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An overview of spermatogonial stem cell physiology, niche and transplantation in fish

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Abstract

Similar to mammals, spermatogenesis in fish is initiated by spermatogonial stem cells (SSCs) which either self-renew or gradually differentiate to produce mature sperm. SSCs are located in a particular testis microenvironment called SSC niche, formed by Sertoli and peritubular myoid cells, the basement membrane and other cellular components/factors from the intertubular compartment that regulate SSCs maintenance and fate. Considering the great variation in testis structure/arrangement across fish species, the study of the niche components is crucial to understand SSCs physiology. Additionally, the germ cell transplantation technique, which has been applied to fish in the last decade, is a unique approach to elucidating important functional aspects of SSCs biology such as: (i) the capacity of SSCs to colonize the testis of recipient species (syngeneic and xenogeneic transplantation) giving rise to donor sperm; (ii) the plasticity of these cells, considering that spermatogonia and oogonia can be derived from SSCs collected from the opposite sex; and (iii) the possibility of genetically manipulating SSCs before transplantation to produce transgenic fish. However, fish SSC isolation and characterization has been limited so far by the lack of specific molecular markers for these cells. Therefore, various research groups are currently investigating specific SSCs markers and, up to date, few proteins have been identified in different spermatogonial populations from distinct fish species (e.g. Notch1, Ly75, Plzf, Oct-4, SGSA-1). Furthermore, the development of a fish SSC culture system would allow the investigation of important regulatory aspects of the SSC physiology in well-defined conditions as well as to *in vitro* amplify these rare cells. Overall, the study of SSC physiology, niche and transplantation in fish has opened up new scenarios for the development of aquaculture and reproductive biotechnologies such as germplasm conservation of endangered or commercially important species and the possibility of generating transgenic fish.

Keywords: spermatogonial stem cell, spermatogonial niche, germ cells transplantation, biotechnology, Nile-tilapia (*Oreochromis niloticus*).

Introduction

Spermatogenesis is a complex process involving a series of cellular changes that ultimately leads to the formation of haploid male gametes (spermatozoa) and includes mitotic, meiotic and post-meiotic phases. In general, in comparison to higher vertebrates the spermatogenic process in fish shows two main differences. First, within the spermatogenic tubules, cytoplasmic extensions of Sertoli cells form the spermatogenic cysts, that involve and nurse a single, clonally and hence synchronously developing group of germ cells deriving from a single type A spermatogonium. Secondly, these cyst-forming Sertoli cells retain their capacity to proliferate in the testes of sexually mature fish (Schulz *et al.*, 2005; Leal *et al.*, 2009). Nevertheless, it is known that in all vertebrates and even in invertebrates, the spermatogenic process is continuously maintained through the self-renewal and differentiation of special stem cells called spermatogonial stem cells (SSCs) that provide the foundation for spermatogenesis (Nóbrega *et al.*, 2009; Schulz *et al.*, 2010). Therefore, the proper balance between the process of self-renewal and differentiation is essential to ensure the continuous homeostasis of spermatogenesis during the reproductive life in males (Alvarenga and França, 2009; Schulz *et al.*, 2010).

SSC activity is regulated within a specific microenvironment in the testes, the spermatogonial stem cell niche which may be influenced by Sertoli and peritubular myoid cells, basement membrane, and other cellular components/factors from the intertubular compartment (Chiarini-Garcia *et al.*, 2003; Hofmann, 2008; Caires *et al.*, 2010; Phillips *et al.*, 2010). Currently, the only means to study SSCs and their niche in fish is by exploiting their stem cell functional properties, such as slow cell-cycling and quiescent nature, through the long-term label-retaining cell approach (Braun *et al.*, 2003), or by studying their functionality and plasticity through spermatogonial transplantation techniques (Okutsu *et al.*, 2006; Lacerda *et al.*, 2010, 2012; Nóbrega *et al.*, 2010). Although the use of SSCs transplantation has been relatively well established in mammals, this methodology has only been adapted to be used in fish at the beginning of the last decade. Studies using SSCs transplantation, have contributed significantly to the understanding of the biological characteristics of fish SSCs and their application to

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developmental biotechnology (Okutsu *et al*., 2007; Yoshizaki *et al*., 2012), as well as to enabling the formation of gene banks, preserving endangered species, and creating opportunities for the genetic improvement of economically important fish species (Lacerda *et al*., 2010), including the novel options for transgenesis (Lacerda *et al*., 2012).

