Characterization of spermatogonial markers in the mature testis of the dogfish (*Scyliorhinus canicula* L.)

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Abstract

In dogfish, spermatogenesis progresses from a restricted germinative zone, which lines the dorsal testicular vessel. Single spermatogonia (A_s), including the spermatogonial stem cells (SSCs), produce successively paired (A_p), undifferentiated (A_{u4} to A_{u512}), and differentiated (A_{d1} to A_{d8}) spermatogonia and preleptotene (PL) spermatocytes through 13 mitoses. Dogfish spermatogonial subpopulations present classical morphological characteristics but cannot be distinguished on the basis of molecular markers. This characterization has been initiated in mammals despite the difficulty to separate each spermatogonial subpopulation. For instance, both glial cell-derived neurotrophic factor family receptor alpha 1 (GFR α 1) and promyelocytic leukemia zinc finger protein (PLZF) are markers of undifferentiated spermatogonia, whereas receptor tyrosine kinase C-kit is a marker of differentiated spermatogonia. The aim of this study is to characterize spermatogonial markers and to differentiate several spermatogonial subpopulations. Dogfish cDNA sequences have been identified and validated by phylogenetic analyses for *gfr α 1*, *plzf*, *pou2*, as well as for high-mobility group box proteins 2 and 3 (*hmb2* and *3*) and for mini-chromosome maintenance protein 6 (*mcm6*). We have used the anatomical advantage of the polarized dogfish testis to analyze the expression of those markers by RT-PCR and *in situ* hybridization. *gfr α 1*, *pou2*, and *plzf* have been detected in the testicular germinative zone, suggesting that spermatogonial markers are relatively well conserved among vertebrates but with a less restricted expression for *plzf*. Moreover, *hmb3* and *mcm6* have been identified as new markers of differentiated spermatogonia. Finally, this first molecular characterization of spermatogonial subpopulations in a chondrichthyan model will be useful for further studies on the SSC niche evolution.

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Introduction

Spermatogenesis is a highly conserved process that makes long-term male fertility possible, thanks to continuous production of spermatozoa from spermatogonial stem cells (SSCs). The future of SSCs is controlled by specific regulating factors in a closed microenvironment called niche, first described by Schofield (1983). In *Drosophila*, the niche controls SSC self-renewal or differentiation commitment by four different mechanisms: symmetric and asymmetric division, reversion of engaged spermatogonia, and SSC loss (Sheng & Matunis 2011). Symmetric and asymmetric divisions have also been reported in mammals (Oatley & Brinster 2008), as well as reversion by intercellular bridge rupture (Nakagawa *et al.* 2010, Yoshida 2012). In the rodent niche, single spermatogonia (A_s including SSCs)

proliferate to form paired spermatogonia (A_p), aligned and undifferentiated spermatogonia (A_{al4-16}), differentiated spermatogonia (A to B), and spermatocytes (De Rooij & Russell 2000, Oatley & Brinster 2008).

In vertebrates and invertebrates, conserved SSC characteristics such as low mitotic activity, mottled chromatin, and contact with the basal membrane have been described for mammals (De Rooij & Russell 2000, Chiarini-Garcia *et al.* 2003), zebrafish (Leal *et al.* 2009), dogfish (Loppion *et al.* 2008), and *Drosophila* (Joti *et al.* 2011, Sheng & Matunis 2011). In mammals, the molecular characterization of the different spermatogonial subpopulations is in progress and a few markers have been characterized. The glial cell-derived neurotrophic factor (GDNF), identified as the main SSC self-renewal control factor, is secreted by the Sertoli cells and binds to the GDNF family receptor alpha 1 (GFR α 1),

