial cysts. The increases in cyst volume and Sertoli cell number per cyst both level off during meiosis/start of spermiogenesis (Matta et al., 2002; Schulz et al., 2005). Intriguingly, this coincides with the formation of occluding or tight junctions between neighbouring Sertoli cells in fish (Silva and Godinho, 1989; Batlouni et al., 2009), establishing a privileged space for the late meiotic/spermiogenic germ cells. The reason for sealing off meiotic and post-meiotic cells may reside in the large number of meiosis- and spermiogenesis-specific germ cell gene products that may have to be shielded from the immune system on the one hand, and may require a specific microenvironment for proper functioning on the other hand.

It seems that in fish spermatogenesis, Sertoli cells are formed by mitosis just in time and in exactly the number required. This tailored Sertoli cell proliferation, first described in the guppy (Billard, 1969b), may be (part of) the background for the observation that cystic spermatogenesis is more efficient than the non-cystic spermatogenesis in mammals, as indicated by parameters like apoptotic germ cell loss (~30% in fish, Matta et al., 2002; approximately 60–80% in rat or mice; Hess and França, 2007), or the number of spermatids formed per Sertoli cell (~100 in guppy, tilapia, or zebrafish; Billard, 1969b; Nóbrega et al., 2009; 8–10 in rats and mice, Hess and França, 2007). Many fish species grow continuously during adulthood, including growth of the testis, so that part of the Sertoli cell proliferation may reflect postpubertal organ growth. Moreover, many species show a seasonally recurring growth-shrinkage cycle of testis weight and volume. In these species, it is possible that also Sertoli cells are lost during the post-spawning involution of the testes. Hence, Sertoli cells would have to be formed anew during the next reproductive season when spermatogonial proliferation is resumed. We speculate that fish testis contains, next to spermatogonial stem cells, a second precursor or stem cell population giving rise to somatic Sertoli cells. Considering that natural and steroid-induced male-to-female sex change is possible in fish (Guiguen et al., 2010), it moreover seems that the somatic precursor cell population can assume male (Sertoli) or female (granulosa) cell fate. Hence, the germ cell-supporting somatic cells in the fish testis show an astonishing developmental plasticity in terms of sexual fate and cell number. However, experimental proof for the existence of a somatic stem cell type, for example by cell lineage tracing experiments (Goenczy and DiNardo, 1996), is not available yet in fish.

Fish Sertoli cells are also astonishing with regard to their efficiency as phagocytotic cells. Usually, macrophages or other phagocytotically active cell types are not present in the tubular compartment of the testis, and phagocytosis is part of the physiological function of Sertoli cells. During spermiogenesis, spermatids shed the so-called residual bodies that contain superfluous cytoplasmic material that is discarded during the 'metamorphosis' to flagellated sperm. A certain level of apoptosis is normal and although fish spermatogenesis is comparatively efficient in this regard, still 30–40% of all germ cells that could be produced theoretically become apoptotic before differentiating to spermatozoa (Billard, 1969b; Vilela et al., 2003). Sertoli cells remove and recycle this material very efficiently, so that cellular debris rarely appears in spermatogenic tubules. Indeed, even when a loss-of-function of a protein required during meiosis leads to apoptotic loss of all spermatocytes in zebrafish (Feitsma et al., 2007), the fraction of apoptotic spermatocytes visible in histological sections from mutant males is only ~10% of the total number of spermatocytes, suggesting a rapid clearance of material phagocytised by Sertoli cells. In addition, in many seasonal breeders, Sertoli cells play an important role in the phagocytosis of residual sperm after the spawning season (Grier and Taylor, 1998; Almeida et al., 2008).

In mammals, formation of occluding junctions between Sertoli cells marks the end of the period of Sertoli cell proliferation and their terminal differentiation, so that Sertoli cells can now support meiotic and post-meiotic germ cell stages in the adluminal part of the germinal epithelium, segregated by occluding junctions from the basal compartment containing spermatogonia. An alike development can be observed in the teleost testis, although adapted to three specific conditions of cystic spermatogenesis. First, maximum cyst volume is attained when germ cells have progressed well into meiosis (pachytene spermatocyte stage) and it is therefore not surprising that Sertoli cell proliferation is observed – although levelling off from more intense proliferation during the spermatogonial phase – until the end of meiosis/beginning of spermiogenesis (Schulz et al., 2005). Strikingly, formation of occluding junctions and termination of Sertoli cell proliferation are temporally related also in different fish species (e.g. Bergmann et al., 1984). Second, terminal differentiation takes place in an asynchronous manner in the fish testis accompanying each spermatogenic wave and reflecting the fact that each cyst functions as an independent Sertoli/germ cell unit that follows its own developmental timing. This is in contrast to a general wave of Sertoli cell differentiation in a relatively short time window in the germinal epithelium of amniote vertebrates. Third, unlike mammals (Setchell, 1986), the Sertoli cell barrier in fish is established at the end of or after meiosis and only haploid germ cells are shielded from the vascular compartment and from the immune system. In a number of species, these junctions restrict the entry of large molecules into the cysts (Abraham et al., 1980; Bergmann et al., 1984; Leal et al., 2009) and in trout, experimental breaching of the barrier leads to the appearance of anti-sperm antibodies (Secombes et al., 1985). However, before occluding junctions are established, Sertoli cells are connected to each other and to germ cells by other types of intercellular junctions (Loir et al., 1995; Pudney, 1993; Grier, 1993; Batlouni et al., 2009).

Finally, the close contact between developing germ cells and Sertoli cells is broken by opening of the cysts, such that the lumen of spermatogenic cysts becomes continuous with the lumen of the spermatogenic tubules. This process is called spermiation and is homologous to the androgen-stimulated (O'Donnell et al., 2000) disintegration of the ectoplasmic specializations that connected late spermatids and Sertoli cells in higher vertebrates. In the literature, the term spermiation is used in two ways in fish: *Sensu strictu* to describe the termination of Sertoli-germ cell contact; in a broader sense to indicate that strippable sperm (milt) is present in the spermatic duct system. In the present paper, the term spermiation is used in the strict sense, while processes related to the hydration/capacitation of sperm refer to the "final maturation" of spermatozoa that takes place, at least in part, outside the testis

in the spermatic duct system are discussed further below (see Section 4.2: steroid hormones).

Considering spermiation *sensu strictu*, disintegration of occluding junctions at the adluminal pole of Sertoli cells may be a mechanism to release sperm into the tubular lumen (Billard et al., 1972). It is not clear yet if a certain loss of Sertoli cells occurs in association with spermiation in fish. In any case, following spermiation Sertoli cells can establish a single-layered epithelium lining the seminiferous tubule, and then were referred to as "lobule boundary cell". However, this was associated with an erroneous identification as Leydig cell homologue, as clarified by Grier and Linton (1977), and the term is no longer used. The epithelium of the spermatic duct system seems to be continuous with the Sertoli cell epithelium lining the lumen in fully mature testis (Billard et al., 1972; van den Hurk et al., 1978).

If at the end of a spawning season residual spermatozoa are left in the testis or spermatic duct system, they are removed by Sertoli cells via phagocytosis (Billard et al., 1972; van den Hurk et al., 1978; Almeida et al., 2008). Neighbouring tubules can show different activities during this period, removal of residual sperm while spermatogonial proliferation is quiescent, or start of spermatogonial proliferation while clearance of residual sperm had been completed. It seems possible that Sertoli cells removing residual sperm produce paracrine factors that prevent resumption of spermatogonial proliferation in the direct vicinity (rainbow trout: Schulz, 1984; Atlantic cod: Almeida et al., 2008).

In summary, piscine Sertoli cells pass sequentially through different stages of activity/differentiation that reflect the developmental stage of the single germ cell clone they take care off. At any given point in time, a fish Sertoli cell is less "busy" than a mammalian Sertoli cell, which is in contact with ~6 different germ cell clones in different stages of development.

2.3. Spermatogenic process – an overview

Spermatogenesis is a highly organized and coordinated process, in which diploid spermatogonia proliferate and differentiate to form mature spermatozoa. The duration of this process is usually shorter in fish than in mammals and is also influenced by the water temperature (Nóbrega et al., 2009). In principle, however, the process is conserved in vertebrates and morpho-functionally can be divided in three different phases: the mitotic or spermatogonial phase with the different generations of spermatogonia (i.e., undifferentiated spermatogonia including the stem cells, and differentiated or differentiating spermatogonia); the meiotic phase with the primary and secondary spermatocytes; and the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating – without further proliferation – into motile, flagellated genome vectors, the spermatozoa. Except for the mitotic phase, in which the number of spermatogonial generations is quite variable between, but determined genetically within species, the other two phases present striking similarities among different vertebrate species (see review by Nóbrega et al., 2009).

In mammals, all somatic and germ cells of the foetus originate from the inner-cell-mass cells of the blastocyst (Kubota and Brinster, 2006). The embryonic or primordial germ cells (PCG) migrate to the genital ridge, colonize the undifferentiated gonads, and are then called gonocytes, which might contain different subpopulations (Ohmura et al., 2004). Gonocytes generate male or female germ cells depending on the sex of the gonad. In an ovary the gonocytes will form oogonial stem cells that eventually all differentiate, and enter the first meiotic division before birth. Hence gonial stem cells are considered to be absent from the mammalian ovary at birth. In the foetal testis, however, after a brief period of proliferation the gonocytes are prevented from entering meiosis by the action of retinoid-degrading enzyme CYP26B1 expressed by Sertoli cells (Bowles et al., 2006). Instead, gonocytes enter a mitotic arrest and become spermatogonial stem cells (SSCs) soon after birth. Throughout postnatal life, SSCs will self-renew themselves or produce daughter cells committed to differentiate into spermatozoa. In adult mammals, recent findings suggest the presence of an SSC subpopulation that is totipotent and able to form different cell types (Guan et al., 2006; Conrad et al. 2008).

Although several candidates are emerging (e.g. RET, OCT4, PLZF, GPR125), at present there are no unique molecular or morphological markers to identify spermatogonial stem cells in vertebrates. Investigations on stem cell biology rely on a functional approach, the spermatogonial transplantation assay that is based on the capacity of transplanted SSCs to populate a recipient's testis.

Based on their morphology (mainly the amount and distribution of heterochromatin) and their presence in the different stages of the seminiferous epithelium cycle, in well studied laboratory rodents (mice, rats, and hamsters) the spermatogonial cells located in the basal compartment of the seminiferous tubules of adult animals can be classified in three different types (De Rooij and Russell, 2000): type A, intermediate, and type B.

Functionally, type A spermatogonia are divided in undifferentiated (A single, A_s; A paired, A_{pr}; A aligned, A_{al}; the latter usually clones of 4, 8, or 16 cells) that are continuously present; and in differentiated spermatogonia (A_1–A_4) that are present at specific stages of the epithelial cycle and committed to form mature sperm following a genetically determined schedule (De Rooij and Russell, 2000). Within the type A spermatogonia, the A_s is classically considered as stem cell. The A_s cells comprise only 0.3% of all germ cells and have a preferential location (niche) in the seminiferous tubules areas adjacent to the interstitium (Chiarini-Garcia and Russell, 2002), often in the vicinity of blood vessels (Yoshida et al., 2007). Due to incomplete cytokinesis, spermatogonial cells resulting from the mitotic (non-self renewal) divisions of A_s cells remain interconnected by cytoplasmic bridges until spermiogenesis/spermiation in all animals. The bridges are responsible for the synchronized development of germ cells derived as clonal cell group from the same stem cell (De Rooij and Russell, 2000). However, the concept of A_s cells being the only type of spermatogonia with stem cell capacity has been challenged recently because undifferentiated spermatogonia type A_{pr} and A_{al} may have the potential to revert to the stem cell fate under certain conditions (Nakagawa et al., 2007). In any case, adequate regulation of the balance between self-renewal and differentiation of SSCs is essential to assure the continuous homeostasis of spermatogenesis in species with continuous spermatogenesis, or to implement the start and end of seasonal reproductive activity.

The number of spermatogonial generations is genetically determined. For instance, mice and rats have six generations of differentiated spermatogonia (A_1–A_4, In, and B), whereas stallions and donkeys have five (A_1–A_3, B_1–B_2). Thus, different species can have different numbers of type A or type B generations, or can lack the intermediate spermatogonia. Within a species, also the number of generations of undifferentiated A_{al} spermatogonia can vary slightly and two or three A_{al} generations can be observed in rodents (De Rooij and Russell, 2000). In laboratory rodents the typical progression from A_S to preleptotene (Pl) primary spermatocytes is as follows, with the number in brackets giving the theoretical cell number that disregards germ cell loss: $A_s \rightarrow A_{pr}$ (2) $\rightarrow A_{al4}$ (4) $\rightarrow A_{al8}$ (8) $\circlearrowright A_1$ (8) $\rightarrow A_2$ (16) $\rightarrow A_3$ (32) $\rightarrow A_4$ (64) $\rightarrow$ In (128) $\rightarrow$ B (256) $\rightarrow$ Pl (512). The step from A_{al8} (8) $\circlearrowright A_1$ (8) is particular since it marks the retinoic acid-dependent differentiation without mitotic division of the last A_{al} generation to A1 spermatogonia. In this way, usually nine mitotic divisions occur in laboratory rodents.