In this context, this review attempts to correlate current findings of reproductive physiology, latest research on the spermatogenic niche and the most recent applied developments for SSC transplantation in various fish species. In the following sections, we will review the spermatogonial stem cell morphology and kinetics, spermatogonial distribution and niche in teleost fishes, as well as a summary of up to date information about fish spermatogonial stem cell transplantation. Finally, we will discuss some advances on SSC phenotypic characterization, cell culture and future perspectives.

Spermatogonial stem cells morphology and kinetics

Spermatogenesis is initiated in the testis when a SSC is triggered to undergo mitosis and form differentiated type of spermatogonia. Different generations of spermatogonia, type A undifferentiated spermatogonia (A_{und}) including SSCs, type A differentiated spermatogonia (A_{diff}), and type B spermatogonia have been identified in zebrafish (*Danio rerio*) and Nile-tilapia (*Oreochromis niloticus*; Fig. 1), and this classification model has been proposed to be used for other fish species (Vilela *et al*., 2003; Leal *et al*., 2009). The number of spermatogonial generations is considered to be species-specific and hence genetically determined (Nóbrega *et al*., 2009; Schulz *et al*., 2010), therefore representing an important parameter to investigate the spermatogenic potential and efficiency in a given species. Morphological differences including nuclear and nucleolus features, size and number of cells per cysts, amount and distribution of heterochromatin and cytoplasmic structure/organelles are, to date, parameters used to identify the different types of spermatogonial generations (Matta *et al*., 2002; Vilela *et al*., 2003; Schulz *et al*., 2005; Leal *et al*., 2009). A single type A spermatogonium (A_{und}) is not connected via cytoplasmic bridges to clone members and is the largest cell of the spermatogenic process in fish, presenting a large clear nucleus with one or two conspicuous nucleoli (Miura, 1999; Schulz and Miura, 2002; Miura *et al*., 2003). Their cytoplasm is poorly differentiated and contains mitochondria associated with a large amount of electron-dense material, called nuage, which contains ribonucleoproteins and RNAs with long half-life times, and includes elements such as vasa mRNA, specifically present in the germ cell lineage (Braat *et al*., 1999; Quagio-Grassiotto and Carvalho, 1999; Knaut *et al*., 2000; Houwing *et al*., 2007; Leal *et al*., 2009; Schulz *et al*., 2010). Spermatogonia type A_{diff} still share some morphological characteristics with A_{und} but have a greatly reduced potential for self-renewal. Irreversible commitment to continue further differentiation, accompanied by changes in various morphological aspects, is observed in type B spermatogonia (Schulz *et al*., 2010).

Spermatogonial distribution and niche

The specific microenvironment in the testes called “spermatogonial stem cell niche” is known to continuously sustain the spermatogenic process throughout the male reproductive life, regulating SSC self-renewal, quiescence and differentiation (Oatley and Brinster, 2012). Despite its crucial role for SSC fate, the cellular and molecular composition of SSC niche has remained unknown for almost all vertebrate species, including fish. In some mammals and zebrafish , the SSC niche has recently been identified within regions of the seminiferous tubules, which are adjacent to the interstitial compartment, preferentially located close to blood vessels (Chiarini-Garcia *et al*., 2001; Yoshida *et al*., 2007; Campos-Junior *et al*., 2012). An important component of this microevironment are somatic cells. In the mammalian testis, Sertoli and peritubular myoid cells regulate SSCs self-renewal by GDNF-GFRA1 and CSF1-CSF1r signaling (Hofmann *et al*., 2005; Hofmann, 2008; Campos-Junior *et al*., 2012). In the zebrafish testes, higher expression of steroidogenesis-related genes and high 11-ketotestosterone (11-KT) levels result in a pro-differentiation microenvironment leading to SSC commitment to differentiation, which indicate a significant role of the Leydig cells and/or their secreted factors in spermatogonial differentiation in fish (Nóbrega *et al*., 2010).

