Cell Tissue Res (2008) 332:533–542
DOI 10.1007/s00441-008-0590-z

REGULAR ARTICLE

Study of the potential spermatogonial stem cell compartment in dogfish testis, *Scyliorhinus canicula* L.

Geraldine Loppion · Amélie Crespel · Anne-Sophie Martinez · Pierrïck Auvray · Pascal Sourdaine

Received: 17 December 2007 / Accepted: 17 January 2008 / Published online: 14 March 2008

Abstract In the lesser-spotted dogfish (*Scyliorhinus canicula*), spermatogenesis takes place within spermatocysts made up of Sertoli cells associated with stage-synchronized germ cells. As shown in testicular cross sections, cysts radiate in maturational order from the germinative area, where they are formed, to the opposite margin of the testis, where spermiation occurs. In the germinative zone, which is located in a specific area between the tunica albuginea of the testis and the dorsal testicular vessel, individual large spermatogonia are surrounded by elongated somatic cells. The aim of this study has been to define whether these spermatogonia share characteristics with spermatogonial stem cells described in vertebrate and non-vertebrate species. We have studied their ultrastructure and their mitotic activity by 5′-bromo-2′-deoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) immunodetection. Additionally, immunodetection of c-Kit receptor, a marker of differentiating spermatogonia in rodents, and of α- and β-spectrins, as constituents of the spectrosome and the fusome, has been performed. Ultrastructurally, nuclei of stage I spermatogonia present the same mottled aspect in dogfish as undifferentiated spermatogonia nuclei in rodents. Moreover, intercellular bridges are not observed in dogfish spermatogonia, although they are present in stage II spermatogonia. BrdU and PCNA immunodetection underlines their low mitotic activity. The presence of a spectrosome-like structure, a cytological marker of the germline stem cells in *Drosophila*, has been observed. Our results constitute the first step in the study of spermatogonial stem cells and their niche in the dogfish.

Keywords Spermatogonia · Spermatogenesis · Lesser-spotted dogfish, *Scyliorhinus canicula* (Elasmobrachii)

G.L. is supported by a CIFRE grant (ANRT and C.RIS Pharma).

G. Loppion · A. Crespel · A.-S. Martinez · P. Sourdaine (✉)
UMR M 100 Ifremer, Physiologie et Ecophysiologie des Mollusques Marins, IFR 146 ICORE,
Université de Caen Basse-Normandie,
14032 Caen, France
e-mail: pascal.sourdaine@unicaen.fr

G. Loppion · P. Auvray
C.RIS Pharma,
54 Rue du Grand Jardin,
35400 Saint-Malo, France

Introduction

Permanent spermatogenesis is ensured by the renewal of spermatogonia from spermatogonial stem cells (De Rooij 2001). The self-renewal and the differentiation of stem cells are tightly linked to their microenvironment or niche. The *Drosophila* model has largely contributed to our understanding of this niche in which the behavior of spermatogonial stem cells is closely dependent on that of their neighboring somatic cells (Kiger et al. 2000; Spradling et al. 2001), such as hub cells, in the distal tip of the testis. Study of the spermatogonial microenvironment in the *Drosophila* testis is made easier by the polarization of the gonad, a characteristic not found in mammals. However, the non-random distribution of undifferentiated spermatogonia along the basal compartment of the seminiferous tubule in rodents suggests that they rely on a niche linked at least to the Sertoli cells and to the interstitium (Chiarini-Garcia et al. 2001, 2003; Hess et al. 2006; Yoshida et al. 2007). Although the characterization of spermatogonial stem cells requires a functional approach, such as transplantation, these cells (or at least undifferentiated spermato-

gonia) can be distinguished from differentiating spermatogonia by using morphological and biochemical criteria and mitotic activity. For example, mouse and rat undifferentiated spermatogonia (A_{single}, A_{paired}, $A_{aligned}$) have mottled chromatin, whereas differentiating spermatogonia have either no heterochromatin in type A1 or increasing levels of heterochromatin (from spermatogonia A2 to Intermediate) along the nuclear envelope (Chiarini-Garcia and Russell 2002; Chiarini-Garcia et al. 2003). Undifferentiated spermatogonial cells can also be characterized by the presence or absence of molecular markers, which in turn can be used to purify and to characterize the spermatogonial stem cells further. For example, mouse spermatogonial stem cells express GFRα-1, the receptor for GDNF (glial cell line-derived neurotrophic factor), and C-ret receptors, which allow their self-renewal by the GDNF secreted from Sertoli cells (Kubota et al. 2004; Hofmann et al. 2005). Another example is the POU-domain transcription factor Oct-3/4, which is expressed in primordial germ cells and spermatogonial stem cells (Pesce et al. 1998; Kubota et al. 2004). The cell membrane c-kit tyrosine kinase receptor for stem cell factor has also been found in primordial germ cell and differentiating types A spermatogonia but not in undifferentiated spermatogonia (Ohta et al. 2000; Sette et al. 2000). In *Drosophila*, visualization of the spectrosome is commonly used as a cytological marker of germline stem cells (Decotto and Spradling 2005; Kai et al. 2005). Spectrosome is composed of α- and β-spectrins, ankyrin, and the adducing-like Hts protein (De Cuevas et al. 1996). It is the precursor of the fusome, a large cytoplasmic structure found in dividing cysts passing through the intercellular bridges (Lin et al. 1994). These two organelles, found in male and female germ cells of *Drosophila*, have also been found in mouse and in *Xenopus* oocytes (Pepling and Spradling 1998; Kloc et al. 2004b).

In the lesser-spotted dogfish (*Scyliorhinus canicula*) and other elasmobranchs, the testis is polarized, and spermatogenesis occurs within spermatocysts (cysts) in which germ cells show synchronous development. The unit structure of the spermatocyst is the spermatoblast (480–500 per cyst), which is composed of one germinal clone associated with one Sertoli cell (Stanley 1966). The genesis of the spermatocysts occurs in the germinative zone, which runs the dorsal length of the testis, and spermatocysts progressively move, in maturational order, toward the opposite margin of the gonad where spermiation occurs. This zonation allows the observation of all stages of spermatogenesis on a transverse section of the testis (Sourdaine and Jégou 1989; Loir et al. 1995). In the germinative zone, located in a specific area between the capsule of the testis and the dorsal testicular vessel, large individual spermatogonia are surrounded by elongated somatic cells. Studies in other elasmobranchs such as the dogfish shark *Squalus acanthias* have investigated the proliferative and apoptotic activities of the pre-meiotic zone, which includes the germinative area (McClusky 2005, 2006; Dubois and Callard 1993; Callard et al. 1995). Recently, in the spotted ray *Torpedo marmorata*, Prisco et al. (2008) have shown that spermatogonia of the early stages present a positive reaction to anti-human type I 3β-hydroxysteroid dehydrogenase (HSD) and to anti-mouse 17β-HSD. Despite these studies, the characterization of potential spermatogonial stem cells within the germinative area calls for further investigation.