The last S-phase in germ cells occurs in preleptotene spermatocytes, after which they enter the very long prophase of the first

meiotic division. The first meiotic prophase is subdivided into stages that carry names describing morphological aspects of the condensing/moving chromosomes: leptonema, zygonema, pachynema, diplonema, diakinesis, and the respective cells are specified as leptotene, zygotene, pachytene, and diplotene spermatocytes, respectively (Cobb and Handel, 1998). During the first meiotic prophase, paternal and maternal chromosomes are aligned. This involves a specific set of meiotic proteins, the synaptonemal complex proteins (Costa and Cooke, 2007), functioning as molecular "zippers" to facilitate the correct positioning of the parental chromosomes for the DNA double strand breaks and their subsequent healing in the context of meiotic cross-overs. Another reason for the long duration of the first meiotic prophase is the intense gene expression required for meiosis-specific proteins on the one hand (Eddy, 1998), and for the mRNAs required during later stages (spermiogenesis) on the other hand, when replacement of histones by protamins renders the germ cell genome inaccessible. Consequently, the mRNAs for many 'late' spermatogenic genes are expressed already in primary spermatocytes, and are stored and protected until use by association with RNA binding proteins (Braun, 1998). Primary spermatocytes are frequently found in testis sections, which reflect the long duration of the first meiotic prophase. They can be identified easily by their relatively large size, and by the characteristic nuclear staining patterns, representing the progressively condensing chromosomes. During the first meiotic division, homologous (maternal and paternal) chromosomes become separated into the nuclei of secondary spermatocytes. These cells are short-lived since they immediately enter the second meiotic cell cycle, skipping DNA-synthesis and leaving the chromosomes largely condensed. During the subsequent M-phase, the sister chromatids become separated into a haploid set in the spermatid nuclei.

Spermatids undergo a final differentiation period known as spermiogenesis, during which they develop a flagellum, the DNA is maximally compacted into a small nucleus, and superfluous cellular material is discarded into the so-called residual body. In higher vertebrates, the Golgi system gives rise to the acrosome that is important during fertilization. In most teleost fish, however, spermatids do not develop an acrosome. In species with distinct sex chromosomes, spermatogenesis-specific genes have the tendency to accumulate on the male-specific chromosome, for example a number of the structural genes required for the formation of the flagellum are on the mammalian Y chromosome (Burgoyne, 1998). The respective mRNAs are required when the germ cell genome has become inaccessible due to maximal chromatin condensation; also, these genes are not available in the nucleus in those 50% of the cells that received the X chromosome during meiosis. In this context, the RNA-binding proteins are relevant that distribute the required mRNAs among clone members, while the cytoplasmic bridges serve as intercellular transport routes for the RNA-protein complexes.

In amniote vertebrates, germ cell clones move during development from the basal compartment of the tubulus where spermatogonia are in contact with the basement membrane, through the tight-junctional barrier between neighbouring Sertoli cells (early spermatocytes), towards the adluminal part (spermatocytes and spermatids) until they are released (spermiation) by Sertoli cells. During spermiogenesis, specific junctions (ectoplasmic specializations) are formed between spermatids and Sertoli cells, which later disintegrate together with the cytoplasmic bridges among spermatids in preparation for spermiation.

2.4. Fish spermatogenesis

In this section, we will discuss the morpho-functional characteristics of the different developmental stages of fish germ cells, from stem cells to spermatozoa. Also, we will provide criteria for the identification of the different germ cell stages and we will propose a nomenclature; in this context, comparisons with the yet better investigated rodent models will be used as well.

We propose to use the same terminology used in higher vertebrates (see above) for fish germ cells, since the available information indicates that similar developmental processes are taking place during spermatogenesis in all vertebrates. For the purpose of comparability between different fish species and between different classes of vertebrates, the use of the same nomenclature for the same cell types is required. Undifferentiated type A spermatogonia (A_{und}) give rise to differentiated type A spermatogonia (A_{diff}), still sharing some morphological characteristics with A_{und} but with a greatly reduced potential for self-renewal. Irreversible commitment to further maturation and a change in a number of morphological aspects gives rise to the more rapidly dividing type B spermatogonia, of which there are usually several generations. The number of generations varies between species (Ando et al., 2000) and is genetically determined. For example, 14 generations were found in the guppy (Billard, 1969b), 10–12 in mosquito fish (Geiser, 1924), 9 in zebrafish (Leal et al., 2009) 6 in mudminnow (Foley, 1927), redfish (Moser, 1967), and rainbow trout (Loir, 1999), or 3 in mullet (Van der Horst, 1978). Cytoplasmic bridges, present between germ cells from the first spermatogonial division, interconnect them to a clonal syncytium in one cyst (Loir et al., 1995).

In species showing several spermatogonial generations, like guppy, zebrafish, or trout, a differentiation can be made based on cell/nuclear size and number of cells per cysts into early and late type B spermatogonia (B_{early} and B_{late}, respectively; see Figs. 2 and 4). When information is available on the exact number of generations, the generations of type B spermatogonia can be numbered (Schulz et al. 2005; Leal et al., 2009). Both in mammals (De Rooij and Russell, 2000) and fish, the type B spermatogonia are dividing more rapidly than the type A spermatogonia, as indicated by the ~5-fold higher mitotic index of B type over A type spermatogonia in African catfish (Schulz et al. 2005). After the final mitosis, type B spermatogonia differentiate into primary (preleptotene) spermatocytes, from where the ensuing developmental stages are: primary spermatocytes (1st meiotic division) → secondary spermatocytes (2nd meiotic division) → spermatids (differentiation without proliferation) → spermatozoa.

The different spermatogonial generations often pose problems as regards their identification and therefore receive particular attention. In general, stem cells are defined as the most undifferentiated cells of a particular lineage. They reside in a niche created by a supporting cell type, Sertoli cells, and the surrounding extracellular matrix. The niche is defined by its specific microenvironment that allows the stem cell to retain its stemness (Hofmann, 2008; Li and Xie, 2005). For different stem cell systems, both symmetric and asymmetric divisions have been described, i.e. the two daughter cells assume similar of different fates, respectively. Although there is no formal proof yet, vertebrate SSCs are considered to divide symmetrically in both self-renewal or differentiating divisions, while the fate assumed would be determined by the somatic environment: if a SSC is exposed to signalling favouring self-renewal, i.e. is in a stem cell niche, a differentiating outcome is unlikely while displacement from the niche, or change of signalling within the niche, would favour differentiation (Kostereva and Hofmann 2008; Oatley and Brinster 2008). Information on specific morpho-functional or molecular characteristics of the SSC niche in the teleost testis is still missing.

One of the undifferentiated traits of SSCs is that they are single cells, i.e. not connected via cytoplasmic bridges to clone members. Accordingly, a stem cell self-renewal division provides single daughter cells, while a pair of spermatogonia joined via a cytoplas-

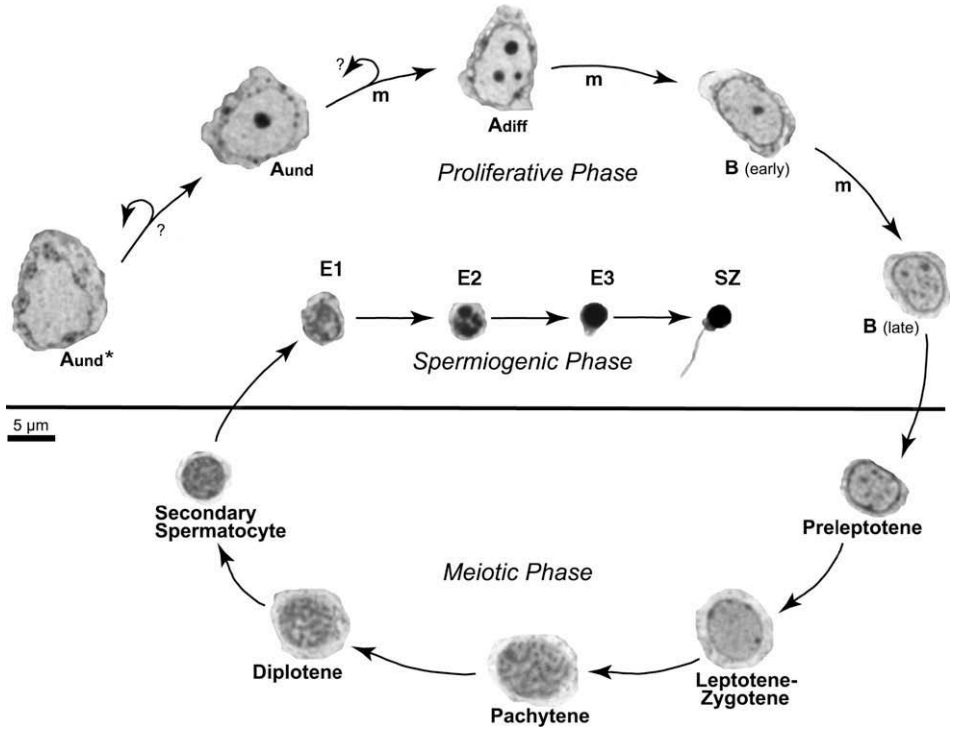

Fig. 2. Step-by-step representation of zebrafish spermatogenesis from type A undifferentiated spermatogonia to spermatozoa, throughout the three phases of the spermatogenic process: proliferative or spermatogonial, meiotic or spermatocytary and spermiogenic. The spermatogonial micrographs show the most characteristic features of each cell type, although there is a range of their morphology and size. Type A undifferentiated* spermatogonia (A_{und}^{*}) (stem cell?); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); type B spermatogonia (B); self-renewal (curved arrows); mitosis (m); early spermatids (E1); intermediate spermatids (E2); final spermatids (E3); and spermatozoa (SZ). A_{und}^{*} and A_{und} are single cells, whereas A_{diff}, B (early) and B (late) are grouped. The first question mark (?) indicates a doubt if A_{und}^{*} and A_{und} are separated by a mitosis or represent different stages of the same cell cycle (see also Fig. 4), while the second question mark indicates uncertainty as regards the "stemness" of A_{und}.

mic bridge emerges from a differentiating mitosis (Oatley and Brinster, 2008). In different species, morphologically and/or functionally different types of single spermatogonia have been described, and it is possible that they also differ in the degree of stemness and/or activity. In the human testis, for example, two types (pale and dark) of single A spermatogonia are present and might play distinct roles as "reserve" versus "active" stem cell, respectively (Schulze, 1979, 1988). Also, recent experiments suggested the existence of "true" and "potential" spermatogonial stem cells in mice testes, since after severe testicular damage (e.g. after chemo- or radiotherapy), undifferentiated spermatogonia (A_{pr} and A_{al}) can act as stem cells (Nakagawa et al., 2007). Finally, a so-called side population among SSCs was described in mice testis (Lassalle et al., 2004; Shimizu et al., 2006), characterized by a differential efflux of a fluorescent dye (Hoechst 33342). However, the properties of the testicular side population are not undisputed (Kubota et al., 2003).

Also in fish unique molecular markers for SSCs are not known yet, although the first candidate has been proposed recently (notch1; Yano et al., 2009). Functional data, such as derived from SSC transplantation studies, are scarce in fish. Transplantable germ cells are present in adult rainbow trout testis (Yano et al., 2009), and primordial germ cells (PGCs) collected at very early stages of ontogenesis from different fish species can be transplanted (e.g. Saito et al., 2008; Yoshizaki et al., 2005), but the recipients have always been fish at early stages of ontogenesis, so that transplanted germ cells joined the recipient's PGCs in their migration to the genital ridges. At present, there is only one proof-of-principle publication reporting the transplantation of SSCs to an adult male recipient (Lacerda et al., 2006). Clearly, this is a very promising approach but at the same time a clear caveat requiring significant research attention since transplantation models are urgently needed for studies on stem cell biology in fish.

A (partially) restricted distribution of spermatogonia provides positional clues for stemness, since at the end of spermatogenic tubules close to the tunica albuginea a very high (e.g. Billard, 1969b), or an elevated (e.g. Matta et al., 2002; Schulz et al., 2005) density of type A_{und} undifferentiated spermatogonia is observed. Exploiting this feature in tilapia, we have analysed tubules close to the tunica albuginea on the ultrastructural level (Fig. 3).