Considering the great variations across fish species, the study of the anatomical distribution of spermatogonia and their relationship to the local microenvironment is quite important. In this regard, Grier (1981) described two different patterns of spermatogonial distribution in the teleost testis. First, in the restricted spermatogonia distribution, spermatogonia are located exclusively in the distal region of the germinal compartment (near the tunica albuginea), which therefore could be considered a particular microenvironment to regulate the spermatogonial fate. This would represent a spatially well-defined SSC niche, compared to other vertebrates. In the second pattern, the unrestricted spermatogonial distribution, SSCs are spread along the germinal compartment throughout the testis, being typical of Cypriniformes, Characiformes, and Salmoniformes (Parenti and Grier, 2004). However, an intermediate pattern also appears to exist between restricted and unrestricted spermatogonial distribution, such as found in Nile-tilapia (*Oreochromis niloticus*; Vilela *et al*., 2003), *Senegalese sole* (Garcia-Lopez *et al*., 2005) or Atlantic cod (*Gadus morhua*; (Almeida *et al*., 2008). In these species, undifferentiated spermatogonia show a preferential, but not exclusive, location close to the tunica albuginea as illustrated for the Nile tilapia (Fig. 2).

Recently, analyzing the topographical distribution of type A spermatogonia in zebrafish, which has an unrestricted spermatogonial distribution, we

found that, similar to mammals (Campos-Junior *et al*., 2012; Costa *et al*., 2012), most of these cells are located in regions of the seminiferous tubules that are adjacent to the interstitial compartment (Nóbrega *et al*., 2010). Moreover, using testes of transgenic zebrafish expressing Gfp in endothelial cells (*fli:egfp*), type A undifferentiated spermatogonia were often found in close association to capillaries surrounding the seminiferous tubules, indicating the existence of a SSC niche in zebrafish, which, next to Sertoli cells, might also be characterized by blood vessel and/or angiocrine factors (Nóbrega *et al*., 2010).

With the exception of some incipient data available for the Japanese eel (Miura *et al*., 2003, 2007) there are practically no studies on the regulation of self-renewal and differentiation of SSCs in fish. In this species, two factors are involved in the control of SSC fate: the PD-ECGF, platelet-derived endothelial cell growth factor and AMH, anti-Müllerian hormone. Under the influence of 17β-estradiol (E2), Sertoli cells produce PD-ECGF, which stimulates the SSC self-renewal. In another way, AMH, also produced by Sertoli cells, is responsible for the inhibition of SSC differentiation and for blocking the proliferation of type B spermatogonia (Miura *et al*., 2002, 2007). Interestingly, the expression of this hormone is suppressed by 11-KT. Other factors such as IGF-I and activin B also seem to interfere in the spermatogonial proliferation and differentiation. Under the influence of 11-KT Sertoli cells produce activin B that stimulates the proliferation of type B spermatogonia, without initiating meiosis. On the other hand, IGF1 induces DNA synthesis in rainbow trout spermatogonia (*Oncorhynchus mykiss*), and is a permissive factor for 11-KT-stimulated spermatogenesis in Japanese eel testis tissue cultures (Miura and Miura, 2003). In Nile-tilapia, *in vitro* studies have demonstrated that IGF and/or hCG (human chorionic gonadotropin) promote spermatogonial mitosis and the onset of meiosis in the presence of 11-KT (Tokalov and Gutzeit, 2005). Furthermore, a recent study reported that recombinant eel GH (growth hormone) induced spermatogonial proliferation in a testis organ culture system, an effect that was independent from the production of steroid hormones or IGF-I (Miura *et al*., 2011). In zebrafish , using short-term adult testes culture Skaar *et al*. (2011) showed that, besides suppressing FSH-stimulated androgen production, recombinant AMH reduces proliferation (inhibition of BrdU incorporation) and prevents differentiation of type A undifferentiated spermatogonia. However, in contrast to results obtained for the Japanese eel, increasing doses of 11-KT did not change AMH mRNA levels significantly which were, on the other hand, down-regulated by FSH (Skaar *et al*., 2011).

In summary, similar to mammals, the survival of fish SSCs occurs as a result of the proper balance of factors originating from the niche. Because spermatogonial niches are dynamic and present high plasticity throughout the development of the individual, they may be influenced by several internal and external factors (Nóbrega *et al*., 2009; Campos-Junior *et al*., 2012). Among these can be mentioned, for example, photoperiod, temperature, pH and ionic concentration of the water, which presumably contribute to determining the seasonality of breeding. Therefore, a better understanding of the regulatory mechanisms operating in the niche probably requires investigations in teleost species living in several different environments.