The aim of this study has been to establish whether dogfish spermatogonia, particularly those of the germinative zone, share some characteristics with spermatogonial stem cells described in other species. We have studied the ultrastructure and mitotic activity of dogfish spermatogonia by in vivo incorporation of the 5′-bromo-2′-deoxyuridine (BrdU) DNA intercalate and by the immunodetection of the proliferating cell nuclear antigen (PCNA). Immunodetection of c-kit, as a spermatogonial molecular marker, and of α- and β-spectrins, as constituents of the spectrosome and the fusome, has also been considered.

Materials and methods

Animals

Mature male lesser-spotted dogfish, *Scyliorhinus canicula* (500–700 g, 54–63 cm), were captured off Cherbourg (Manche, France) by using the facilities of the Lycée Maritime et Aquacole de Cherbourg and were stored in large natural seawater tanks at the Centre de Recherches en Environnement Côtier (Luc sur Mer, France). Fish were allowed to acclimate for at least 2 weeks before tissue sampling or experimentation. All the in vivo experiments were performed according to ethical guidelines. For the BrdU labeling experiments, animals (n=3) were injected intraperitoneally with BrdU (Sigma-Aldrich, Saint-Quentin Fallavier, France; 100 mg/kg) diluted in Gautron's buffer (GB), consisting of 280 mM NaCl, 3.2 mM $CaCl_2$, 3 mM KCl, 3.8 mM $MgCl_2.2H_2O$, 1 mM Na_2HPO_4, 4.6 mM D-glucose, 20 mM HEPES, 330 mM urea, 58 mM trimethylamine oxide, pH 7.8, 890 mOsm/kg), during the month of December. Following injection, animals were kept in running seawater tanks for 1–3 weeks. Dogfish were killed by sectioning the spinal cord and pithed. Testes were removed and stored in ice-cold GB and then cut transversely into 2-mm slices. According the experiment, testicular sections were dissected further into four zones (zone A: germinative area and spermatocysts with spermatogonia; zone B: spermatocysts with spermatocytes; zone C: spermatocysts with round and elongate spermatids;

zone D: mature spermatids) under a stereomicroscope as previously described (Loir and Sourdaine 1994).

Electron microscopy

Testicular fragments (1 mm^3) were fixed in a cacodylate solution (0.3 M cacodylate, 2% glutaraldehyde, 2% saccharose, 0.01% $CaCl_2$, $2H_2O$, 876 mOsm, pH 7.8) for 3 h at room temperature and then washed overnight in cacodylate buffer (0.3 M cacodylate, 8% saccharose, 0.01% $CaCl_2$, $2H_2O$, 850 mOsm, pH 7.8). After post-fixation in 2% osmium tetroxide in cacodylate buffer for 2 h at 4°C, samples were dehydrated and embedded in Epon. Ultra-thin sections were cut with a diamond knife, stained in aqueous solution of uranyl acetate and 2% lead citrate, and examined in a JEOL 1011 transmission electron microscope.

Western blotting

For protein extraction, testicular zones were crushed in liquid nitrogen and homogenized in ice-cold extraction-buffer (50 mM TRIS pH 7.4, 20 μM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor mix) and sonicated (80 W). The lysate was centrifuged for 30 min at 15,000*g* and desalted in Microcons Y10.

Proteins (50 μg) were resolved by 8% or 10% SDS-polyacrylamide gel electrophoresis and then blotted on a polyvinylidene dilfuoride membrane (GE Healthcare, Orsay, France). After a 1-h blocking step in 5% non-fat dry milk in TRIS buffer (pH 7.4), the membrane was probed overnight at 4°C with the human α− and β-spectrin mouse monoclonal antibody (SBSP2; Abcam, Cambridge, UK), diluted 1:100, or with the human c-kit rabbit polyclonal antibody (C-19; Tebu-bio, Le Perray en Yvelines, France), diluted 1:200, or with the recombinant rat PCNA mouse monoclonal antibody (PC10; Invitrogen, Eragny sur Oise, France), diluted 1:500. Reactions were detected after a 2-h incubation with the corresponding peroxidase-conjugated secondary antibody: the rabbit anti-mouse immunoglobulin (Dako france, Trappes, France), diluted 1:1000; the polyclonal swine anti-rabbit immunoglobulin (P 0399, Dako france, Trappes, France), diluted 1:400. The peroxidase reaction was developed with DAB (3,3′-diaminobenzidine; Sigma-Aldrich, Saint-Quentin Fallavier, France).

Immunohistochemistry

Testicular sections of dogfish were fixed in Davidson solution (10% glycerol, 20% formol, 30% ethanol, 30% filtered seawater, 10% acetic acid) at 4°C for 24 h, embedded in paraffin wax, and then sliced at a thickness between 4 and 10 μm.

PCNA immunohistochemistry was performed with an avidin-biotin-peroxidase PCNA staining kit containing the PC10 antibody (Invitrogen, Eragny sur Oise, France) on 4-μm tissue sections from testes taken in January and February. Sections were treated with 0.3% H_2O_2/1× PBS (pH 7.4) for 30 min to inactivate endogenous peroxidases, washed in 1× PBS (pH 7.4), and incubated with 1% bovine serum albumin (BSA)/1× PBS for 10 min. The sections were then incubated with the antibody for 2 h at room temperature, washed several times in 1× PBS, and subseqently incubated with streptavidin-peroxidase complex for 10 min, followed by DAB for 10 min. Negative controls were generated by omitting the primary antibody and by serial dilution of this antibody.

For c-kit immunohistochemistry, 5-μm tissue sections were heated twice in 10 mM sodium citrate buffer (pH 6) for 90 s each time in a microwave oven (600 W), cooled, rinsed in 1× PBS, and incubated in 0.3% H_2O_2 in 1× PBS for 30 min, followed by 0.1% Triton X-100/1× PBS (PBT) containing 1% BSA for 30 min. The sections were subsequently incubated overnight at 4°C with the human c-kit rabbit polyclonal antibody (C-19, Tebu-bio, Le Perray en Yvelines, France) diluted 1:50 in 1% BSA/PBT, washed several times in PBT, and incubated for 2 h at room temperature with the horseradish peroxidase (HRP)-conjugated polyclonal donkey anti-rabbit IgG H&L (ab7083, Abcam, Cambridge, UK) diluted 1:250 in 1% BSA/PBT, followed by DAB. Positive controls were performed on testicular sections of mouse and rat, and negative controls were performed by saturating the primary antibody with c-kit (C-19) peptide (Tebu-bio, Le Perray en Yvelines, France; 10× more concentrated than the antibody).

BrdU detection was carried out with the BrdU labeling and detection kit II (Roche Diagnostics, Meylan, France) following the manufacturer's instructions with minor modifications. Sections were incubated with 0.3% H_2O_2/1× PBS for 30 min, washed in PBS before a blocking step in 1% BSA/1× PBS for 30 min, incubated with the anti-BrdU mouse monoclonal antibody diluted 1:100 for 1 h at 37°C, washed several times with 1× PBS, and incubated with HRP-conjugated rabbit anti-mouse immunoglobulins (Dako france, Trappes, France) diluted 1:200, followed by DAB.