Different from mammals, spermatogonia in teleosts are not in direct contact with the basal lamina, and are always completely surrounded by Sertoli cells. Morphometric studies have shown a striking and steady decrease in germ cell volume in tilapia (approximately 2300 to 160 μm^3) from A_{und} spermatogonia to the last generation of B spermatogonia (Schulz et al. 2005). Simultaneously, germ cell number (1 to ~120 cells) as well as Sertoli cell number increased (1.4 to 3.6 cells) per spermatogonial cyst. We can therefore conclude that tilapia spermatogonia go through 7 rounds of mitosis before differentiating into spermatocytes, i.e. there are eight generations of spermatogonia. These results are a good illustration of the dynamics of spermatogenic cysts in fish and how morphometric investigations can help our understanding about the functional aspects of spermatogenesis.

Besides the nuclear size, that also decreases dramatically (~550 to 80 μm^3) from type A_{und} to type B spermatogonia, the amount and distribution of chromatin and cytoplasmic structure/organelles also changes in tilapias. In this regard, the two types of A_{und} spermatogonia differ in tilapia. The most undifferentiated type is most frequently found close to the tunica albuginea (Fig. 3A) while another type of A_{und} (Fig. 3B) can also be found at a certain distance from the tunica; both cell types are large single cells surrounded by

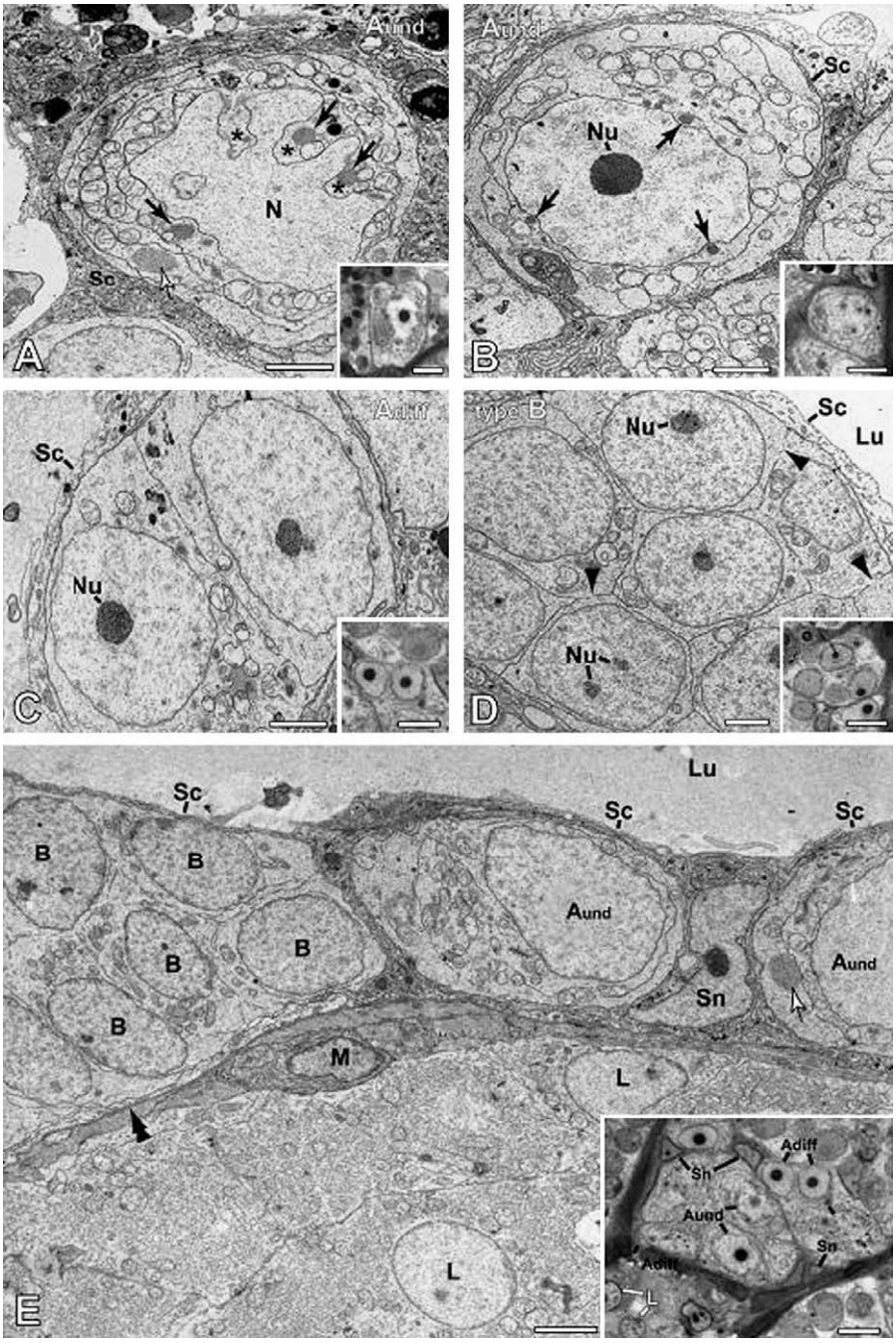

Fig. 3. Characterization of tilapia spermatogonial types under electron and light (insets) microscopy. A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiated spermatogonia; B, type B spermatogonia; Sc, Sertoli cell cytoplasm; Sn, Sertoli cell nucleus; N, nucleus; Nu, nucleoli; asterisks, nuclear folds; black arrows, "*nuages*"; white arrow, low electron dense "*nuage*" far from the nucleus; Lu, lumen; arrowheads, spermatogonial membrane opening; L, Leydig cell nucleus; M, myoid cell nucleus; double arrowhead, basal membrane of the seminiferous tubules. Bar, 1.5 μm; insets bar, 7 μm.

Sertoli cells. However, it seems that the cell with an exclusive location close to the tunica has a slightly smaller nucleus, less heterochromatin, a more convoluted nuclear envelope, and more mitochondria that are closer to the nucleus and surrounded by smooth endoplasmic reticulum (Fig. 3A). Particularly interesting is that these cells present large amounts of electron-dense material called "nuage", usually located between the nuclear envelope and the mitochondria. It is assumed that this dense material is composed of ribonucleoproteins and mRNA species showing a long half-life time, including *vasa* or *piwi* mRNA, specific products of the germ cell lineage (Knaut et al., 2000; Houwing et al. 2007). Intriguingly, "nuage" is one of the cellular characteristics of PGCs, so that this cell type may be closest to the ontogenetically earliest germ cell type. Apparently, the second type A_{und} (Fig. 3B) spermatogonium is the second largest germ cell type in tilapia. The main differences to the previous cell type is a reduced amount of "nuage", a more smooth outline of the nuclear membrane, an increased amount of heterochromatin, and a prominent nucleolus (Fig. 3B). It is not known if these two types represent different phenotypes (e.g. during different phases of the cell cycle) of the same cell type, or if they are separated by a mitotic division/differentiation. The somewhat broader distribution of the larger type A_{und}

spermatogonia suggests that in any case a displacement from the tunica takes place. Future studies will have to show if both cell types have (similar?) stem cell capacity, or if one of them can be considered as a reserve stem cell, or even a more primitive germ cell (PGC/gonocyte?).

A similar situation appears to exist in zebrafish (Leal et al., in press) although due to the unrestricted distribution of spermatogonia, there are no positional clues. However, careful analysis of the morphology of spermatogonia revealed that there are two similar types of single A_{und} spermatogonia (Figs. 2 and 4) as in tilapia, although in zebrafish there seems to be no size difference between these cells, both being larger than all other germ cell types.

Type A_{diff} spermatogonia are found in cysts with two to eight germ cells (Figs. 3C and 4). Their nuclei are round to oval, they contain no or very little nuage-like material, show one or more nucleoli, and are somewhat smaller than the A_{und} spermatogonia. With the latter cells, they share a low amount of heterochromatin in the nucleus, and a relatively large cytoplasmic volume (Figs. 1B, 2, and 4).

Type B spermatogonia are present in several generations. These cells occur in cysts with 16 or more germ cells, the number depending on the species-specific number of mitotic cell cycles that are completed before the germ cells differentiate into primary spermatocytes. The B spermatogonia show a progressively decreasing cell and nuclear volume, with a small rim of cytoplasm surrounding an oval to round nucleus that gradually shows more heterochromatin (Fig. 4).

After geometrical expansion of the spermatogonia number, the last type B spermatogonia differentiate into spermatocytes to enter meiosis, a sequence of two specialized cell cycles. Meiosis I—reductional, where the homologous chromosomes are segregate into secondary spermatocytes; and the meiosis II—equational, where the sister chromatids segregate, resulting in four haploid cells harboring one copy of each chromosome. One of the aims of meiosis is to generate genetic diversity through two events, first the recombination (crossing-over) and then the segregation of homologous chromosomes.

Under light microscopy, the criteria used to identify the meiotic cells are based mainly on nuclear characteristics, such as, size (diameter), shape, degree of chromosome condensation, and associated metaphasic figures (Figs. 1B and 2). In leptotene/zygotene zebrafish spermatocytes, the nucleus is larger (~5.5 μm) and more round in comparison with the last type B spermatogonia, the chromatin is clear and shows small dots of heterochromatin outlining the nuclear membrane (Figs. 1B and 2). Pachytene spermatocytes are the most frequent meiotic germ cells (the approximate duration of pachytene in zebrafish is 12 h—unpublished result), and has a large (~6.5 μm) and dense nucleus, with the chromosomes appearing as bold lines from the nuclear membrane to the center of the nucleus (Figs. 1B and 2). Diplotenic primary spermatocytes are often found together with metaphasic figures (metaphase I); the chromosomes reach their maximum degree of condensation and are found close to the nuclear membrane (Figs. 1B and 2). Sometimes, in the diplotenic primary spermatocytes, the outline of the nuclear membrane is not clear, or has disappeared already.

The secondary spermatocytes, resulting from meiosis I, are rare because they quickly enter meiosis II. They are small cells with a dense nucleus (~ 4 μm), usually found together with metaphasic figures (metaphase II) (Fig. 1B, 2). A fine description of meiotic germ cells using light and electron microscopy can be found for

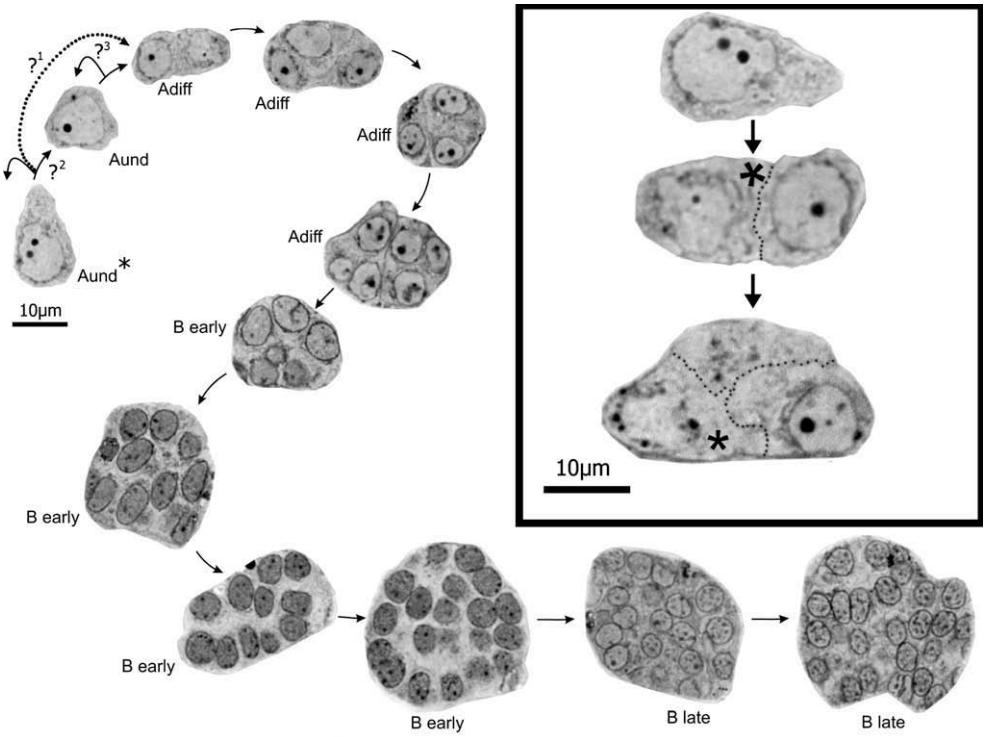

Fig. 4. Spermatogonial generations in zebrafish. The terminology to address the spermatogonial generations in zebrafish was based mainly on nuclear (heterochromatin amount) and nucleolar characteristics, and the number of germ cells inside the cyst. Using high resolution light microscopy applied to araldite-embedded sections, we propose five types of spermatogonia: type A undifferentiated* (A_{und}^{*}), type A undifferentiated (A_{und}), type A differentiated (A_{diff}), type B early (B_{early}) and type B late (B_{late}). The curved arrows indicate self-renewal while the simple arrows indicate mitosis. The first question mark ($?^1$) indicates a doubt as regards a possible asymmetric division of A_{und}^{*} (in the inset, note the stippled line towards a pair of spermatogonia). The second question mark ($?^2$) refers to the uncertainty if type A_{und}^{*} is separated from A_{und} by mitosis or if they represent different stages of the same cell cycle. The third question mark ($?^3$) indicates a doubt as regards the "stemness"of A_{und}. Inset: The asterisks show undifferentiated spermatogonia belonging to cysts with other types of spermatogonia. This observation might indicate that asymmetrical divisions can occur but requires further studies in teleosts.

several species of fish (see Pudney, 1995; Quagio-Grassiotto and Carvalho, 1999; Schulz et al., 2005). Histomorphometric analyses from tilapia testis (Schulz et al., 2005) have demonstrated that volume changes of meiotic germ cells were similar to those observed in mammals (França and Russell, 1998). However, in mammals, diplotene spermatocytes show the maximum volume (França and Russell, 1998) whereas in tilapia, pachytene spermatocytes are the biggest cell. We do not have an explanation for the shift to pachytene, or even zygotene in guppies, in teleosts at present.