SSC transplantation

Germ cell transplantation is a very important reproductive technique developed by Brinster and colleagues in 1994 (Brinster and Zimmermann, 1994). Initially described in mammals, it consists of the transference of donor germ cells into the gonads of a recipient animal for the production of gametes from the donor. It is generally accepted that only SSCs are able to colonize the niche and re-establish spermatogenesis in the recipient testis. Therefore, germ cell transplantation not only provides a unique opportunity for gaining new insights into spermatogenesis and the biology of the stem cell niche, but also presents a unique functional bioassay to test the competence of putative SSCs. Therefore, over the years, the technique has gained increasing scientific interest due to its large potential for application in animal reproduction (Honaramooz and Yang, 2011). Although the use of germ cell transplantation has been relatively well established in mammals, the technique started to be applied in fish species only recently (Ciruna *et al*., 2002; Takeuchi *et al*., 2003, 2004). In this scenario, we established a new means of achieving SSCs transplantation in Nile tilapia, in which spermatogonia were transplanted through the urogenital papilla of adult recipient fish (Lacerda *et al*., 2006, 2010). In these studies we demonstrated that isolated donor spermatogonia, injected into the testis of a busulfan-treated adult fish are able to shortly resume spermatogenesis and produce sperm in the recipient testis, which also sired progeny with the donor genotype. Besides that, cryopreserved Nile tilapia spermatogonia were also able to efficiently proliferate and differentiate into spermatozoa in recipient testes after transplantation (Lacerda *et al*., 2010).

SSCs transplantation technology in fish originated a new avenue of research in reproductive biotechnology leading to important advances in the study of the physiology and biology of SSCs, preservation of valuable and endangered fish genetic resources and applications for breeding programs and aquaculture production (Yoshizaki *et al*., 2011). Genetic manipulation of spermatogonial cells followed by transplantation also allows for the production of progeny possessing a modified donor genome (Yoshizaki *et al*., 2011). Alternatively, germ cells from various commercially valuable fish species can be xenotransplanted to recipient fish species that undergo fast sexual maturation, thereby allowing an advantageous commercial production of those species

with long life cycles (Lacerda *et al*., 2012). Moreover, species with high commercial value associated with high production costs due to large rearing space requirements, maintenance difficulties, stunted reproductive capabilities in captivity, or seasonal variations in reproduction, can potentially produce gametes using surrogate recipients from species easier to rear in captivity (Lacerda *et al*., 2012).

As listed in Table 1, several freshwater and marine fish species have been used for germ cell transplantation. In these studies, primordial germ cells (PGCs), spermatogonial stem cells (SSCs) and oogonia were the germ line cells successfully collected from different donor species and transplanted into embryos, larvae or adult fish to attempt donor offspring production (Table 1). Each transplantation method holds specific characteristics, advantages and disadvantages. In the studies using embryos (Table 1A) only one donor PGC is necessary to generate millions of gametes in the recipient gonad and, after a long period of time (up to two years, depending on the species), the germ line transmission success is close to 100% (Saito *et al*., 2008). The use of larval recipients (Table 1B) has similar characteristics as in techniques involving embryos, such as long time required for sperm production and high efficiency in donor genetic transmission (Okutsu *et al*., 2007). However, using adult fish as recipients (Table 1C) results in faster donor sperm production while the rates of germ line transmission so far show a lower efficiency (Majhi *et al*., 2009; Lacerda *et al*., 2010). Different from mammals, the sexual plasticity in fish offers fascinating biotechnological possibilities considering that spermatogonia and oogonia can produce gametes from the opposite sex, i.e., oocytes and sperm, respectively. Consequently, in some species (carrying the X and Y chromosomes), spermatogonial transplantation into female recipients was proposed as a novel methodology to produce Y eggs (Okutsu *et al*., 2006; Nóbrega *et al*., 2010; Yoshizaki *et al*., 2010a), which could then be used to generate all-male populations for basic biological and aquaculture purposes. The opposite is also possible since oogonia isolated from ovaries can differentiate into fully functional X sperm (Yoshizaki *et al*., 2010b; Wong *et al*., 2011).