In all cases, sections were counterstained with 0.5% hematoxylin and examined on a BX50 Olympus microscope with a Spot RTKE camera.

Immunofluorescence detection of spectrins

For analysis of spectrosome and fusome, 10-μm testicular histological sections were incubated for 5 min in PBT and

then in 1% BSA/PBT for 30 min to reduce background staining and non-specific binding, respectively. Incubation with the monoclonal mouse anti-human α- and β-spectrins antibody (SB-SP2; Tebu-bio, Le Perray en Yvelines, France) diluted 1:50 was carried out in 1% BSA/PBT overnight at 4°C, followed by several washes in PBT. Sections were incubated for 2 h at room temperature with fluorescein-isothiocyanate-conjugated polyclonal anti-mouse IgM (Tebu-bio, Le Perray en Yvelines, France) diluted 1:100 in 1% BSA/PBT. Subsequently, tissue sections were stained with 4,6-diamidino-2-phenylindole diluted 1:6,000, washed in PBT, mounted in mounting medium (70% glycerol, 30% PBS, 2% N-propyl-gallate), and examined under a confocal microscope (FV 1000).

Results

Stages of spermatogonial spermatocysts

Based on Loir et al. (1995), the classification of the spermatogonial stages used in this work is the following. Stage I, corresponds to large isolated spermatogonia (10.23±0.28 μm in nuclear diameter) of the germinative zone, surrounded by elongated somatic cells and isolated from the more advanced stage by a dense matrix. Stage II, outside the germinative area, can be subdivided into stage IIa presenting clusters of spermatogonia (7.71±0.21 μm in nuclear diameter) and somatic cells (Sertoli cells), stage IIb in which both types of cells regroup progressively to form spherical spermatocysts without a lumen, and stage IIc in which spermatogonia and Sertoli cells are alternately disposed as a single layer around a lumen. Stage III corresponds to spermatocysts with a single layer of Sertoli cell nuclei around the central lumen and a single layer (stage IIIa) or two layers (stage IIIb) of spermatogonia toward the basal lamina. Stage IV has four layers of spermatogonia and Sertoli cell nuclei remaining at the adluminal position. Stage V presents five layers of spermatogonia and Sertoli cell nuclei, which are at an adluminal (majority) or at an intermediate position. Stage VI exhibits about six layers of spermatogonia (corresponding to pre-leptotene spermatocytes) and Sertoli cell nuclei are in an intermediate (majority) or basal position.

Ultrastructure

Spermatogonia in stage I (Fig. 1a) occur singly or in groups of two and are surrounded by somatic cells. The nucleus of a spermatogonium has mottled chromatin with a nucleolus. The cytoplasm contains numerous mitochondria, which are dispersed throughout the cytoplasm. Associated with the mitochondria, dense material, as a germinal "nuage", can be observed. No intercellular bridge has been observed between spermatogonia at this stage. In stage II, spermatogonia have nuclei of a similar appearance to those in stage

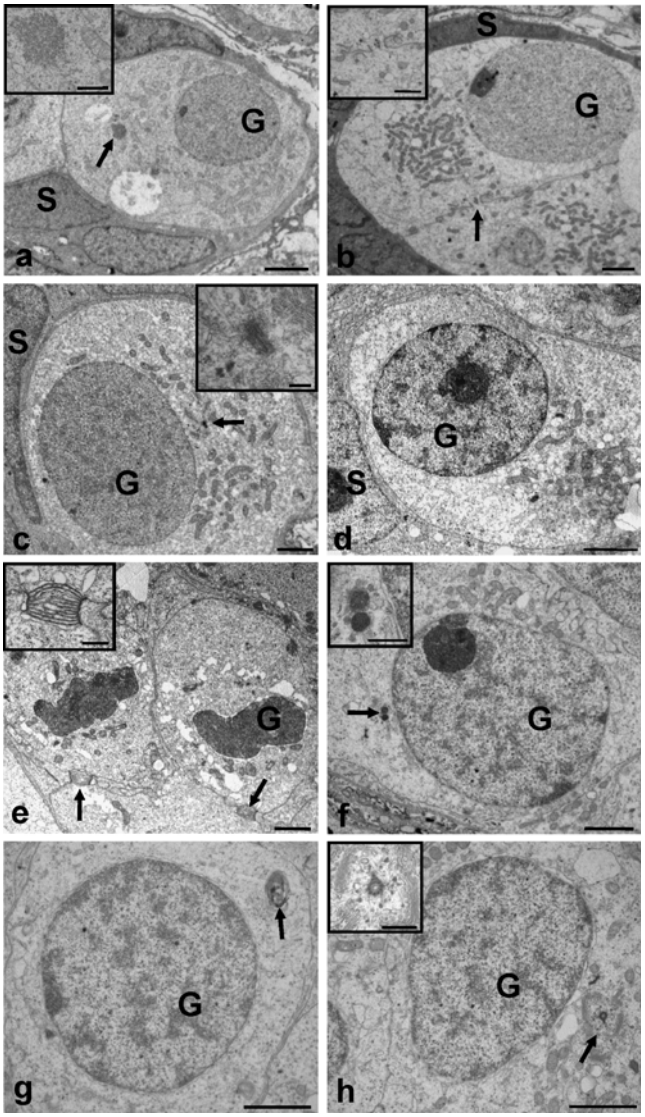

Fig. 1 Electron micrographs of spermatogonia (*G*) of the lesser-spotted dogfish (*S* Sertoli cells). In stage I, spermatogonia (**a**) are surrounded by somatic cells; their nuclei have mottled chromatin with a nucleolus, and dense material associated with the mitochondria (germinal nuage) can be observed (*arrow*, *inset*). In stages IIa (**b**) and IIc (**c**), the nuclei of spermatogonia have a similar appearance to that in stage I. Intercellular bridges are observed between paired spermatogonia (**b**, *arrow*, *inset*), and a structure with a tubular appearance (**c**, *arrow*, *inset*) is also visible. In stage III, flecks of heterochromatin increase in the nuclei (**d**, stage IIIb) and spermatogonia in division present intercellular bridges closed by multiple transverse cisternae (**e**, *arrow*, *inset*). In stage IV, nuclei of spermatogonia have increasing amounts of heterochromatin lining the nuclear envelope (**f**–**h**) and grouped mitochondria and various organelles lie within their cytoplasm, such as a chromatoid body-like structure (**f**, *arrow*, *inset*), autophagic vacuoles (**g**, *arrow*), or centriole associated with the Golgi complexes (**h**, *arrow*, *inset*). *Bars* 2.5 μm (**a**–**f**), 2 μm (**g**, **h**), 500 nm (*insets* **a**, **b**, **e**, **f**, **h**), 200 nm (*inset* **c**)

I, and mitochondria seem grouped to one pole of the cell. However, intercellular bridges are observed between paired spermatogonia (Fig. 1b, stage IIa), and dense material of a tubular appearance lies between the Golgi complexes (Fig. 1c, stage IIc). In stage III, flecks of heterochromatin increase in the nuclei (Fig. 1d, stage IIIb), and spermatogonia undergoing mitosis present intercellular bridges with multiple parallel transverse cisternae (Fig. 1e). In stage IV, the nuclei of spermatogonia show increasing amounts of heterochromatin lining the nuclear envelope (Fig. 1f–h). Grouped mitochondria and various organelles are also observed within the cytoplasm, such as the chromatoid body-like structure (Fig. 1f), autophagic vacuoles (Fig. 1g), or the centriole associated with the Golgi complexes (Fig. 1h).