Spermiogenesis consists of series of morphological changes that lead to the differentiation of spermatids into spermatozoa. The changes include nuclear condensation, elimination of organelles and cytoplasm, flagellum formation, and the rearrangement of cellular organelles along the spermatozoon cytoplasm (Jamieson, 1991). These remarkable modifications during spermiogenesis may explain the cyst volume decrease during this phase in tilapia (Schulz et al., 2005). Moreover, apoptotic loss of germ cell may occur in this phase.

It is possible to characterize three types of spermatids with respect to the nuclear condensation: E1 (early spermatids), E2 (intermediate spermatids), and E3 (final spermatids; Fig. 1B, 2; Schulz et al., 2005). Also, in fish, three types of spermiogenesis (type I, II, and III) have been described (Mattei, 1970; Quagio-Grassiotto and Oliveira 2008) based on the orientation of the flagellum to the nucleus, and on whether or not a nuclear rotation occurs. Type I is characterized by a perpendicular flagellum in relation to the nucleus with nuclear rotation; in type II, the flagellum develops parallel to the nucleus without nuclear rotation, and in type III, the flagellum is central without nuclear rotation (Mattei, 1970; Quagio-Grassiotto and Oliveira 2008). These patterns are reflected in the spermatozoa structure, and highly conserved within taxonomic units, and are, therefore, a powerful tool for phylogenetic analyses in fish (Jamieson, 1991; Quagio-Grassiotto and Oliveira 2008).

In teleost fish spermatozoa generally have no acrosome, and the impenetrable chorion is pierced by a micropyle that gives access to the membrane of the oocyte. Spermatozoa often show a spherical nucleus with homogenous, highly condensed chromatin, a nuclear fossa, a midpiece of variable size with or without a cytoplasmic channel, and one or two long flagella (Jamieson 1991). Moreover, fish spermatozoa can be classified into two forms, aquasperm and introsperm, according the external or internal mode of fertilization, respectively (Jamieson, 1991).

At the end of spermiogenesis, when intercellular bridges are broken and spermatozoa are individualized, the junctional complex between the cyst-forming Sertoli cells undergoes a dynamic remodeling that culminates into the cyst opening and hence the release of spermatozoa into the tubular lumen (Fig. 1B). It is not clear if in fish the release of spermatozoa is facilitated by myoid cells or/and the Sertoli cell cytoskeleton.

In some species, the release of germ cells occurs already at the spermatid (or spermatocyte) stage, and spermiogenesis (and meiosis) is (are) completed in the tubular lumen. This type of spermatogenesis is called semicystic spermatogenesis (Mattei, 1993) and is found in different taxonomic groups, such as Opheliidae (Mattei, 1993), Scorpaenidae (Muñoz et al., 2002), Bleniidae (Lahnsteiner and Patzner, 1990), Corydoradinae (Spadella et al., 2007), and Soleidae (García-López et al., 2005 and Kamstra and Schulz, unpublished observation). In semicystic spermatogenesis, Sertoli cells form earlier than in other species an epithelial lining of the spermatogenic tubules. However, the fact that individualized spermatids from different germ cell clones proceed through spermiogenesis in the tubular lumen seems but a variation of the timing of germ cell individualization.

There are cases, however, where spermatozoa are not "individualised" during release. Instead, sperm bundles are formed within the cyst when Sertoli cells secrete a capsule around a group of spermatids, thereby creating spermatophores (Grier, 1984). In the case of spermatozeugmata, on the other hand, Sertoli cells enlarge and send projections around the gametes to anchor them at the luminal margin of the cyst (Grier, 1993; Pudney, 1993). Also junctions between differentiating spermatids and Sertoli cells have been described (mosquito fish; Arenas et al. 1995). Interestingly, in this species spermatids undergo an elongation process, which is atypical for most teleosts but typically seen in mammals. Since also in those cartilaginous fish showing elongated spermatids ectoplasmic specializations were found (Stanley and Lambert 1985), the two features may be functionally connected.

The fluid, in which spermatozoa are suspended in the tubular lumen after spermiation, is produced by Sertoli cells (Billard et al. 1972; Setchell, 1986). In some species, like catfish (Loir et al., 1989), seminal vesicles are present as differentiation of the spermatic duct. In African catfish, the seminal vesicles are tubular structures that develop from caudal part of immature testis where spermatogenic tissue merges into spermatic duct system (van den Hurk et al., 1987); seminal vesicle tissue develops under the influence of rising androgen levels during puberty (Cavaco et al., 2001). Their columnar, single-layered epithelium is homologous to Sertoli cells (Loir et al., 1989) and expresses high levels of FSHR (Bogerd et al., 2001) while the thin lamina propria, on which they reside, contains some cells that show morphological characteristics of Leydig cells (van den Hurk et al., 1987). The seminal vesicles produce a fluid characterised by an acid pH value; the Leydig cell-like population may be a source for pheromones (Resink et al., 1987).

Data dealing with the timing of spermatogenetic events are scarce. The duration of meiosis plus spermiogenesis varies from 7 to 21 days in 4 tropical species (Silva and Godinho, 1983, Sinha et al., 1983) to 1–3 months in species living in temperate and cold zones (Louie and Dixon, 1972; Billard, 1983). At 25 °C, the total duration of spermatogenesis in guppy is 36 days (Billard, 1969b).

3. A complex and changing testicular transcriptome during maturation

Different approaches, such as analysis of candidate gene expression or suppressive subtractive hybridization (SSH) cDNA libraries served to study the dynamic gene expression patterns associated with the spermatogenic process. More recently, global testicular transcriptome analyses have been carried out using arrays that allow a high throughput and cost effective description of the temporal changes in expression of hundreds or thousands of genes simultaneously. LeGac and collaborators (Mazurais et al., 2005) analyzed gene expression in trout testes assigned to histologically defined stages of gonadal maturation, from stage I (immature) to stage IV (spermiogenesis). The initial data, obtained using 1152 trout cDNAs spotted on macroarrays, supported the view that the transition from prepubertal to maturing testis was associated with changes in the expression of numerous genes. In addition, gene expression profiles analysis showed that differentially expressed genes were segregating into five distinct clusters, some of them being enriched in genes involved in the same biological process. Cluster A included up-regulated genes linked to protein modification and cell organisation and biogenesis. Cluster B grouped genes transiently over-expressed at stage II as spermatogonia type B actively proliferate; different biological processes were present but none seemed significantly over-represented in this cluster. Cluster C included genes up-regulated from stage II onwards; the genes are involved in proliferation, protein modification and biosynthesis. Clusters D and E were up regulated during the meiotic and post-meiotic stages III and IV, respectively. As expected, gene annotation was associated with mitotic/meiotic progression and with

protein synthesis/catabolism, protein modification, and cell organisation and biogenesis.

Hence, changes in gene expression detected by global transcriptome analysis required further investigations to determine if changes in gene expression occurred within a numerically stable cell population and/or were the result of the proliferation of specific cell populations. To determine the cellular origin of gene expression, transcriptome data were collected from spermiating trout and from isolated germ cell populations enriched in spermatogonia, spermatocytes, or haploid cells (Rolland et al., submitted for publication). This study used a cDNA microarray (GEO datasets platform GPL3650) designed by the AGENAE consortium and carrying more gene representatives (9152 contigs with a UniGene annotation). The classification of about 8000 expression values, obtained for each of the 50 testicular samples, again clearly displayed distinct transcriptomes in relation to the cell types or stages of gonadal maturation. In addition, cDNA microarrays data were supplemented by Q-PCR and in situ hybridisation (ISH) approaches to address the interactions within and between complex gene networks.

Genes could be classified in at least 8 main clusters that reflect major expression profiles (A–H; see Fig. 5). For instance, the transition from the gonadal stages I to VIII was marked by changes of the expression pattern of genes encoding extra-cellular matrix proteins (cluster C). This observation is in agreement with the tubular reorganization observed within the testis during the reproductive cycle. In addition, the expression levels of cell cycle and mitotic spindle regulatory genes increased in relation to the rapid mitotic activity of the type B spermatogonia (cluster F and G). Genes encoding growth factors were differently expressed throughout the spermatogenetic process (cluster B). For instance, two members of the TGFβ superfamily, anti-Müllerian hormone (AMH) and gonadal soma derived factor (GSDF), were dramatically repressed in the Sertoli cells as germ cell differentiation occurred. A very interesting observation was that genes involved in the repression of DNA recombination were up-regulated during the same period, suggesting that meiotic processes are postponed in spermatogonia (cluster F). Transcripts encoding meiotic and post-meiotic proteins (sycp1, sycp3, dyneins) were significantly over-represented in clusters G and H and their levels of expression increased simultaneously with the appearance of large numbers of spermatocytes and spermatids. Another cluster in C contained genes that were highly expressed only at spermiation (Fig. 5).

4. Extrinsic regulation of spermatogenesis

4.1. Pituitary hormones and growth factors

The gonadotropins, LH and FSH, are the most important pituitary hormones regulating testicular physiology. Two points are of great relevance for their biological activity, the specificity, with which the gonadotropins interact with their receptors and the cellular site(s) of receptor expression. In mammals, FSH and LH interact with their receptors (FSHR and LHR, respectively) in a highly specific manner with little overlap in biological activities at physiological hormone concentrations. LH regulates Leydig cell sex steroid production; FSH regulates Sertoli cell activities, such as the structural, nutritional, and regulatory (paracrine) support of germ cell development (Huhtaniemi and Themmen, 2005). Both, receptor specificity and site of expression, contribute to the impression that the biological activities of fish gonadotropins seem less clearly separated. In most cases where the binding characteristics of FSHR and LHR have been characterized using hormones from the same (or very closely related) species (African catfish: Garcia-Lopez et al., 2008; Channel catfish: Sampath Kumar et al., 2000; Japanese eel: Kazeto et al., 2008; zebrafish: So et al., 2005; but with the possible exception of rainbow trout: Sambroni et al., 2007), the FSHR showed a preference for FSH, but was also activated by LH. However, cross-activation of the FSHR requires high LH concentrations and may be restricted to periods with peak LH plasma levels, such as during the spawning season. From an evolutionary point of view, it is interesting to note that an avian FSHR behaved in a