Spermatogonial stem cell characterization and culture

After transplantation only a small population of the type A spermatogonia is capable of colonizing and continuously maintaining donor spermatogenesis in recipient testis, and these cells therefore are retrospectively identified as the SSC population (Okutsu *et al*., 2006; Yano *et al*., 2008). To date, little is known about the biology of SSC in non-mammalian vertebrates including fish, and in addition, due to limited availability of and difficult accessibility to these cells, their physiological properties are still poorly understood. In this context, the appropriate identification and isolation of SSCs is essential for the efficiency of transplantation as well as to better understand the proliferation and differentiation behavior of these cells after transplantation. The isolation and more detailed study of SSCs in fish has been limited primarily by the lack of specific molecular markers for these cells.

In mammals, mainly in rodents, several molecular markers (cell surface markers, RNA-binding proteins, zinc finger proteins, cytokines, cell cycle proteins and others) have been characterized in order to identify SSCs and their early progenitors (reviewed by Phillips *et al*., 2010 and Kolasa *et al*., 2012). In contrast, few studies address the spermatogonial phenotype characterization in fish. Although some candidates have been proposed (Table 2), unique molecular markers for fish SSCs are not known yet. In the testes of teleost fish, early spermatogonia have been characterized mainly by morphological criteria. For instance, according to high resolution light microscopy characteristics, two types of single undifferentiated spermatogonia differ in Nile-tilapia testis (Fig. 1). The most undifferentiated type (A_{und1}) is most frequently found in regions of the seminiferous tubule close to the tunica albuginea while the second type (A_{und2}) can be found at a certain distance from the tunica (Fig. 2). Currently, we have investigated the expression of proteins which are considered to be markers of rodents SSCs in the Nile-tilapia testis. Our findings indicate that the expression profile of Nile-tilapia SSC is similar to that of rodents (unpublished data), which suggests a conserved role for some molecules signaling in SSCs at the onset of vertebrate spermatogenesis. Recently, a strategy to purify the type A spermatogonia through flow cytometric cell sorting, based only on their morphological characteristics (forward light scatter and side light scatter), was successfully established in fish (Kise *et al*., 2012).

The isolation or enrichment of SSCs using specific molecular markers would provide new and auspicious dimensions for spermatogonial transplantation in fish and could advance studies on both, basic and applied biology of fish spermatogonia. Also in this context, the establishment of a culture system of fish SSCs or co-cultivating these cells with somatic cells (e.g. Sertoli cells) would allow to investigate important regulatory and functional aspects of SSC biology in well-defined conditions, for example, to study the effect of various factors which can influence or determine the destination (self-renewal or differentiation) of these cells. So far, the basic techniques for the long-term *in vitro* culture of spermatogonia have been established for Zebrafish (Kawasaki *et al*., 2012), rainbow trout (Shikina and Yoshizaki, 2010) and Medaka (Hong *et al*., 2004). Furthermore, such a culture system would allow the development of strategies to *in vitro* amplify SSC numbers of rare, endangered, or commercially valuable fish species (Shikina *et al*., 2008; Shikina and Yoshizaki, 2010). These cells can be cryopreserved and subsequently transplanted to closely related recipient fish species (Lacerda *et al*., 2010; Yoshizaki *et al*., 2010b).

Table 1. Studies on germ cell transplantation in fish using embryo (A), newly-hatched larvae (B) and adult as recipient model (C).