Immunoblotting

The PCNA monoclonal antibody detected only one band of about 31 kDa (Fig. 2a). The c-kit polyclonal antibody detected several bands, among which two (apparent molecular weight of 56 and 52 kDa) were more strongly detected (Fig. 2b). The bands of about 190, 114, 83, 52, and 41 kDa seemed to be specific because they were not revealed by immunoblotting when the antibody was preincubated with the peptide antigen, whereas the band of about 56 kDa persisted (data not shown). The immunoblotting with spectrins antibody did not allow us to visualize protein bands (data not shown).

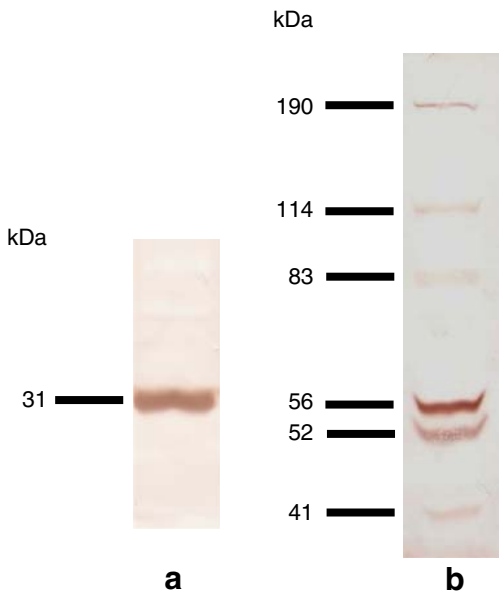

Fig. 2 Western blot analysis of 50 μg testicular proteins resolved by 10% (**a**) or 8% (**b**) SDS-polyacrylamide gel electrophoresis. **a** Recombinant rat PCNA mouse monoclonal antibody (PC10), diluted 1:500, reacts with a single band of about 31 kDa. **b** Human c-kit rabbit polyclonal antibody (C-19), diluted 1:200, reacts with several bands of about 41, 52, 56, 83, 114, and 190 kDa. Only the band of about 56 kDa persists when the c-kit antibody is preincubated with the peptide antigen (not shown)

Mitotic activity

PCNA and BrdU immunodetections were used to determine spermatogonial mitotic activity. In transverse sections of the testis, PCNA immunodetection was restricted to the zone containing cysts with spermatogonia and to the epigonal tissue. According to the spermatogonia of the germinative zone, PCNA either was not detected or was detected in the cytoplasm or nuclei (Fig. 3a). In stages IIa to IIb–c, PCNA was immunolocalized in nuclei of the Sertoli cells and spermatogonia (Fig. 3b,c). About 25% and 80% of spermatogonia in stages I and IIa, respectively, were nuclear-labeled. In stage III (Fig. 3d), the nuclei of Sertoli cells have no further labeling, whereas PCNA remained immunodetected in the nuclei of spermatogonia until stages V-VI (Fig. 3e, f). No PCNA immunostaining was observed in cysts containing primary spermatocytes or in germ cells at more advanced stages (data not shown). The nuclei of cells of the epigonal tissue, the haematopoietic tissue used as a positive control, were PCNA-immunopositive (data not shown).

The proliferation of spermatogonia was also followed for 3 weeks after a single BrdU injection. After 1 week, BrdU was immunodetected in the cytoplasm of spermatogonia in stage I (Fig. 4a) and in stage II (Fig. 4c) but not in stage III (Fig. 4e). After 3 weeks, the cytoplasm of spermatogonia in stage I (Fig. 4b) was still labeled, whereas the nuclei of Sertoli cells (also noted after 2 weeks) and of spermatogonia in stages II (Fig. 4d) and the nuclei of spermatogonia in stages III (Fig. 4f) were labeled. BrdU was also immunodetected in nuclei of spermatogonia until stage VI (data not shown). In the epigonal tissue, BrdU was immunodetected in nuclei of cells from the first week (Fig. 4g) and after 3 weeks (Fig. 4h).

Localization of c-kit

In the germinative zone, spermatogonia showed a positive reaction for anti c-kit, which appeared localized in the cytoplasm and inside or in the vicinity of the cell membrane (Fig. 5a). The same labeling pattern was observed in spermatogonia at stage II (Fig. 5b). When the antibody used for the c-kit detection was blocked by preincubation with the peptide antigen, no labeling was observed (Fig. 5c).

Localization of α- and β-spectrins

Immunofluorescence detection of α- and β-spectrins in testis showed a general juxtamembranous staining of Sertoli