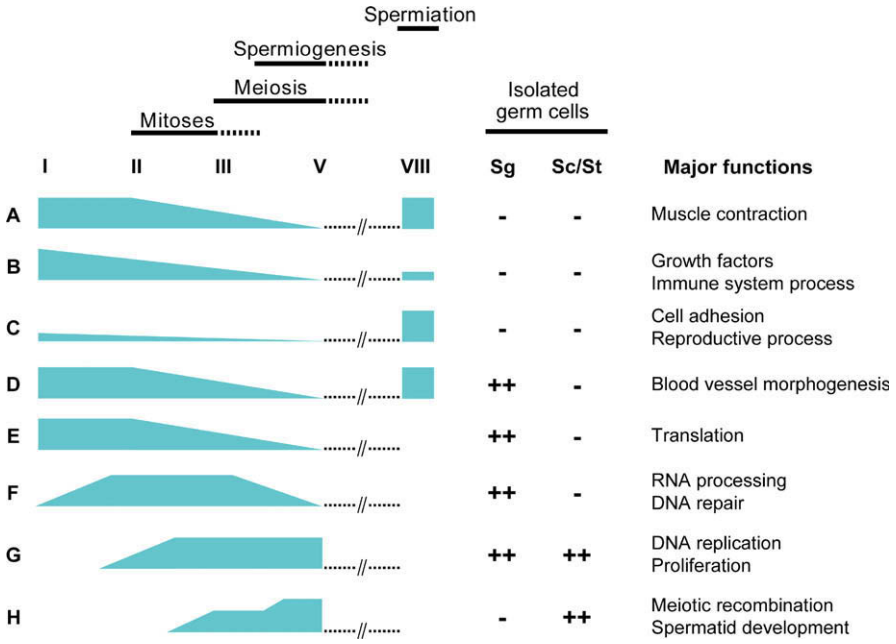

Fig. 5. Gene expression clusters of male gonad development in the rainbow trout. Different development stages of male gonad (I–VIII) together with isolated germ cells (Sg, spermatogonia; Sc, primary spermatocytes; St, spermatids) were used to establish a comprehensive data set of gene expression during trout spermatogenesis. More than 3000 differentially expressed genes were grouped according to their expression profiles in both developing testes and isolated germ cells (expression clusters A–H). A representative expression pattern of each cluster is schematized during gonad maturation whereas – and ++ indicate a low and a high expression in isolated germ cells, respectively. Major functions, as evidenced through a GeneOntology term enrichment analysis, are also indicated for each cluster.

similar manner as regards the cross-activation by LH (Wakabayashi et al., 1997). The LHR too can be cross-activated by FSH, but the required FSH concentrations (close to 1 μg/ml in rainbow trout and African catfish) are far above maximum plasma levels, so that the LHR can be considered as LH-specific physiologically.

Still, piscine FSHs are potent steroidogenic hormones (Planas et al., 1993). One possibility to explain FSH-mediated stimulation of Leydig cell steroidogenesis is to assume that Leydig cells in fish, different from their mammalian counterparts, express the FSHR gene. An antibody raised against the eel FSHR detected immunoreactive material on both, Leydig and Sertoli cells (Ohta et al., 2007). In African catfish, both Leydig and Sertoli cells expressed the FSHR gene, while LHR gene expression was restricted to Leydig cells (Garcia-Lopez et al., 2008). Taken together, the data on receptor pharmacology and localization suggest that Leydig cell steroidogenesis is directly regulated by LH and by FSH, while Sertoli cell functions are predominantly regulated by FSH, although high LH concentrations, such as during the spawning season, might cross-activate the FSHR.

Data on LH plasma levels are available from a number of fish species, but much less data has been published on circulating FSH levels, and as regards males, information is restricted to salmonid species only (Campbell et al., 2003; Gomez et al., 1999; Prat et al., 1996). In these species, plasma LH levels were very low or undetectable during the start of testis development (slow and rapid proliferation of spermatogonia), became detectable when germ cells entered meiosis, but did not increase clearly until close to the spawning season. FSH levels, on the other hand, showed a transient increase in association with spermatogonial proliferation, then reincreased with spermiation *sensu strictu*, and decreased before the spawning season started. Campbell et al. (2003) reported that elevated circulating levels of androgens and FSH coincided in male Chinook salmon with active spermatogonial proliferation and Ohta et al. (2007) demonstrated that suppressing the steroidogenic activity of FSH abolished the stimulatory effect of FSH on spermatogonial proliferation in Japanese eel. Hence, the present knowledge in fish indicates that FSH-mediated steroidogenesis stimulates early stages of spermatogenesis.

What do we know about the possible roles of FSH as a direct regulator of Sertoli cells? Prominent FSH activities in mammals are: stimulation of Sertoli cell proliferation during the prepubertal period; induction of terminal differentiation of Sertoli cells together with androgens and thyroid hormones during puberty; regulation of growth factor release, which in turn modulates spermatogenesis; or support of terminal phases of spermiogenesis and spermiation. Many of these functions were uncovered by analysing the effects of experimentally induced or pathologically characterized FSHR loss-of-function animal models or patients (Huhtaniemi and Themmen, 2005). However, information on FSH effects on fish spermatogenesis that is not related to steroidogenesis is not available so far. It is tempting to speculate that elevated FSH plasma levels during the start of spermatogonial proliferation may be functionally related to proliferation of Sertoli cells, considering that the number of these cells increases in association with the mitotic expansion of spermatogonial cysts in tilapia and African catfish (Schulz et al., 2005).

A few growth factors have been identified that are produced by Sertoli cells and that regulate germ cell proliferation behaviour (see Section 4.2 below), such as the TGFβ family members activin (Miura and Miura, 2001), GSDF (Sawatari et al., 2007), AMH (Miura et al., 2002) and its receptor (Morinaga et al., 2007), or an orthologue of platelet-derived endothelial cell growth factor (PD-ECGF; Miura et al., 2003). While in some cases information is available on the modulation of expression of these growth factor genes by androgens (activin, AMH) or estrogens (PD-ECGF), no information is available as regards the role of FSH in regulating growth factor expression in fish. In mammals, for example, FSH stimulates AMH expression in the prepubertal but not in the adult testis (Lukas-Croisier et al., 2003) and AMH exerts an inhibitory effect on Leydig cell differentiation (Salva et al., 2004) and androgen production (Fynn-Thompson et al., 2003). It will be interesting to study in fish these endo- and paracrine mechanisms. We anticipate that technical advances (e.g. production of recombinant FSH and establishment of testis cell/tissue culture approaches for more species) will allow significant progress in the near future considering the role of FSH in fish spermatogenesis.

Other pituitary hormones next to LH and/or FSH can modulate testicular steroidogenesis, such as growth hormone (Singh et al., 1988), and cross-talk between the reproductive and growth axes occurs via sex steroids that modulate pituitary GH release (Melamed et al., 1995). In pubertal Chinook salmon, the start of spermatogonial proliferation coincided with elevated levels of FSH, androgens, and of insulin-like growth factor 1 (IGF1; Campbell et al., 2003), a growth factor released by the liver in response to GH stimulation. In addition to a possibly systemic action, binding studies revealed the presence of GH receptors in trout testis and also enriched Sertoli cell population (LeGac et al., 1993; Gomez et al., 1998). Also IGF1 mRNA expression and IGF-I receptors were demonstrated on germ and Sertoli cells in trout (LeGac et al., 1996) and tilapia testis (Berishvili et al., 2006), and experimental evidence supports the role of IGF1 in proliferation and/or differentiation of spermatogonia (Loir and LeGac, 1994; Vinas and Piferrer, 2008). Also, it has been proposed that GH, directly and/or through locally produced IGF1, can modulate testicular steroidogenesis (LeGac et al., 1996) and/or germ cell proliferation (Loir, 1999). Finally, it is interesting to note that IGF1 plays a permissive role as regards the stimulatory effects of androgens on spermatogenesis in Japanese eel (Miura and Miura, 2001). In general it appears that regulatory input to spermatogenesis via gonadotropins and sex steroids can be modulated or fine-tuned by other pituitary hormones and local signalling in the testis. The mechanisms involved are largely unknown but may involve the differential expression, per spermatogenic cyst, of hormone and growth factor receptors.

4.2. Steroid hormones

The sex steroids, progestagens, androgens, and estrogens are mainly produced in the gonads. Plasma levels of steroid hormones show important variations during male gonad maturation. In general, estrogens are considered 'female' hormones but are formed in male vertebrates as well (Schlinger and Arnold 1992; Hess et al. 1995; Betka and Callard 1998). In male teleosts, 17β-estradiol (E2) is present in blood serum in rather low concentrations (Miura et al. 1999; Amer et al. 2001; Chaves-Pozo et al., 2007). E2 plasma levels show a transitory elevation at the beginning of the reproductive cycle in trout (Gomez et al., 1999). Androgens (testosterone, T; 11-ketotestosterone, 11-KT) increase gradually as spermatogenesis proceeds and decrease at spermiation. During the reproductive cycle of salmonid fish, there are two peaks of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) blood plasma levels. A big peak is observed in the spawning season, another small peak during the progression of spermatogonial proliferation. The small peak of DHP was known in salmonid fish for some time (Depeche and Sire, 1982; Scott and Sumpter, 1989; Vizziano et al., 1996). Taken together, these data suggest that steroid hormones have important and distinct roles in controlling fish spermatogenesis. These roles will be discussed below.

4.2.1. Estrogens

Estrogens bind to nuclear receptors that act as ligand inducible transcription factors. Three estrogen receptor subtypes (alpha, beta1 and beta2) are expressed in fish and the male gonad is a

major site of expression. Estrogen receptors expression has been reported in the somatic testicular cells and in haploid male germ cells (Miura et al., 1999; Chang et al. 1999; Bouma and Nagler, 2001; Wu et al., 2001; Menuet et al., 2002; Ito et al., 2007).

These findings suggest that estrogens have an important role in regulating gene expression in the testis. Administration of E2 to maturing sea bream males resulted in the identification of numerous estrogen dependent genes in the testis (Pinto et al., 2006). For many of these genes, there is no evidence that they are relevant for testicular functions, although they could be considered as biomarkers of estrogen exposure. Another group of estrogen dependent genes may be more relevant since they were involved in different biological processes including cell proliferation (PCNA, fibrinogen B and G), lipid metabolism, protein metabolism and folding, transport, and cell communication (Pinto et al. 2006).

In another study, LeGac and collaborators (unpublished results) have administrated E2 implants to prepubertal male trout (stage I/II with spermatogonia only), resulting in E2 plasma levels of 1–8 ng/ml, and analysed global transcriptome changes in the testis. Although the treatment significantly up-regulated 80 transcripts and down-regulated 33 other transcripts, gene expression variations did not exceed 1.5- to 3-fold with the exception of the vitellogenin encoding gene (up to 10- to 30-fold induction), a well known biomarker for oestrogen exposition. E2 had significant regulatory effects (direct or indirect) on the expression of genes important for the regulation of the steroidogenesis (i.e. Star, 3βHSD, aromatase A and B) and for spermatogenesis: increased expression of genes encoding for retinol binding proteins I and II suggest that estrogens control the homeostasis of retinoic acid within the testis. Retinoic acid availability is required for the proliferation and differentiation of the undifferentiated spermatogonia in mouse (Zhou et al., 2008), although it seems less relevant in zebrafish (Alsop et al., 2008). In addition, the AMH gene was down-regulated in oestrogen treated trout, which supports the involvement of estrogens in the stimulation of the spermatogonial stem cell renewal in male as initially proposed in eel (Miura et al., 1999). In medaka, administration of low doses of ethinyl estradiol resulted in a similar stimulatory effect of estrogens on spermatogonial stem cell renewal, whereas high doses had an inhibitory effect (Song and Gutzeit, 2003). In gilthead seabream, supraphysiological doses (>100 ng/ml) of estrogens inhibited proliferation, induced apoptosis of undifferentiated spermatogonia, accelerated late events in spermatogenesis and induced post-spawning processes such as infiltration of acidophilic granulocytes (Chaves-Pozo et al., 2007). In maturing fish, high oestrogen doses reduced the seminal fluid volume, increased sperm density, and may lead to sterility (Lahnsteiner et al., 2006).

4.2.2. Androgens

Two androgen receptor subtypes (α and β) have been described in fish and they are all predominantly expressed in the gonad (Takeo and Yamashita, 1999; Todo et al., 1999; Ikeuchi et al., 2001). The AR was expressed in Sertoli and interstitial cells but not in germ cells (Ikeuchi et al., 2001), suggesting that androgens develop biological activity via the testicular somatic cells.

Androgens strongly influence testicular gene expression. In prepubertal trout, both T and 11-KT treatments (inducing blood plasma levels of 15–30 ng/ml) have similar effects on a common group of 155 up-regulated and 20 down-regulated genes (LeGac et al., 2008; Rolland et al., submitted for publication). Some of these genes are expressed in Sertoli cells and have been previously demonstrated to regulate spermatogenesis and steroidogenesis. For instance, expression of the AMH gene that inhibits differentiation of spermatogonia is significantly suppressed by T and 11-KT. Numerous transcription factors of known significance for testis development were regulated by T or 11-KT treatment (see Table 1 for selected examples). Of note is that androgen treatment also increased numerous transcripts involved in muscle development and function. In view of the known myogenic action of androgens, it is therefore interesting to note that the peritubular cells in the testis are myoid cells with contractile characteristics, known androgen-responsive target cells in higher vertebrates.