	Donor		Recipient		Results of transplantation	Reference
	Species	Germ cell	Species	Age		
A	Zebrafish (*Danio rerio*)	PGC[1]	zebrafish (*Danio rerio*)	blastula-stage of fish embryos	donnor derived offspring	Ciruna *et al*., 2002, Saito et al., 2010
	Pearl danio (*Danio albolineatus*)	PGC	zebrafish (*Danio rerio*)	blastula-stage of fish embryos	male and female donor gametes	Saito *et al*., 2008, 2010
	Goldfish (*Carassius auratus*)	PGC	zebrafish (*Danio rerio*)	blastula-stage of fish embryos	donor sperm production	Saito *et al*., 2008, 2010
	Loach (*Misgurnus anguillicaudatus*)	PGC	zebrafish (*Danio rerio*)	blastula-stage of fish embryos	donor sperm production	Saito *et al*., 2008, 2010
	Japanese eel (*Anguilla japonica*)	PGC	zebrafish (*Danio rerio*)	blastula-stage of fish embryos	colonization of the gonads	Saito *et al*., 2011
B	GFP-transgenic trout (*Oncorhynchus mykiss*)	PGCs	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	donor derived offspring	Takeuchi *et al*., 2003
	GFP-transgenic trout (*Oncorhynchus mykiss*)	PGCs	masu salmon (*Oncorhynchus masou*)	newly hatched fish larvae	donor derived offspring	Takeuchi *et al*., 2004
	GFP-transgenic trout (*Oncorhynchus mykiss*)	SSCs[2]	male/female wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	donor derived offspring	Okutsu *et al*., 2006
	GFP-transgenic trout (*Oncorhynchus mykiss*)	SSCs	sterile triploid salmon (*Oncorhynchus masou*)	newly hatched fish larvae	only trout sperm/eggs production	Okutsu *et al*., 2007
	GFP-transgenic trout (*Oncorhynchus mykiss*)	Cryopreserved PGC	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	male and female donor gametes	Kobayashi *et al*., 2007
	Nibe croaker (*Nibea mitsukurii*)	SSCs	nibe croaker (*Nibea mitsukurii*)	3- to 6-mm larvae	spermatocyte cysts	Takeuchi *et al*., 2009
	Nibe croaker (*Nibea mitsukurii*)	SSCs	chub mackerel (*Scomber japonicas*)	5.3-mm larvae	spermatogonial cysts	Yazawa *et al*., 2010
	Wild-type trout (*Oncorhynchus mykiss*)	SSCs	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	colonization of the gonads	Kise *et al*., 2012
	Masu salmon (*Oncorhynchus masou*)	SSCs	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	colonization of the gonads	Kise *et al*., 2012
	Japanese char (*Salvelinus leucomaenis*)	SSCs	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	colonization of the gonads	Kise *et al*., 2012
	Nibe croaker (*Nibea mitsukurii*)	SSCs	nibe croaker (*Nibea mitsukurii*)	4-mm larvae	colonization of the gonads	Kise *et al*., 2012
	Immature yellowtail (*Seriola quinqueradiata*)	SSCs	yellowtail (*Seriola quinqueradiata*)	8-day-old sterile larvae	donor derived offspring	Morita *et al*., 2012
	Transgenic zebrafish (*Danio rerio*)	oogonia	zebrafish (*Danio rerio*)	2-wk-old sterile hybrid larvae	donor derived offspring	Wong *et al*., 2011
	GFP-transgenic trout	oogonia	wild-type trout (*Oncorhynchus mykiss*)	newly hatched fish larvae	oogonia-derived sperm production	Yoshizaki *et al*., 2010a
C	Nile tilapia (*Oreochromis niloticus*)	SSCs and cryopreserved SSCs	strains of Nile tilapia (*Oreochromis niloticus*)	adult fish (busulfan-treated animals)	donor derived offspring	Lacerda *et al*., 2010
	Juvenile pejerrey (*Odontesthes bonariensis*)	SSCs	patagonian pejerrey (*Odontesthes hatchery*)	adult fish (busulfan-treated animals)	donor derived offspring	Majhi *et al*., 2009
	Zebrafish (*Danio rerio*)	SSCs	male zebrafish (*Danio rerio*)	adult fish (busulfan-treated animals)	incomplete donor spermatogenesis	Nóbrega *et al*., 2010
	Zebrafish (*Danio rerio*)	SSCs	female zebrafish (*Danio rerio*)	adult fish (busulfan-treated animals)	oocyte production	Nóbrega *et al*., 2010

[1] Primordial germ cells, [2] Spermatogonial stem cells.

Table 2. Spermatogonial cell markers in fish testes.

Marker	Specification	Species investigated	Expressed by	Reference
Notch1	Notch homolog protein 1	Rainbow trout	Type-A spermatogonia	Yano *et al*., 2009
Ly75 (CD205)	Lymphocyte antigen 75	Rainbow trout	Predominantly type-A spermatogonia	Nagasawa *et al*., 2010
		Pacific bluefin tuna	Type-A spermatogonia	Nagasawa et al., 2012
Plzf	Promyelocytic leukaemia zinc finger	Zebrafish	Early Spermatogonia	Ozaki *et al*., 2011
Pou5f1 (Oct-4)	POU domain, class 5, transcription factor 1	Medaka	Type-A spermatogonia	Sanchez-Sanchez *et al*., 2010
SGSA-1	Spermatogonia specific-antigen-1 (*unknown identity*)	Japanese eel	Early Spermatogonia	Kobayashi *et al*., 1998