expressed by spermatogonia, and signals through the Ret receptor tyrosine kinase (RET; Naughton *et al.* 2005, Oatley & Brinster 2008). Additional cell surface markers of undifferentiated spermatogonia have been identified, such as thymus antigen 1 (THY1; Kubota *et al.* 2004). Transcriptional factors promyelocytic leukemia zinc finger protein (PLZF) and Pou domain class 5 homeobox 1 (POU5F1) are other important factors involved in regulating SSCs fate in mammals (Buaas *et al.* 2004, Tenenhaus Dann *et al.* 2008) as they promote their self-renewal and stimulate the expression of pluripotency factors like REDD1 (Hobbs *et al.* 2010). On the other hand, some factors such as the tyrosine kinase receptor C-KIT or the spermatogenesis- and oogenesis-specific basic helix-loop-helix protein 1 and 2 (SOHLH1/2) are known to promote the proliferation and differentiation of spermatogonial progenitors (Feng *et al.* 2000, Ballow *et al.* 2006, Prabhu *et al.* 2006). Such markers have been used to establish a molecular signature of different spermatogonial subpopulations previously identified on the basis of their morphology (Chiarini-Garcia *et al.* 2003, Hermann *et al.* 2010). Thus, mouse undifferentiated spermatogonia A_s , A_p , and A_{al} are $GFR\alpha 1^+/PLZF^+/C-KIT^-$ and differentiating spermatogonia A and B are $GFR\alpha 1^-/PLZF^-/C-KIT^+$. Even if the relationship between the molecular signature of undifferentiated spermatogonia and their self-renewing activity remains to be further clarified, $GFR\alpha 1^+/Nanos2^+$ spermatogonial populations are known to largely correspond to A_s and A_p spermatogonia, which are potential stem cells in the undisturbed testis. Next, these cells give rise to transient amplifying Neurog3⁺ Aal spermatogonia, which differentiate to Kit⁺A1 spermatogonia (for review: Yoshida (2012)). However, undifferentiated mouse spermatogonia exhibit internal heterogeneity in expression of genes or in behavior (Yoshida 2012). Characterization of the different subpopulations of undifferentiated spermatogonia and of their microenvironment remains to be improved.

The anatomy of the dogfish testis makes it an interesting model to study the microenvironment controlling spermatogenesis. Furthermore, spermatogenesis has been well described and is divided into 18 stages (Stanley 1966, Holstein 1969, Loir & Sourdaire 1994, Loir *et al.* 1995). Spermatogenesis proceeds within spermatocysts (cysts) where germ cell development is synchronous. Cysts are composed of spermatoblasts (480–500 per cyst) in which one Sertoli cell is associated with one spermatogonium initially and with 64 spermatids in the post-meiotic stages. Cysts are formed after a proliferating phase for both spermatogonia and Sertoli precursors upon leaving the germinative zone constituting the potential stem cell compartment (Loppion *et al.* 2008), and cysts then proceed in maturational order toward the opposite margin of the gonad. Zonation thus allows to observe all spermatogenesis stages on a cross section of the testis and to dissect the pre-meiotic state

(zone A, stages I–VI), meiotic state (zone B, stages VII–X), early spermiogenesis (zone C, stages XI and XII), and late spermiogenesis (zone D, stages XIII–VVII) regions (Loir & Sourdaire 1994, Loir *et al.* 1995). Zone A can be further dissected to separate the area A0, which corresponds to the germinative zone (stage I) and the remaining area, A-. This area corresponds to the progenitor proliferating area (stage II), the cyst formation (stage IIIa) coinciding with the end of Sertoli cell divisions (Stanley 1966, Holstein 1969, Loppion *et al.* 2008), and to the further four mitosis of differentiated spermatogonia leading to preleptotene (PL) spermatocytes (stages IIIb, IV, V, and VI). In this model, the number of spermatogonial divisions from stage I to stage VI was estimated to be 13 by Holstein (1969) on the basis of the number of spermatoblasts per cyst. Our previous observations (Loppion *et al.* 2008) have suggested that the germinative zone located between the testicular capsule and the main testicular blood vessel contains single spermatogonia, paired spermatogonia, and (at least) clusters of four interconnected spermatogonia. Therefore, undifferentiated spermatogonia were found to be distributed from stage I (germinative zone) to stage II, which could correspond to the successive types of A_s , A_p , and A_{al} described for rodents. Moreover, the phylogenetic position of Chondrichthyans, the progress in sequence database production for several elasmobranchs (Coolen *et al.* 2007, Venkatesh *et al.* 2007, King *et al.* 2011, Tan *et al.* 2012, Quan *et al.* 2013), and the possibility to perform transcriptome (Redon *et al.* 2010) and proteome (Loppion *et al.* 2010) analyses makes the dogfish an excellent reference species to study and understand the evolution of the SSC niche.

The aim of this study is to improve the molecular characterization of spermatogonial subpopulations in dogfish. This characterization includes mammalian established markers such as $GFR\alpha 1$, POU5F1, and PLZF and dogfish previously highlighted sequences (Redon *et al.* 2010) such as mini-chromosome maintenance protein 6 (*mcm6*) and the high-mobility group box proteins 2 and 3 (*hmgb2* and *hmgb3*). Phylogenetic analysis, real-time PCR, and *in situ* hybridization experiments were performed and allowed us to show that *gfrα1*, *pou5f1*, and *plzf* are expressed in the SSC niche of the dogfish testis, but with differences in their expression patterns compared with mammals, and that *hmgb2* and *mcm6* are potential markers to discriminate potential SSCs from differentiating spermatogonia.