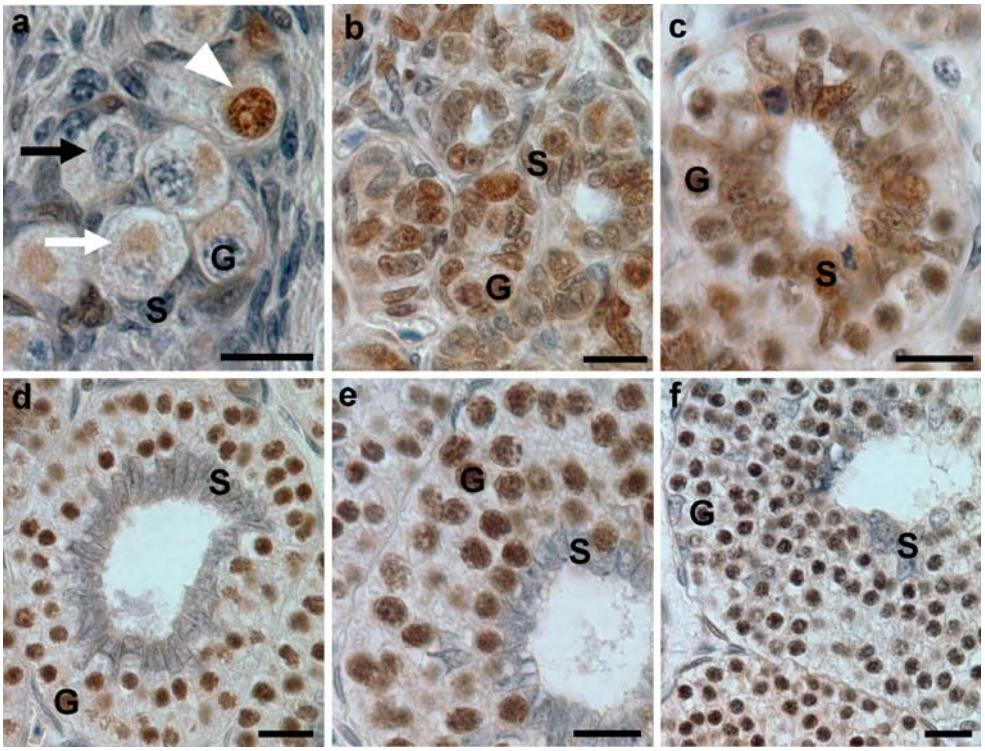

Fig. 3 Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the lesser-spotted dogfish testis (*S* Sertoli cells, *G* spermatogonia, *C* spermatocytes). The spermatogonia of the germinative zone (**a**) present nuclear (*white arrowhead*), cytoplasmic (*white arrow*), or no immunolabeling (*black arrow*). In stages IIa and IIb-c (**b**, **c**), Sertoli cells and spermatogonia are immunolabeled. From stage III to stages V-VI (**d–f**), only the nucleus of the spermatogonia is immunolabeled. *Bars* 40 μm

cell and spermatogonia in the various stages. More precisely, in some spermatogonia of the germinative zone, an intense fluorescent circular area (possibly the spectrosome) was noted (Fig. 6a). In stage II, the intense fluorescence in the spermatogonia presents an elongated form suggesting fusome-like structures (Fig. 6b). Additionally, an electron-microscopic observation of an intercellular bridge between spermatogonia of stage IIc revealed the

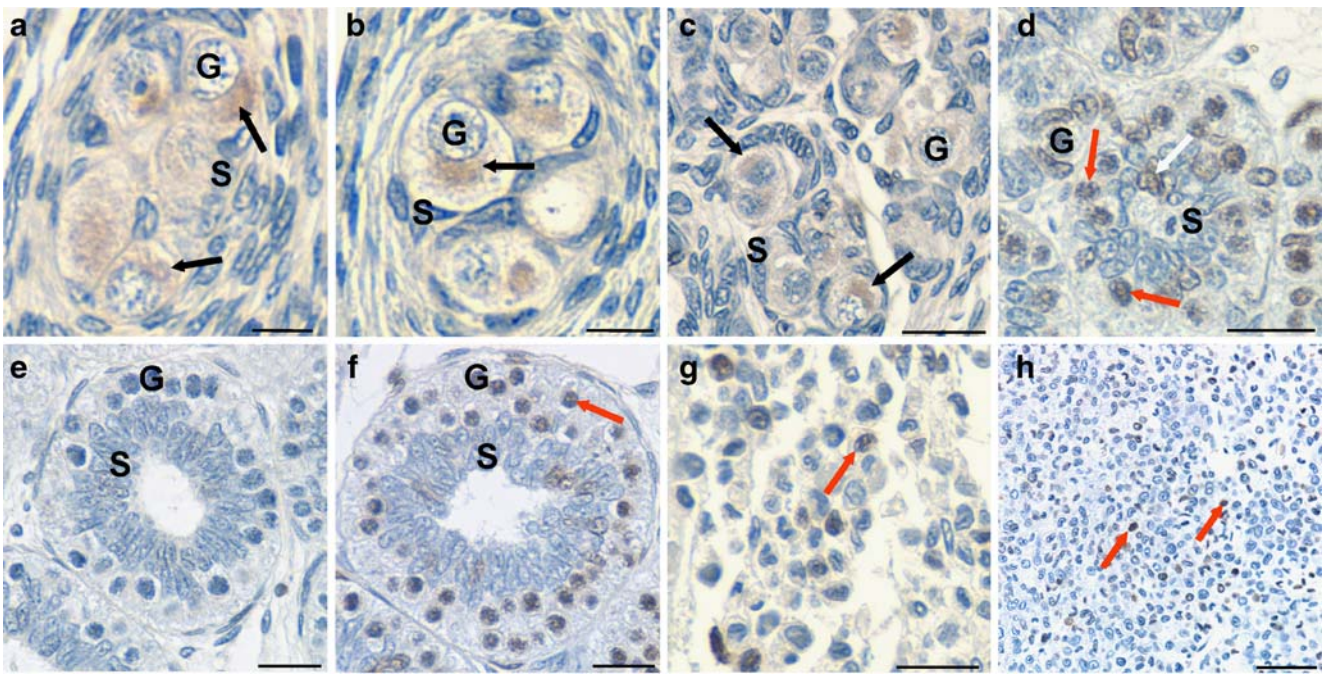

Fig. 4 BrdU immunodetection in the lesser-spotted dogfish testis after one in vivo injection (*S* Sertoli cells, *G* spermatogonia). At 1 week after injection (**a**, **c**, **e**, **g**), BrdU was immunodetected in the cytoplasm of spermatogonia (*black arrows*) in stage I (**a**) and in stage IIa (**c**) but not in stage IIIa (**e**) and in the nuclei of epigonal tissue cells (**g** *red arrow*). After 3 weeks (**b**, **d**, **f**, **h**), BrdU had accumulated in the cytoplasm of spermatogonia (*black arrows*) of the germinative zone (**b**), in the nuclei of spermatogonia (*red arrows*) and of Sertoli cells (*white arrow*) of cysts at stage IIc (**d**), in the nuclei of spermatogonia (*red arrow*) of cysts at stage IIIb (**f**), and in nuclei of cells of the epigonal tissue (**h** *red arrows*). *Bars* 10 μm (**a**, **b**), 25 μm (**c–g**), 50 μm (**h**)

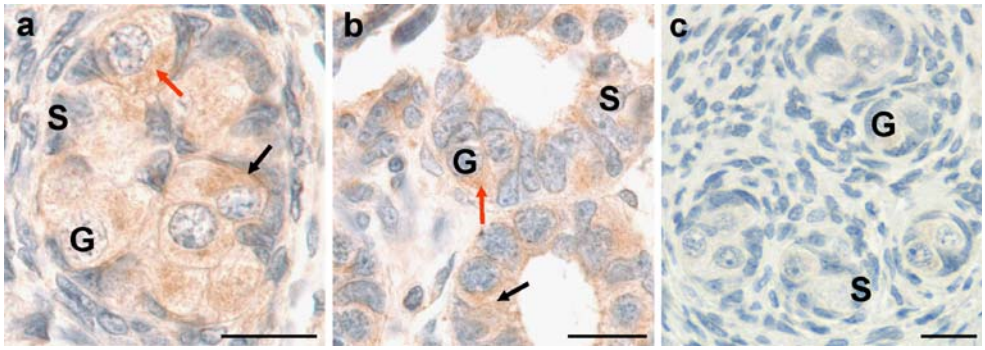

Fig. 5 Immunohistochemical detection of c-kit in the lesser-spotted dogfish testis (*S* Sertoli cells, *G* spermatogonia). In the germinative zone (**a**), spermatogonia present juxtamembranous (*black arrow*) and cytoplasmic (*red arrow*) immunolabeling. Cell membrane (*black arrow*) and cytoplasmic (*red arrow*) staining is also observed in spermatogonia of cysts at stage IIc (**b**). Negative control was performed by using the blocking peptide (**c**). *Bars* 10 μm (**a**), 25 μm (**b**, **c**)

presence of cytoplasmic filaments, which could have partially constituted the fusome (Fig. 6c).