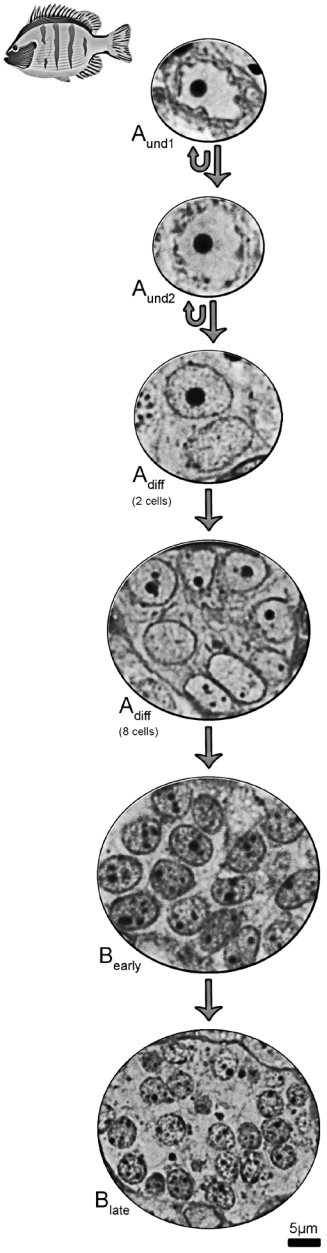

Figure 1. Spermatogonial generations in the Nile tilapia. Two types of single undifferentiated spermatogonia are observed in Nile-tilapia testis: A_{und1} and A_{und2}. These cells possess the capacity of self-renew (curved arrows) or differentiate (arrows). Undifferentiated spermatogonia divide to form type A differentiated spermatogonia (A_{diff}) that exist in clones of 2 to 8 germ cells inside the spermatogenic cysts. Ultimately, type A differentiated spermatogonia give rise to type B spermatogonia (B_{early}) which after undergoing successive mitotic divisions (B_{late}) enter in the meiotic phase.