Beside similar responses, T and 11-KT also affected a subset of genes differentially. LeGac and colleagues (unpublished results) observed that 56 genes were up-regulated by 11-KT but not by T, and 260 genes were up-regulated by T but not by 11-KT. This finding indicates the possibly specific effects of each of these androgens. In mice, different classes of androgen receptor binding DNA response elements exist which might be an essential feature of differential androgen target gene regulation (McPhaul and Young, 2001).

Many transcripts that changed after sex steroid treatment displayed an interesting pattern of expression during testis maturation (Rolland et al., submitted for publication). For example, 3 subgroups of androgen-responsive genes are up-regulated respectively, in the prepubertal stage I, at the initiation of rapid spermatogonial proliferation (stage II), and in meiotic/post meiotic stages (stages III–V). This suggests that these particular steroid-regulated genes play a significant role during testis maturation and spermatogenesis. Furthermore, most of these maturational changes in gene expression appear highly correlated with the changes in circulating androgen levels. For instance, some genes are up-regulated when androgen levels increased moderately during the initiation of spermatogenesis; others are over-expressed in parallel to the large increase in androgen levels occurring around spawning time (Rolland et al., submitted for publication). Indeed, a large proportion of genes up-regulated by androgen treatment is found over-expressed at stage VIII (cluster C, Fig. 5). Since several of these genes code for ion or water transporters (Atp1b1a, Aqp4, Kcnk15, Slc26a4, Slc27a11), androgens might be of particular importance for milt hydration or for providing a proper environment for sperm maturation/conservation. This study may serve as a basis for hypothesis-driven research and provide insight as regards gene networks and pathways under sex steroid control in the trout testis. Combined with the in situ localization of gene expression and the search for steroid responsive elements in their regulatory sequences, such approaches will progressively allow for the comprehension of gene regulations involved at the key steps of spermatogenesis.

Androgens are effective in supporting either the whole process of spermatogenesis, or at least some steps such as spermatogonial multiplication and spermatocyte formation (guppy) or maturation (killifish) (Remacle, 1976; Billard et al., 1982; Fostier et al., 1983; Billard, 1986; Nagahama, 1994; Borg, 1994). They may also participate in the initiation of puberty (Miura et al., 1991a,b; Cavaco et al., 1998). In addition, androgens induces spermiation in some species but were clearly less effective than progestins (Ueda et al., 1985).

4.2.3. Progestins

Nuclear and membrane-bound progestin receptors types are highly expressed in fish gonads (Todo et al., 2000; Zhu et al., 2003). The highest levels of circulating progestins are observed during the entire spermiation process and these hormones have been involved in the regulation of several testicular functions. Progestins as DHP or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) advance and induce spermiation in Salmonidae and Cyprinidae (Ueda et al., 1985), increase milt production (Baynes and Scott, 1985; Yueh and Chang, 1997), and stimulate spermatozoa motility (Miura et al., 1992; Tubbs and Thomas, 2008). Besides, the involvement of DHP in meiosis was recently reported in male eel (Miura et al., 2006).

Table 1
Transcription factors relevant for testis development were up- or down-regulated in immature rainbow trout testis following androgen treatment in vivo.

Up regulated
Doublesex- and mab-3-related transcription factor 1: DMRT1 is known as an important factor in sex differentiation. DMRT1 is essential for testicular development in mice and might play a role in medaka spermatogenesis. DMRT1 expression was inhibited by E2 and stimulated by T and 11KT, while DHP had no significant effect. When testis maturation proceeded until after the initiation of meiosis, DMRT1 expression was under-expressed in germ cells
Transcription factor AP-1 (Proto-oncogene c-jun): c-jun and c-fos (components of the transcription factor AP1) regulate cellular growth and differentiation and also exert regulatory roles in steroidogenesis and spermatogenesis. In trout, c-jun transcript was under-expressed in germ cell populations as compared to total testis, and to decrease in stage III compared to stage I-II of testis development; it is up regulated after 11KT, T, or DHP treatment but not modified by E2
Transcription factor E2F4 (E2F-4): The E2F family plays a crucial role in cell cycle control and suppresses proliferation-associated genes. In trout, this transcript was over expressed in germ cells, increased in stage III (start of meiosis), and was up-regulated after T or 11KT, but not after DHP treatment
LIM domain transcription factor LMO4 interacts with Smad proteins and modulates TGFp signalling in epithelial cells; it takes part in the negative regulation of estrogen receptor alpha transactivation functions. In our experiments, its expression is very stable during testis maturation, but is up regulated after T or 11KT treatment
Nuclear receptor 0B1 (DAX-1) functions as a negative regulator of steroidogenesis during the development and adult function of the reproductive axis; it is also suggested as a negative co-regulator of nuclear receptors, including androgen and progesterone receptor.
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1): PPAR are ligand-inducible transcription factors implicated in lipid transport and metabolism; in mammals PPARγ could play a major role in testicular somatic cells. In trout testis we found that the PPAR co-activator is under expressed in germ cells, compared to whole testis. It is up-regulated after T, 11KT, and DHP treatments.
Transcription factor SOX-8: this SRY-related HMG-box protein may be involved in male sex determination and AMH regulation but its role during spermatogenesis is not known. It shows only limited variation in expression during testis maturation but is up-regulated after androgen and DHP treatment
Nuclear matrix transcription factor 4 (Cas-associated zinc finger protein) regulates transcription from matrix metalloproteinase promoters and from the type-I collagen promoter. It may suppress BMP2 signalling, and impaired spermatogenesis was found in Nmp4-disrupted mice. We found that this factor is over-expressed in testis in stage III–V, compared to stage I and to germ cell populations. It is up-regulated after T or11KT, but not after E2 or DHP treatment
Down regulated
T-box transcription factor TBX1 (Testis-specific T-box protein): Involved in early developmental gene regulation, TBX1 has a second phase of expression in adult testis. In our experiments, TBX1 was under-expressed in isolated germ cells compared to the whole testis, and its expression decreased from stage III to VIII. It is down-regulated after T and 11KT treatments, also after DHP treatment but to a lesser extent

4.3. Molecular regulation of spermatogenesis

4.3.1. Spermatogonial stem cell renewal

As mentioned earlier, spermatogonial mitosis can be categorized in slow spermatogonial renewal and rapid proliferation of differentiated spermatogonia towards meiosis (Clermont, 1972). Both kinds of spermatogonial mitosis are regulated by different mechanisms by steroid hormones.

Evidence obtained in different vertebrate classes indicates that spermatogonial renewal is regulated by E2. In Japanese eel, the role of E2 in spermatogenesis was investigated using in vitro and in vivo experimental approaches (Miura et al., 1999). Slow renewal mitosis of eel spermatogonia was promoted by implanting E2, but was suppressed by tamoxifen (an oestrogen receptor antagonist). In vitro, 10 pg/ml of E2 was sufficient to induce spermatogonial renewal divisions in cultured testicular tissue, confirming the in vivo observations. E2 treatments did not, however, promote rapid spermatogonial proliferation and meiosis. In Japanese huchen (*Hucho perryi*), E2 similarly promoted spermatogonial stem cell renewal in vitro (Amer et al. 2001). In medaka, administration of low doses of estrogenic compounds resulted in a similar effect whereas high doses had an inhibitory effect (Song and Gutzeit, 2003). These findings clearly indicate that oestrogen is also a 'male' hormone, and plays an important role in SSC renewal. This function of estrogens is not only known from teleost fish, but also from other vertebrates, such as mammals (Kula 1988), amphibians (Minucci et al., 1997), and reptiles (Chieffi et al., 2002). Furthermore, in female teleosts oogonial renewal proliferation is regulated by E2 (Miura et al., 2007).

Generally, E2 induces target gene expression via its nuclear receptor, and the factors translated from oestrogen target genes affect biological processes. The molecular mechanisms of oestrogen-induced spermatogonial renewal were analyzed in Japanese eel (Miura et al., 2003, 2007). Eel spermatogenesis related substance 34 (eSRS34), a homologue of mammalian platelet derived-endothelial cell growth factor (PD-ECGF), was identified by expression screening as gene up-regulated by E2 and functionally analysed using eel testis tissue and cell culture systems. Recombinant eSRS34 induced spermatogonial stem cell renewal, while addition of a specific anti-eSRS34 antibody prevented spermatogonial stem cell renewal induced by E2. These results indicate that eSRS34 is a 'spermatogonial stem cell renewal factor' (Fig. 6). Fibrinogens (FgB and FgG) are known as positive regulators of cell proliferation in wound healing and in tumours (Staton et al., 2003). In sea bream testis, FgB and FgG are up-regulated by E2 stimulation (Pinto et al., 2006), and also may be regulators of spermatogonial renewal like eSRS34.

Other genes have been involved in the proliferation of fish spermatogonial stem cells. A new member of the TGFβ family, GSDF, increases PGC numbers and promotes the mitotic activity of the spermatogonia in prepubertal trout (Sawatari et al., 2007). However, a duplicated copy of the GSDF gene has been identified recently in salmonids (GSDF2). The GSDF2 transcripts are restricted to the testis and the Sertoli cells. Contrary to the first gene copy, GSDF2 encodes a protein containing all the conserved cysteine residues known to be important for the function of the TGFβ family members (Lareyre et al., 2008). To date, it is not known whether the two GSDF factors have distinct or redundant functions in trout.

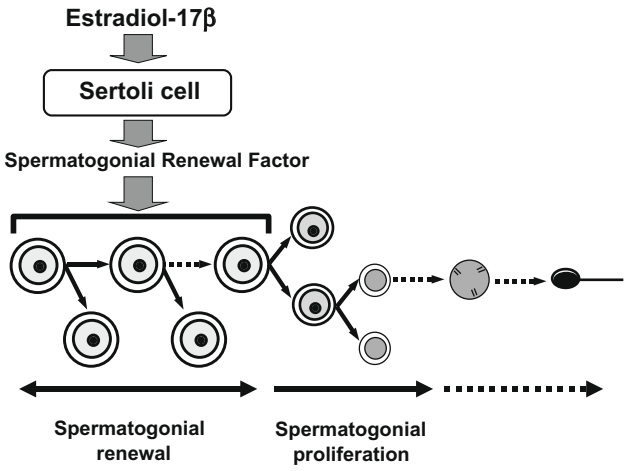

Fig. 6. A schematic summary of possible oestrogen-dependent control mechanisms of spermatogonial renewal in fish.

4.3.2. Spermatogonial proliferation toward meiosis

Upon gonadotropic stimulation, spermatogonial mitosis switches from the slow self-renewal pathway to rapid proliferation toward meiosis. We refer to this point as the initiation of spermatogenesis. As discussed above, FSH plays a major regulatory role during early stages of spermatogenesis, while LH is mainly involved in the final stages of maturation. In agreement with this notion, FSH was the key gonadotropin for initiation of spermatogenesis using eel testicular organ culture (Ohta et al., 2007) (Fig. 7).

The eel Fsh receptor is expressed by Leydig cells and by Sertoli cells surrounding type A and early type B spermatogonia. In an in vitro organ culture, recombinant eel Fsh (r-eFsh) induced complete spermatogenesis from the proliferation of spermatogonia to spermiogenesis during 36 days of culture; also spermatozoa were observed in the testicular fragments. Spermatogenesis induced by r-eFsh was inhibited by trilostane, a specific inhibitor of 3β-hydroxysteroid dehydrogenase. Thus, the main function of FSH during the initiation of spermatogonial proliferation is stimulating the production of spermatogenesis-inducing steroids, such as 11-ketotestosterone (Miura et al., 1991a), the major androgen of teleost fish. In fact, FSH can induce the production of 11-ketotestosterone in testis in vitro (Kamei et al., 2005, Ohta et al., 2007). Future work will have to show if FSH has additional functions next to stimulating Leydig cell steroid production, for example in relation to FSH receptor expressing Sertoli cells.

11-Ketotestostrone was first identified by Idler et al. (1961) as a major androgenic steroid in the male sockeye salmon (*Oncorhynchus nerka*). In various teleost fish, this steroid has since then been shown to be synthesized in the testis following GTHs stimulation and high levels were detected in the serum during spermatogenesis (Billard et al. 1982). The function of 11-ketotestosterone for spermatogenesis became clear for the first time using an eel testicular organ culture system (Miura et al., 1991a). When 11-KT was added to the organ culture, spermatogenesis was induced from the proliferation of spermatogonia to spermiogenesis. This action of 11-KT is not limited to the Japanese eel but has been recognized also in goldfish (Kobayashi et al., 1991) and Japanese huchen (Amer et al., 2001). These findings indicate that 11-KT is one of the factors involved in the initiation of spermatogonial proliferation toward meiosis. However, it is believed that the action of 11-KT is mediated by other factors produced by Sertoli cells that, in contrast to germ cells, express androgen receptor protein (Ikeuchi et al., 2001). It is possible that some of these factors are growth factors, such as IGF1 and activin B. IGFs are known to be mediators of growth hormone action in vertebrates. Although IGF1 is also necessary for the regulation of eel spermatogenesis, its role is to support the action of 11-KT. More specifically, in Japanese eel 11-KT is necessary for the induction of spermatogenesis, whereas IGF1 is necessary for the continuation of the process (Nader et al. 1999).