Discussion

In *Scyliorhinus canicula*, spermatogonia of the germinative zone (stage I) are isolated and apparently not connected by intercellular bridges and have a nucleus with chromatin of a mottled appearance and a poorly differentiated cytoplasm with few organelles, except for numerous mitochondria. These ultrastructural features have also been described for rodent undifferentiated spermatogonia comprising the successive spermatogonial types of A_{single}, A_{paired}, and $A_{aligned}$ (Chiarini-Garcia et al. 2001, 2003; Chiarini-Garcia and Russell 2002), which constitute the potential stem cell compartment (De Rooij and Grootegoed 1998; Nakagawa et al. 2007). A germinal nuage associated with mitochondria has been observed in the cytoplasm of stage I spermatogonia. This structure, which might correspond to various identities and/or names (Kloc et al. 2004a), is also found in the cytoplasm of type A spermatogonia of *Synbranchus marmoratus* (Lo Nostro et al. 2003) and of the cardinal fish (Fishelson et al. 2006) and in primary spermatogonia of the catfish (Batlouni et al. 2006) and in rodent primary spermatocytes (for a review, see Parvinen 2005). Cytoplasmic bridges have been observed between spermatogonia from the early stage IIa before the start of the lumen formation of the cyst (stage IIc) and before that of spermatoblast formation (stage IIIa), whereas the nuclear aspect of these spermatogonia remains mottled. Such intercellular bridges have been observed between spermatogonia of *Squalus acanthias* but at a later stage (Pudney1995). Our observation suggests that spermatogonia are interconnected after their first division, as in the case of rodent A_{paired} spermatogonia. This suggests that, in the dogfish, germ cells form a syncytium with their sister cells of not only the same spermatoblast but of the entire spermatocyst population. This is also consistent with the number of 13 spermatogonial divisions proposed by Holstein (1969). In the following spermatogonial stages (III-IV), the diversity of organelles increases in the cytoplasm and flecks of heterochromatin increase in nuclei with larger amounts lining the nuclear envelope, as observed in other species such as rodents (Chiarini-Garcia and Russell 2002). However, contrary to the description for the spotted ray *Torpedo marmorata* (Prisco et

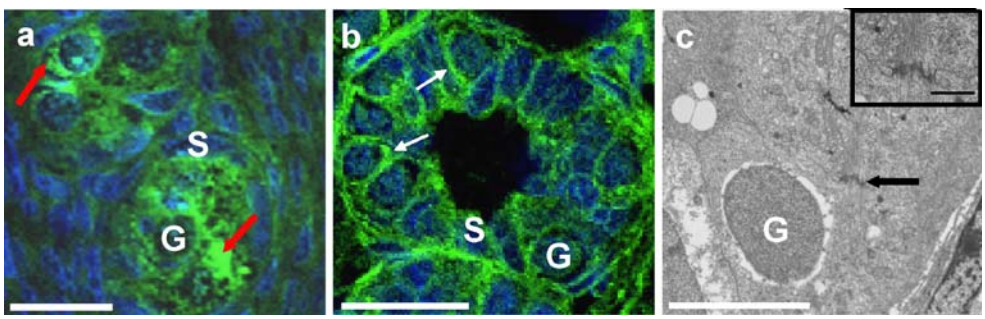

Fig. 6 Immunofluorescence detection of α- and β-spectrins in the lesser-spotted dogfish testis (*S* Sertoli cells, *G* spermatogonia). In the germinative zone (**a**), some spermatogonia have a dense circular area of intense labeling (*red arrows*). In stage II (**b**), spermatogonia present elongated labeling (*white arrow*). By electron microscopy (**c**), a fusome-like structure made of cytoplasmic filaments passing through an intercellular bridge between two spermatogonia in stage II is visible (*black arrow*, *inset*). *Bars* 50 μm (**a**, **b**), 5 μm (**c**), 1 μm (enlargement)

al. 2008), the spermatogonia of *Scyliorhinus canicula* do not seem to show the typical ultrastructural aspect of steroid-producing cells. Moreover, during mitosis of spermatogonia, we have observed parallel structures within intercellular bridges. These structures have also been reported by Dym and Fawcett (1971) and they probably limit intercellular traffic of organelles.

Because stem cells are defined by their low mitotic activity (Ehmcke et al. 2006), we have looked at the localization of PCNA, as a marker of proliferation, in spermatogonia of the dogfish. PCNA is synthesized during the G1 phase of the cell cycle and is an auxiliary protein of DNA polymerase during DNA replication (Chapman and Wolgemuth 1994). In mammals, the molecular weight of PCNA is 36 kDa (Chapman and Wolgemuth 1994), but in the Japanese eel, a second form of 32 kDa has also been identified (Miura et al. 2002), which corresponds to the apparent molecular weight observed in our study for PCNA of *Scyliorhinus canicula*. In comparison with spermatogonia at stage II, those at stage I have a lower nuclear immunolocalization of PCNA reflecting a lower spermatogonial mitotic activity in the germinative zone. In stages II, in which cystic organization takes place, both spermatogonial and Sertoli cell nuclei present PCNA immunolabeling, and from the stage IIIa coinciding with spermatoblast formation, only spermatogonia remain labeled until stage VI. These results partially differ with those observed for *Squalus acanthias* (McClusky 2005) in which neither of the two cell types is simultaneously PCNA labeled in the germinal zone, which corresponds to stage II in *S. canicula* (the germinal ridge of *S. acanthias* corresponding to stage I). Furthermore, based on PCNA analysis, a seasonal change in the proliferation of spermatogonia of the germinal ridge has been observed in *S. acanthias* with an active period from April to August (McClusky 2005). On the other hand, *S. canicula* presents uninterrupted spermatogenesis throughout the year, even if a peak in the number of spermatocysts containing spermatocytes and spermatids (April to October) or in sperm reserves (March to May) has been reported (Dobson 1974; Garnier et al. 1999). Consequently, we cannot exclude that, for *S. canicula*, the mitotic activity of spermatogonia in stage I slightly varies during the season. To complete the observation obtained with PCNA labeling, the mitotic activity of spermatogonia has also been followed by in vivo BrdU incorporation. At 3 weeks after injection, no nuclear BrdU labeling is observed in stage I spermatogonia, suggesting low mitotic activity, whereas spermatogonia from stage II to stage VI present nuclear staining that reflects their proliferation phase. Both PCNA and BrdU experiments have shown a low proliferation activity of stage I spermatogonia, which is a characteristic of stem cells (Ehmcke et al. 2006).