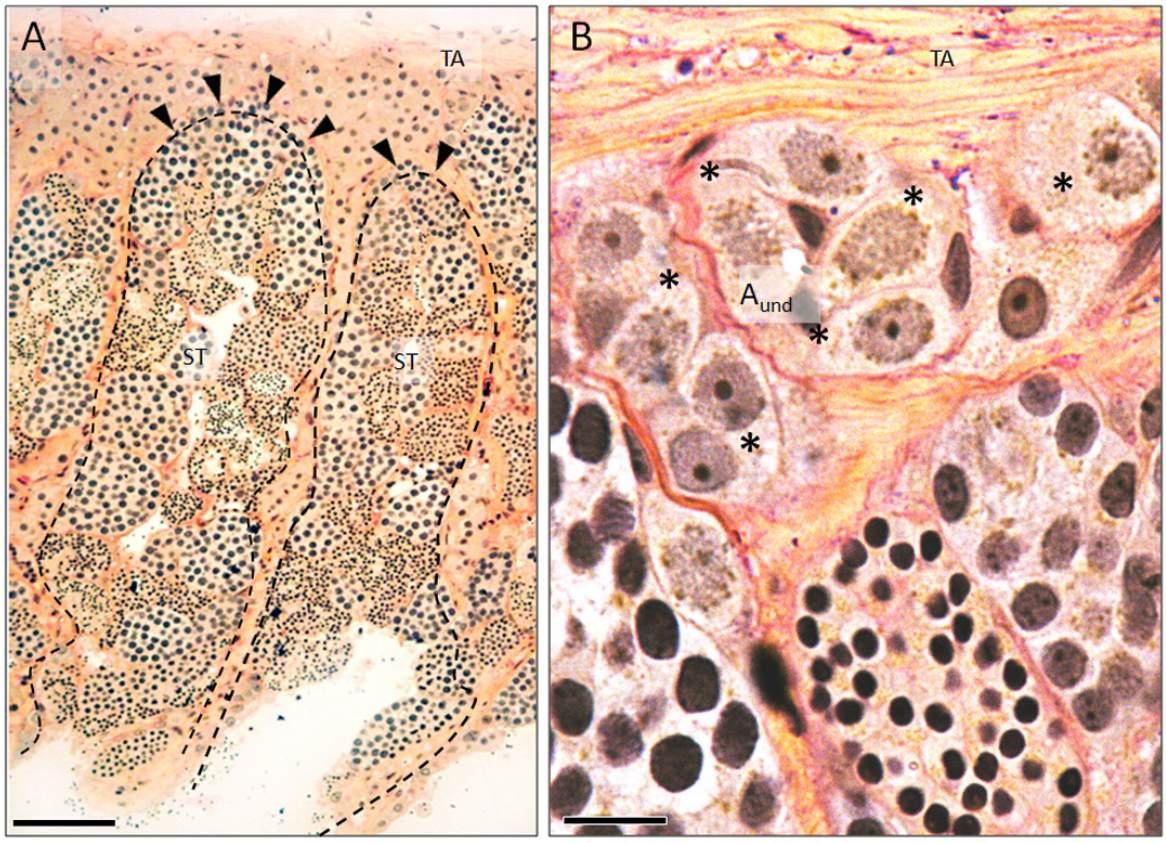

Figure 2. Distribution of type A spermatogonia in the germinal compartment of Nile tilapia testis. (A) In this species, seminiferous tubule (ST) show a radial arrangement in the testicular parenchyma and, at the distal regions, they present a blind ending (arrowheads) near to the tunica albuginea (TA). At this region (B), a high density of type A undifferentiated spermatogonia (A_{und}) is observed (asterisk). Bars A=100 µm, B=15 µm.

Future perspectives

Spermatogonial stem cells represent the adult germ line component with the most promising biotechnological possibilities for reproductive intervention in fish. Further knowledge on SSC physiology and niche characteristics is required and sought to impact the future use of the transplantation approach, a central element of the state of the art in fish reproductive technologies that are currently being developed. In such a promising scenario, we can expect significant advances in commercial fish production and the recovery of endangered fish populations in the future. This optimistic view is based on the high plasticity of fish SSCs to assimilate into spermatogenic stem cell systems across a wide range of species and the relatively uncomplicated techniques involved, all of which makes technological transfer realistic.

SSC transplantation into immunocompromised mouse testis has been the only so far accepted functional assay for mammalian SSCs (Brinster and Zimmermann, 1994; Kubota and Brinster, 2006). Current knowledge with fish transplantation constitutes a body of evidences indicating that SSC physiology is conserved in phylogenetically distant vertebrate groups. Hence, standard general SSC assays, based on transplantation, are constantly being developed and improved for several species. In that respect, Nile-tilapia is becoming an established model of recipient animal in fish SSC assays, in part because of the excellent advantages of that species, such as easy adaptation to captivity, rapid body growth and rapid achievement of sexual maturity presenting reproductive organs with an appropriate size that allows simple manipulations for transplantation and other related reproductive procedures (Lacerda *et al*., 2010, 2012). The discovery and establishment of new testicular cell markers in fish will allow further advances in our understanding of SSC physiology. To this end, the generation of a transgenic fish with an engineered, constitutively expressed reporter gene would be instrumental. Thus, unequivocal identity of donor SSCs could be easily established after transplantation into a recipient fish testis. This approach, combined with the use of highly sensitive separation methods, such as FACS (Fluorescence-activated cell sorting) and MACS (Magnetic-activated cell sorting), will guarantee that fish SSC phenotype will continue to be characterized, as

it was originally accomplished in mammalian murine species (Shinohara *et al*., 1999, 2000; Kubota *et al*., 2003, 2004). Presumably, important aspects of SSC self-renewal physiology are conserved, but further investigations are required to determine if any deviations from patterns known in mammals do exist, discovering novel fish specific signaling pathways. This knowledge would be valuable to design better methods of *in vitro* SSC population expansion, a prerequisite for SSC-based genetic manipulations in fish (Lacerda *et al*., 2012). Additionally, advances in SSC culture will expand our knowledge on SSC biology.

It seems evident that the central aspects highlighted on this review (SSC molecular identity, niche) represent highly interconnected basic knowledge units that convey in very useful biotechnological methods such as SSC transplantation. In the end, transplantation technologies will be included in the tool kit of reproductive management of many commercially relevant or endangered species. Furthermore, the rearing of fish species with complex life cycles and zootechnical traits in small inexpensive confinement facilities, using well adapted foster species, as well as new methods for fish gamete generation, are but some of the feasible possibilities that the study of SSC physiology brings to fish reproduction, on behalf of protein production for human consumption from aquaculture systems.

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