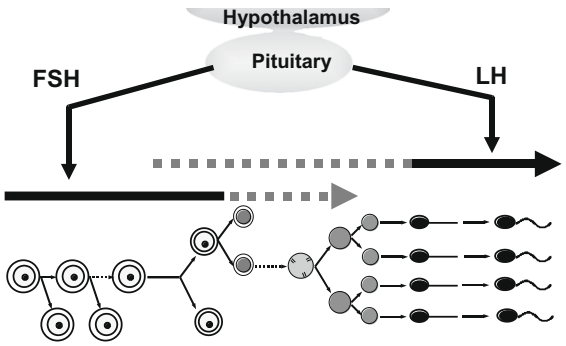

Fig. 7. A schematic summary of the roles of FSH, mainly regulating processes during the mitotic phase, and of LH, mainly regulating processes during the spermiogenic phase, on fish spermatogenesis.

How does 11-KT initiate spermatogonial proliferation in fish? In Japanese eel, two members of the TGFβ superfamily, AMH (Miura et al., 2002) and activin B (Miura et al. 1995a), have important roles during the initiation of spermatogenesis induced by 11-KT.

Activin B is a dimeric growth factor composed of two activinβ B subunits. In the Japanese eel, activin B was found in the testis at the initiation of spermatogenesis after hCG stimulation, with its expression site restricted to Sertoli cells. Both transcription and translation of eel activin B were induced by 11-KT stimulation in vitro. Further, activin B induced proliferation of spermatogonia, but was unable to induce meiosis or further developmental steps (Miura et al., 1995a).

AMH, also known as Müllerian inhibiting substance, is secreted as intercellular signalling protein. It was identified and later purified on the basis of its ability to induce regression of the female genitalia primordium, the Müllerian duct, in mammalian embryos (Balanchard and Josso, 1974). In mammals, AMH is produced by Sertoli cells of the foetal and prepubertal testis and by ovarian granulosa cells after birth (Balanchard and Josso, 1974; Vigier et al., 1984). AMH was isolated from eel testis as gene down-regulated by human chorionic gonadotropin (hCG) treatment using gene expression screening. AMH was expressed in Sertoli cells of immature testes, but was strongly down-regulated after gonadotropin stimulation. Expression of AMH mRNA was suppressed by 11-KT in vitro. To examine the function of AMH in spermatogenesis, recombinant eel AMH (eAMH) was added to a testicular organ culture system. Spermatogonial proliferation induced by 11-KT in vitro was suppressed by recombinant eAMH. Furthermore, addition of a specific anti-eAMH antibody induced spermatogonial proliferation in a germ cell/somatic cell co-culture system. This data set indicates that eAMH prevents the initiation of spermatogenesis and, therefore, suppression of AMH expression is necessary to initiate spermatogenesis. Hence, AMH is a spermatogenesis-preventing substance. Down-regulation of AMH mRNA during the progression of spermatogenesis has been reported in some teleost species (Rodríguez-Marí et al., 2005; Halm et al., 2007; Pala et al., 2008), which supports our hypothesis as regards the role of AMH. Taken together, it seems possible that the two TGFβ family members AMH and activin play opposing roles with regard to the differentiation of rapidly proliferating spermatogonia, activin B being a stimulatory, and AMH an inhibitory factor (Fig. 8).

AMH is also related to the control of gamete development before initiation of spermatogenesis in fish. In medaka, AMH stimulated germ cell proliferation already before hatching just after primordial germ cells had reached the gonadal primordium (Shiraishi et al., 2008). In medaka, a loss-of-function mutation of the AMH receptor not only resulted in excessive proliferation of the PGCs before hatching but also a premature initiation of meiosis, and prevented germ cell proliferation during male sex differentiation after hatching (Morinaga et al., 2007), so that AMH may also act as a meiosis preventing substance. Thus, AMH is multi-functional factor for reproduction in fish. It would be interesting to investigate in higher vertebrates if the phylogenetically old functions of AMH have been conserved, at least to some extent.

4.3.3. Initiation of meiosis

Following mitotic proliferation, type B spermatogonia enter the meiotic prophase and differentiate into primary spermatocytes. DHP, a major progestin in teleost fish, is an indispensable hormone for the initiation of meiosis in spermatogenesis.

Progestins are sex steroid hormones important for female reproduction. In mammals, the principal physiological action of progestin is to prepare the reproductive tract for pregnancy and

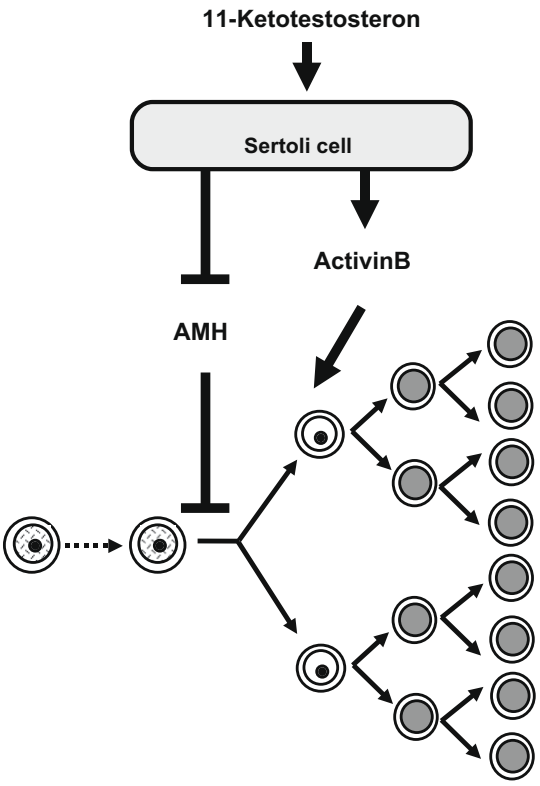

Fig. 8. A schematic summary of the possible control mechanisms at the initiation of spermatogonial proliferation toward meiosis in fish. 11-KT; 11-ketotestosterone, AMH; anti-Müllerian hormone.

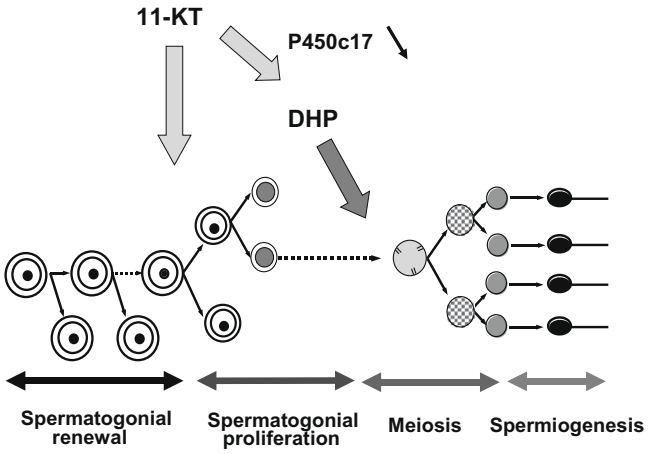

Fig. 9. A schematic summary of the relationship between 11-ketotestosterone (11-KT) and DHP (17α,20β-dihydroxy-4-pregnen-3-one) in regulating fish spermatogenesis. 11-KT can inhibit the expression of P450c17, facilitating DHP production via an increased availability of substrate for DHP synthesis.

to provide nutritive support for the embryo during gestation (Thomas, 1998). In all vertebrates, progestin also plays important roles in gametogenesis. In females, progestins regulate oocyte maturation by binding to an oocyte plasma membrane receptor. Progestin binding inhibits oocyte adenylate cyclase, resulting in reduced cyclic AMP dependent protein kinase activity. This induces the activation of "maturation promoting factor" (MPF) via Cdc25, and thereby the resumption of division I of meiosis (Nagahama, 1997).

During the reproductive cycle of male salmonid fish, there are two peaks of DHP blood plasma levels. A big peak is observed in the spawning season, another small peak during the progression of spermatogonial proliferation; the spawning-associated peak will be discussed later. The small peak of DHP was known in salmonid for some time (Depeche and Sire, 1982; Scott and Sumpter, 1989), but its role had not been clarified clearly yet. Recently, a role for DHP in early spermatogenesis became clear using the eel testis tissue/cell culture systems (Miura et al., 2007).

DHP and its nuclear receptors were present in the testis at an early stage of spermatogenesis, and DHP was shown to induce DNA replication in spermatogonia. Although 11-ketotestosterone also stimulated DNA synthesis in spermatogonia, as mentioned above, antibodies against DHP prevented DNA replication when added during the period when meiosis was initiated. DHP treatment also induced the expression of meiosis specific markers, such as Dmcl and Spo11. Furthermore, Spo11 expression and synaptonemal complexes, specific features of the meiotic prophase, were detected in testicular fragments cultured with DHP in some germ cells that showed morphological characteristics of undifferentiated spermatogonia. These data indicate that DHP induces spermatogonia to enter the meiotic prophase (Fig. 9).

Recently, DHP was shown to induce meiosis not only in spermatogenesis, but also during early oogenesis in Japanese huchen, *Hucho perryi*, and common carp, *Cyprinus carpio* (Miura et al., 2007). In huchen and carp ovarian organ culture, DHP induced DNA synthesis of oogonia, and the expression of Spo11 and synaptonemal complex proteins in some germ cells. Therefore, DHP is also implicated in the regulation of early oogenesis from oogonial proliferation to initiation of the first meiotic division. These data from male and female gonad tissue and cell culture systems suggest that DHP, a progestin, is an essential factor for the initiation of meiosis in both spermatogenesis and oogenesis.

What are the molecular mechanisms by which DHP initiates meiosis in fish? Although we attempted to clone key factors regulated by DHP stimulation (Ozaki et al., 2006), the mechanism has not been clarified yet. The zebrafish loss-of-function mutant for a DNA-repair enzyme, the Mlh1 mutant male, is completely infertile due to a block in meiosis I (Feitsma et al., 2007). Such mutant lines may be suitable models to further study DHP-regulated control mechanisms of meiosis.

4.3.4. Final maturation of male gametes

During the breeding season of male teleost fish, numerous plasma hormone levels show remarkable changes which are initiated by an increase in LH secretion (Prat et al., 1996). LH secretion induces an increase in the production of the testicular steroids, such as 11-KT and DHP or 20β-S (the latter mostly in marine species). 11-KT injections induced spermiation in goldfish and some salmonids, and DHP injections had similar effects in several salmonids (Ueda et al., 1985; Milla et al., 2008) and eel (Miura et al., 1991b). Recently, it has been proposed that 11-deoxycorticosterone participates in the control of milt fluidity as well (Milla et al., 2008). While LH and these steroids clearly are involved in regulating spermiation in fish, the mechanism of action of these hormones on milt hydration, sperm migration to the sperm duct, or increase in milt volume, are still unclear.

In some species, spermatozoa released from Sertoli cells after completion of spermiogenesis are not yet capable of fertilizing eggs. In salmonids, spermatozoa in the testis and in the sperm duct are immotile. Dilution with fresh water induces motility of spermatozoa collected from the sperm duct while testicular spermatozoa remain immotile after dilution. Thus, spermatozoa acquire the ability of motility during their passage through the sperm duct.

Sperm maturation, the phase during which non-functional gametes develop into mature spermatozoa (fully capable of vigorous motility and fertilization) involves physiological but no morphological changes. In salmonids, sperm maturation (the acquisition

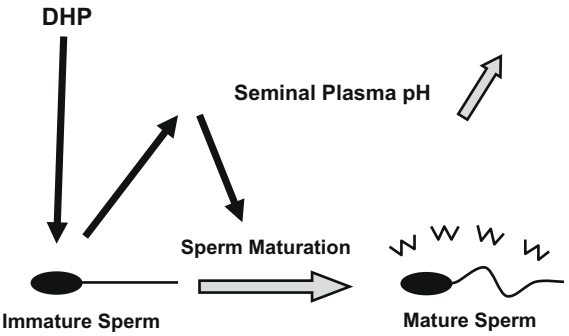

Fig. 10. A schematic summary of the possible control mechanisms of sperm maturation in fish.

of sperm motility) has been induced by increasing the seminal plasma pH (approximately to pH 8.0) in the sperm duct, which results in elevation of intra-sperm cAMP levels (Morisawa and Morisawa, 1988; Miura et al. ,1992). Similar results have been reported for Japanese eel spermatozoa by Miura et al. (1995b) and Ohta et al. (1997).