Spermatogonial stem cells can be identified by their phenotypic characteristics, which have largely been studied in rodents. For example, spermatogonial stem cells are GRFα-1$^+$/oct-3/4$^+$/Thy-1$^+$/c-Ret$^+$/c-kit$^-$ (Kubota et al. 2003; Pesce et al. 1998). We have focused on the expression of c-kit in the testis of *Scyliorhinus canicula*. C-kit, a transmembrane tyrosine kinase receptor, plays an important role in the development of primordial germ cells and in the maintenance of differentiated spermatogonia (Ohta et al. 2000; Loveland and Schlatt 1997). Results of Western blot analysis indicate that the polyclonal antibody recognizes five specific protein bands. In the rodent testis, several forms of c-kit receptor have been recorded with an apparent molecular weight of 124 and 160 kDa in rat (Dym et al. 1995) and of 33, 48, and 150 kDa in mice (Feng et al. 1997). The proteins of about 114 kDa and 190 kDa might respectively be an immature and a mature form of c-Kit, as has been observed in rat testis (124 kDa and 160 kDa, respectively; Dym et al. 1995) in which the mature form is fully glycolysated (Nocka et al. 1989). The protein bands of about 83, 41, and 52 kDa might correspond to truncated forms of c-kit and/or be the result of proteolytic cleavages during sample preparation. In rodents, c-kit protein expression is higher in differentiated type A spermatogonia and in pachytene spermatocytes than in undifferentiated type A spermatogonia. This expression pattern is similar whatever the batch of antibody used (Vincent et al. 1998; Schrans-Stassen et al. 1999; Prabhu et al. 2006). In our study, we have observed c-kit immunolabeling in the cytoplasm and possibly in the cell membrane of stage I spermatogonia. Recently, Raucci and Di Fiore (2007) have shown that c-kit receptor protein is expressed in both types of spermatogonia distinguishable in *Rana esculenta* but with intensities varying according to the reproductive period. Although this study has shown, for the first time, that spermatogonia of elasmobranchs can express the c-kit receptor, its use as a marker of differentiating spermatogonia in the dogfish requires additional study. Recent studies of the control of spermatogonial differentiation in mice by growth factors highlight the pathways involving the spermatogonial markers. Thus, He et al. (2007) have shown that *Gfr–α1* inactivation induces the differentiation of spermatogonia as evidenced by elevated expression of c-kit and decreased expression of Oct-3/4. The role of Plzf in the repression of c-kit expression has also been shown (Filipponi et al. 2007). During our study, we have been unable to detect Oct-3/4 by Western blotting and immunohistochemistry with a human Oct-3/4 rabbit polyclonal antibody. Further characterization of dogfish cellular markers is needed to explore the control of spermatogonial cell fate by growth factors.

Spectrins are the major components of two cytoplasmic structures, the spectrosome and the fusome (de Cuevas et al. 1996). These structures are found in germ cells in

vertebrate and non-vertebrate species (Pepling and Spradling 1998; Lin et al. 1994; Kloc et al. 2004b) and particularly in *Drosophila* (Lin et al. 1994; de Cuevas et al. 1996; Hime et al. 1996). The spectrosome, a fusome precursor, is localized in germinal stem cells (Deng and Lin 1997; Pepling and Spradling 1998), whereas the fusome, found in dividing germ cells shows a branched appearance because it passes through intercellular bridges (Lin et al. 1994). The immunofluorescence detection of spectrins, performed on testis of *Scyliorhinus canicula*, has revealed the presence of a spherical structure in the spermatogonia of the germinative zone; this might correspond to the spectrosome. On the other hand, stage II spermatogonia present an elongated form that might be the fusome. This hypothesis is reinforced by electron-microscopic observations of filaments passing through intercellular bridges between stage II spermatogonia. This study has shown that the spectrosome and fusome could be distinctive morphological markers of undifferentiated and differentiating spermatogonia in the dogfish, as used in *Drosophila* (Kai et al. 2005), and as also observed in *Xenopus* (Kloc et al. 2004b).

In conclusion, this study has shown that spermatogonia of the germinative zone possess some characteristics of spermatogonial stem cells, such as low mitotic activity shown by the lack of nuclear PCNA and BrdU incorporation, a spectrosome, a nucleus with chromatin of mottled appearance, and a poorly differentiated cytoplasm. Because this zone is easily distinguishable in dogfish testis, *Scyliorhinus canicula* might represent an interesting model for a comparative evolutionary study of the spermatogonial stem niche.

Acknowledgements The authors are grateful to Mr. Sechet and Mr. Rousseville of the Lycée Maritime et Aquacole de Cherbourg for their cooperation in collecting dogfish and to the staff, particularly Dr. Gimazane, Mr. Bucaille and Mr. Guyon, of the Centre de Recherches en Environnement Côtier (Luc sur Mer) for the care given to animals. Thanks are also extended to Miss Adeline for preparing the histological material, to Mr. Goux of CMAbio for his help with the electronic microscopy, and to Mr. Joubel for the final proofreading of the manuscript.

References

Batlouni SR, Romagosa E, Borella MI (2006) The reproductive cycle of male catfish *Pseudoplatystoma fasciatum* (Teleostei, Pimelodidae) revealed by changes of the germinal epithelium. An approach addressed to aquaculture. Anim Reprod Sci 96:116–132

Callard GV, Jorgensen JC, Redding JM (1995) Biochemical analysis of programmed cell death during premeiotic stages of spermatogenesis in vivo and in vitro. Dev Genet 16:140–147

Chapman DL, Wolgemuth DJ (1994) Expression of proliferating cell nuclear antigen in the mouse germ line and surrounding somatic cells suggests both proliferation-dependent and -independent modes of function. Int J Dev Biol 38:491–497

Chiarini-Garcia H, Russell LD (2002) Characterization of mouse spermatogonia by transmission electron microscopy. Reproduction 123:567–577

Chiarini-Garcia H, Hornick JR, Griswold MD, Russell LD (2001) Distribution of type a spermatogonia in the mouse is not random. Biol Reprod 65:1179–1185

Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in seminiferous tubules. Reproduction 126:669–680

Decotto E, Spradling AC (2005) The *Drosophila* ovarian and testis stem cell niches: similar somatic stem cell and signals. Dev Cell 9:501–510

De Cuevas M, Lee JK, Spradling AC (1996) α-Spectrin is required for germline cell division and differentiation in the *Drosophila* ovary. Development 122:3959–3968

Deng W, Lin H (1997) Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in *Drosophila*. Dev Biol 189:79–94

De Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121:347–354

De Rooij DG, Grootegoed JA (1998) Spermatogonial stem cells. Curr Opin Cell Biol 10:694–701