Sperm maturation is also regulated by the endocrine system. In some teleosts including Japanese eel, it has been suggested that DHP regulates sperm maturation (Miura et al., 1991b, 1992). It seems that DHP action is mediated through an increase in the seminal plasma pH, which in turn increases the cAMP content in sperm, thereby allowing the acquisition of sperm motility (Miura et al., 1991b, 1992, 1995b; Fig. 10). However, progestins may have a direct action on sperm motility since 20β-S binds to sperm plasma membrane and stimulates sperm hypermotility in Atlantic croaker and seatrout (Tubbs and Thomas, 2008). A membrane bound progestins receptor (mPRα) has been localized on the plasma membrane of the spermatozoon mid piece in Atlantic croaker (Thomas et al., 2005).

Thus, estrogenic, androgenic, and progestagenic sex steroids, are important regulators for the progression of spermatogenesis from spermatogonial stem cell renewal to sperm maturation.

5. Intrinsic regulation of spermatogenesis

Experimental approaches depriving the testis from crucial extrinsic input, e.g. hypophysectomy (Billard, 1969a; Khan et al., 1986), or testis cell/tissue culture in the absence of hormones/growth factors demonstrated that the development beyond the slow self-renewal of undifferentiated type A spermatogonia requires extrinsic regulatory input in fish. Similar conclusions can be drawn from experimental models in mammals, where loss of the pituitary gonadotropins for different reasons (e.g. Kendall et al., 1995; Seminara et al., 2003) resulted in a failure of pubertal maturation. Still, interspecies germ cell transplantation experiments showed that the timing of the different developmental steps constituting spermatogenesis is determined by the germ cell genome (França et al., 1998), i.e. reflects cell autonomous processes. It appears therefore that hormones/growth factors do not implement a step-wise regulation of germ cell development, but rather are responsible for regulating the balance and transition between the different micro-environments that are required for the germ cells to execute their autonomous developmental program.

The complexity of this developmental program can be exemplified through a short side-step to yeast. From the nearly 6200 genes in the genome of *Saccharomyces cerevisiae* (Goffeau et al., 1996), 1600 are expressed differentially during meiosis. When deleting 300 of these genes one by one (Rabitsch et al., 2001), ~10% were found to be required for proper segregation of the meiotic chromosomes only. Using the relation of 10% of 1600 meiosis-related genes being required for meiosis, 160 genes represent ~2.5% of the yeast genome. Calculating with the estimated 37,460 genes in the zebrafish genome, 2.5% represented more than 900 potentially meiosis-specific genes in zebrafish; additional spermatogenesis-specific genes may function during the mitotic and the spermiogenic phases in multicellular organisms. Some of the spermatogenesis-specific genes seem to be highly conserved, as exemplified by the rescue of a meiotic defect in a *Drosophila* mutant by the homologous human gene (Xu et al., 2003). In mammals, several cases of compromised gene functions derived from mutagenesis screens, targeted disruption of candidate genes, or from the genetic analysis of infertility patients, were characterised with regard to their spermatogenesis phenotypes. These germ cell-autonomous processes are often based on germ cell-specific (variants of) cell cycle regulators, transcription factors, or enzymes sometimes referred to as chauvinist genes that, when suffering a loss-of-function mutation, lead to an arrest of spermatogenesis at a specific stage, thereby identifying the essential function(s) of the gene product. The respective information has been reviewed comprehensively (De Rooij and de Boer, 2003). The information available in fish is reviewed below.

The gene *dead end* (*dnd*) encodes an RNA binding protein that is a component of the germ plasm in zebrafish, and is specifically expressed in primordial germ cells (PGCs). Dnd protein is localized to perinuclear germ granules (nuage) within PGCs. Knockdown of *dnd* mRNA results in failure of PGCs to migrate towards the genital ridges and subsequent PGC apoptosis, resulting in a germ cell-free, sterile gonad where the somatic compartment differentiates as a testis (Slanchev et al., 2005; Weidinger et al., 2003). A similar phenotype as regards the loss of all PGCs has been observed in mice where a point mutation resulted in a premature stop codon of the murine *dnd* orthologue gene (Youngren et al., 2005).

At a later stage in zebrafish ontogenesis, loss-of-function mutations of the two germ cell-specific members of the Piwi subclass of the Argonaut family of proteins result in complete germ cell loss as well (Houwing et al., 2008; Houwing et al., 2007). Akin phenotypes have been described in mice, although loss of these proteins (called MIWI and MILI, respectively) still allows survival of spermatogonia and apoptosis occurs at the spermatocyte stage (Deng and Lin, 2002). However, ablation of an additional mouse variant of the Argonaut family, MIWI2, also affects SSC maintenance (Carmell et al., 2007). In zebrafish, the *piwi* orthologues are *ziwi* and *zili*, coding for enzymes involved in the metabolism of a germ line-specific class of non-coding, small RNAs, the Piwi-interacting (pi) RNAs. Most piRNAs are involved in transposon silencing, possibly by modulating the DNA methylation pattern and/or by piRNA-guided breakdown of transposon-derived sequences, while some of them might also target non-repetitive, exonic sequences (Houwing et al., 2008). Loss of Ziwi protein results in the apoptotic loss of all germ cells at 3–4 weeks of age and, in contrast to wild-type siblings, mutant germ cells were not able to enter differentiation (spermatogenesis) until they became apoptotic. The present model assumes that Ziwi and Zili collaborate to amplify the piRNAs required for transposon silencing; still, Ziwi alone seems capable to generate low levels of piRNAs (Houwing et al., 2008). Therefore, it may not be surprising that germ cell loss in *zili* occurs at a later stage (6–7 weeks of age) than in *ziwi* loss-of-function mutants. Similar to Ziwi, germ cells cannot enter the differentiation path (spermatogenesis) in the absence of Zili until entering apoptosis.

The last example discussed in the context of germ cell autonomous regulation of spermatogenesis refers to a gene, *mlh1*, operating during meiosis. In leptotene spermatocytes, a large number of double-strand breaks are generated in the chromosomes (Marcon and Moens, 2005). The homologues are still apart, but begin to search for homology. Synapsis, close association of homologues

by binding of synaptonemal complex proteins, starts in zygotene and is completed in pachytene. During synapsis the double-strand breaks are repaired. A fraction of these, on average one or two breaks per pair, is repaired via homologous recombination with a nonsister chromatid of the homolog. These crossing-over sites become visible in diplotene, when desynapsis takes place, and are stabilized as chiasmata, the only sites that keep the chromosome pair together. The mutL complex involved in meiosis consists of Mlh1 and Mlh3 and is present in distinct foci on the synapsed elements during pachytene. In mice, the MLH1/MLH3 foci coincide in timing, number, and position with the presumptive sites of crossing over (Marcon and Moens 2005). The idea is therefore that the complex stabilizes a limited number of recombination sites for repair by crossing over, while the other sites will be repaired via different mechanisms. *Mlh1* and *mlh3* knockout mice have very similar phenotypes in respect to meiosis, males are sterile and lack spermatozoa completely. A very similar phenotype has been found in zebrafish lacking a functional Mlh1 protein (Feitsma et al., 2007). The arrest in spermatogenesis was localized to metaphase I and primary spermatocytes accumulated at this arrest point, showing irregular meiotic figures in the nuclei and massive apoptosis. Surprisingly, females were fully fertile, but their progeny shows high rates of dysmorphology and mortality within the first days of development caused by aneuploidy, resulting from meiosis I chromosomal missegregation.

6. Future aspects and research directions

Spermatogenesis provides motile cellular vectors through an evolutionary conserved process that has been placed under the control of the brain-pituitary system in vertebrates. The master control exerted by the vertebrate endocrine system over testis maturation (puberty) and adult spermatogenesis, whether continuous or intermittent in seasonally reproducing species, provides numerous evolutionary advantages, such as silencing of the reproductive system until somatic development and/or seasonal conditions are sufficiently favourable for an individual to "afford" reproduction. Moreover, integrating (re-)activation of spermatogenesis, via its dependency on sex steroids, with reproductive behaviour and secondary sexual characters, allows for sexual selection to have an impact. Seemingly wasteful (ornamental) competitive traits, often present in males, reflect the individual's competence to recruit resources from the environment, involve several genes spread over the genome, and hence can serve as genome quality indicators that are used during mate choice. In this way deleterious alleles are efficiently removed, and advantageous ones rapidly fixed in a population (Clutton-Brock, 2007; Siller, 2001).

What are the most important steps in spermatogenesis under control of the endocrine system? With the information available at present, there seem to be three points where reproductive hormones play a critical regulatory role: (i) the balance between self-renewal and differentiation of SSCs; (ii) the transition of type A spermatogonia to rapidly proliferating type B spermatogonia, and (iii) the entry into meiosis. During later developmental stages, on the other hand, the endocrine system seems to assume a permissive rather than stimulatory role, enabling Sertoli cells and possibly other somatic cells to generate the microenvironment germ cells require to proceed through meiosis and spermiogenesis.

The sensitivity of the earlier stages for stimulatory input via the endocrine system is a characteristic feature for vertebrates and makes this period very interesting from the point of view of basic biology. Knowledge on the regulatory mechanisms is only beginning to emerge, and respective information will contribute significantly to our understanding of (spermatogonial) stem cell biology. Moreover, the recent finding that SSCs are pluripotent cells in mice and men will further boost research into understanding the balance between SSC self-renewal and differentiation, from a basic biology, comparative, but also from an applied (e.g. medical, veterinary, conservation biology) point of view.

In addition, the early stages of spermatogenesis are of great interest for research fields, such as aquaculture biotechnology and ecotoxicology. Surface freshwater bodies, for example, contain biologically significant amounts of estrogens, oestrogen-like compounds, anti-androgens, and other agonists/antagonists of nuclear receptors, and detrimental effects on spermatogenesis of these compounds, termed endocrine disrupting chemicals, have been described in wild populations of fish and in experimental models (Hutchinson et al., 2006). In aquaculture biotechnology, precocious male maturation is commonly observed problem in different species, with adverse effects on growth and flesh composition, for example (see Taranger et al., 2010). Detailed knowledge about the underlying processes triggering and sustaining testis maturation will be of great relevance for developing approaches to control male puberty, and for estimating the impact of, and the requirement for monitoring/regulating the presence of endocrine disrupting chemicals in surface freshwater bodies.

Improving our knowledge on the early stages of spermatogenesis sensitive to exogenous stimulatory input, we need experimental systems suitable to investigate, for example, SSC biology in fish. Until now, the SSC transplantation assay is the only system available in vertebrates, has been adapted for tilapia already, and should be developed for other species as well. For example, we known that 11-KT suppresses Sertoli cell AMH expression and thereby facilitates the start of spermatogenesis, but since also the receptor for AMH is expressed by Sertoli cells, we in fact only know about the initial autocrine loop of this signalling system that will probably consist of at least one other signalling molecule produced by Sertoli cells, a respective receptor on germ cells, and the germ cell's response system to this signalling. Considering that in the fruitfly, different regulatory systems function in parallel to control SSC behaviour (Li and Xie, 2005), including signalling from SSCs to the somatic cells, and assuming an at least as complex regulatory network in the vertebrate stem cell niche in the testis, we can safely predict that there is a lot to learn.

In order to learn more about SSCs and other stages of germ cell development as well as their interaction with somatic cells of the testis, we need to enrich or purify these cell types. This is not only required for transplantation assays to investigate SSC biology, but also for transcriptomic or proteomic profiling of the different cell types. Possible approaches include the generation of transgenic lines expressing fluorescent proteins under the control of promoter/enhancer sequences that are specific for the cell types or developmental stages of interest. Also, double or triple transgenic lines expressing distinct reporter genes will be excellently suited for cell lineage tracing studies, or for investigating the interaction of different cell types during testis differentiation and adult functioning. Finally, the complete process of spermatogenesis can be studied in fish using ex vivo tissue culture systems (eel: Miura et al., 1991; goldfish: Remacle 1976; tilapia: Tokalov and Gutzeit, 2005). Similar systems should be developed for model species like medaka and zebrafish, in order to study the direct effects of hormones/growth factors on spermatogenesis, possibly including the live-imaging of developmental processes with tissue from transgenic animals expressing fluorescent proteins and modern imaging devices.

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