Dobson S (1974) Endocrine control of reproduction in the male *Scyliorhinus canicula*. PhD thesis, University of Wales

Dubois W, Callard GV (1993) Culture of intact Sertoli/germ cell units and isolated Sertoli cells from *Squalus* testis. II. Stimulatory effects of insulin and IGF-I on DNA synthesis in premeiotic stages. J Exp Zool 267:233–244

Dym M, Fawcett DW (1971) Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 4:195–215

Dym M, Jia MC, Dirami G, Price JM, Rabin SJ, Mocchetti I, Ravindranath N (1995) Expression of c-kit receptor and its autophosphorylation in immature rat type A spermatogonia. Biol Reprod 52:8–19

Ehmcke J, Wistuba J, Schlatt S (2006) Spermatogonial stem cells: questions, models and perspectives. Hum Reprod Update 12:275–282

Feng H, Sandlow JI, Sandra A (1997) Expression and function of the c-kit proto-oncogene protein in mouse sperm. Biol Reprod 57:194–203

Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S (2007) Repression of kit expression by Plzf in germ cells. Mol Cell Biol 27:6770–6781

Fishelson L, Delarea Y, Gon O (2006) Testis structure, spermatogenesis, spermatocytogenesis, and sperm structure in cardinal fish (Apogonidae, Perciformes). Anat Embryol (Berl) 211:31–46

Garnier DH, Sourdaine P, Jégou B (1999) Seasonal variations in sex steroids and male sexual characteristics in *Scyliorhinus canicula*. Gen Comp Endocrinol 116:281–290

He Z, Jiang J, Hofmann MC, Dym M (2007) Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod 77:723–733

Hess RA, Cooke PS, Hofmann MC, Murphy KM (2006) Mechanistic insights into the regulation of the spermatogonial stem cell niche. Cell Cycle 5:1164–1170

Hime GR, Brill JA, Fuller MT (1996) Assembly of ring canals in the male germ line from structural components of the contractile ring. J Cell Sci 109:2779–2788

Hofmann MC, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279:114–124

Holstein AF (1969) Zur Frage der lokalen Steuerung der Spermatogenese beim Dornhai (*Squalus acanthias* L.). Z Zellforsch 93:265–281

Kai T, Williams D, Spradling AC (2005) The expression profile of purified *Drosophila* germline stem cells. Dev Biol 283:486–502

Kiger A, White-Cooper H, Fuller MT (2000) Somatic support cells restrict germline stem cell self-renewal and promote differentiation. Nature 407:750–754

Kloc M, Bilinski S, Etkin LD (2004a) The Balbiani body and germ cell determinants: 150 years later. Curr Top Dev Biol 59:1–36

Kloc M, Bilinski S, Dougherty MT, Brey EM, Etkin LD (2004b) Formation, architecture and polarity of female germline cyst in *Xenopus*. Dev Biol 266:43–61

Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci 100:6487–6492

Kubota H, Avarbock MR, Brinster RL (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 101:16489–16494

Lin H, Yue L, Spradling AC (1994) The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. Development 120:947–956

Lo Nostro FL, Grier H, Meijide FJ, Guerrero GA (2003) Ultrastructure of testis in *Synbranchus marmoratus* (Teleostei, Synbranchidae): the germinal compartment. Tissue Cell 35:121–132

Loir M, Sourdaine P (1994) Testes cells: isolation and culture. In: Hochachka PW, Mommsen TP (eds) Biochemistry and molecular biology of fishes: analytical techniques. Elsevier, New York, pp. 219–272

Loir M, Sourdaine P, Mendis-Handagama SM, Jégou B (1995) Cell-cell interactions in the testis of teleosts and elasmobranchs. Microsc Res Tech 32:533–552

Loveland KL, Schlatt S (1997) Stem cell factor and c-kit in the mammalian testis: lessons originating from mother nature's gene knockouts. J Endocrinol 153:337–344

McClusky LM (2005) Stage and season effects on cell cycle and apoptotic activities of germ cells and Sertoli cells during spermatogenesis in the spiny dogfish (*Squalus acanthias*). Reproduction 129:89–102

McClusky LM (2006) Stage-dependency of apoptosis and the blood-testis barrier in the dogfish shark (*Squalus acanthias*): cadmium-induced changes as assessed by vital fluorescence techniques. Cell Tissue Res 325:541–553

Miura C, Miura T, Yamashita M (2002) PCNA protein expression during spermatogenesis of the Japanese eel (*Anguilla japonica*). Zool Sci 19:87–91

Nakagawa T, Nabeshima Y, Yoshida S (2007) Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12:195–206

Nocka K, Majumder S, Chabot B, Ray P, Cervone M, Bernstein A, Besmer P (1989) Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice—evidence for impaired c-kit kinase in mutant mice. Genes Dev 3:816–826

Ohta H, Yomogida K, Dohmae K, Nishimune Y (2000) Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 127:2125–2131

Parvinen M (2005) The chromatoid body in spermatogenesis. Int J Androl 28:189–201

Pepling ME, Spradling AC (1998) Female mouse germ cells form synchronously dividing cysts. Development 125:3323–3328

Pesce M, Gross MK, Schöler HR (1998) In line with our ancestors: Oct-4 and the mammalian germ. BioEssay 20:722–732

Prabhu SM, Meistrich ML, McLaughlin EA, Roman SD, Warne S, Mendis S, Itman C, Loveland KL (2006) Expression of c-Kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. Reproduction 131:489–499

Prisco M, Liguoro A, Richiarri L, Del Giudice G, Angelini F, Andreuccetti P (2008) Immunolocalization of 3beta-HSD and 17beta-HSD in the testis of the spotted ray *Torpedo marmorata*. Gen Comp Endocrinol 155:157–163

Pudney J (1995) Spermatogenesis in nonmammalian vertebrates. Micros Res Tech 32:459–497

Raucci F, Di Fiore M (2007) The c-kit receptor protein in the testis of green frog *Rana esculenta*: seasonal changes in relationship to testosterone titre and spermatogonial proliferation. Reproduction 133:51–60

Schrans-Stassen B, Van De Kant HJ, De Rooij DG, van Pelt AM (1999) Differential expression of c-*kit* in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140:5894–5900

Sette C, Dolci S, Geremia R, Rossi P (2000) The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. Int J Dev Biol 44:599–608

Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98–104

Sourdaine P, Jégou B (1989) Dissociation and identification of intact seminiferous lobules from the testis of dogfish (*Scyliorhinus canicula*). Cell Tissue Res 255:199–207

Stanley HP (1966) The structure and development of seminiferous follicule in *Scyliorhinus canicula* and *Torpedo marmorata* (Elasmobranchii). Z Zellforsch 75:453–468

Vincent S, Segretain D, Nishikawa S, Nishikawa SI, Sage J, Cuzin F, Rassoulzadegan M (1998) Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. Development 125:4585–4593

Yoshida S, Sukeno M, Nabeshima YI (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726