Materials and methods

Animals

Mature male dogfish *Scyliorhinus canicula* were captured from Cherbourg (English channel, France) using the facilities of the Lycée Maritime et Aquacole and stored in natural seawater

tanks at the Centre de Recherches en Environnement Côtier (Luc-sur-Mer, France). The fish were allowed to acclimate for at least 2 weeks before tissue sampling and were killed by severing the spinal cord and pithing (except when brain was collected). Testes, brain, kidney, eye, muscle, spleen, epididymis, liver, gill, and epigonal organ (the lymphomyeloid tissue of elasmobranchs) were sampled and directly transferred into ice-cold Tri-Reagent with the exception of the testes that were transferred into ice-cold Gauthron's buffer (Loir & Sourdaïne 1994) complemented with 58 mM TMAO. Fresh testicular cross sections were arrayed into five zones (A0, A-, B, C, and D) under a stereomicroscope, as described previously (Loir & Sourdaïne 1994).

Identification and in silico analyses of dogfish sequences

Sequences of interest were obtained from a dogfish testicular suppressive and subtractive cDNA bank (Redon *et al.* 2010), from a dogfish embryonic and juvenile cDNA bank (Redon *et al.* 2010, Quan *et al.* 2013), and from a dogfish gonadic RNA sequencing bank (ANR PhyloFish project currently ongoing) using the TBLASTN algorithm. Phylogenetic analyses were performed using additional amino acid sequences selected by means of the TBLASTN algorithm from ESTs, mRNA, or genome databases from the NCBI Institute (accession numbers are indicated in Fig. 1). Amino acid sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) Software (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and then shortened to exclude divergent extremities. Phylogenetic trees were built using the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.1 (Tamura *et al.* 2011) and two methods (maximum likelihood and neighbor-joining methods). The reliability of the inferred trees was estimated by applying the bootstrap procedure with 1000 replications. Protein domains were subsequently identified and annotated by means of the Simple Modular Architecture Research Tool (SMART) Software version 7 (<http://smart.embl-heidelberg.de/>; Schultz *et al.* (1998) and Letunic *et al.* (2012)). The conservation of protein domains between the dogfish and other species (mice, chicken, xenopus, and zebrafish) was assessed with ClustalW Multiple alignment version 1.4 (Thompson *et al.* 1994) and is indicated as a percentage of identity in Fig. 1.

RNA extraction and purification

Fresh tissue samples (100 mg) were ground up, total mRNA were extracted in 1 ml Tri-Reagent with conical pestles and needles, complemented with 200 µl 1-bromo-3-chloropropane (Sigma), and homogenized and centrifuged for 15 min at 4 °C at 13 500 g. Aqueous phases were transferred to new tubes with 500 µl isopropanol, centrifuged, and pellets were rinsed with 75% ethanol and air-dried. Total RNA were re-suspended in DEPC-treated water and quantified with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Les Ulis Courtaboeuf, France). All glassware and solutions containing RNA were RNase free.

Real-time PCR

To prevent any DNA contamination, 1 µg total RNA was treated with 2 U RQ1 DNase (Promega) in a 10 µl volume. Random hexamers (250 ng) were added and total RNA was denatured for 5 min at 70 °C and then RT was immediately carried out for 1 h at 37 °C with 500 nM dNTP, 25 U RNasin, 200 U M-MLV-RT, and M-MLV RT buffer (Promega) in a final 25 µl volume. Real-time PCR was performed on an iCycler apparatus (Bio-Rad) in triplicate and repeated using cDNA from three or six different animals. Real-time PCR consisted of 5 µl 1/20 diluted cDNA, 70 nM primers, and Absolute Blue SYBR Green Fluorescein mix (Thermo Scientific) with the following cycling parameters: 1×(95 °C, 30 s); 45×((95 °C, 30 s) and (60 °C, 45 s)); and 80×(55+1 °C, 10 s). Results were established with the iCycler Software (IQTH 3.1 Bio-Rad), efficiency of PCR was assessed using appropriate dilutions series, and single amplification formation was confirmed on melting curves. cDNA results were normalized against 5S RNA by means of the comparative Ct method (Livak & Schmittgen 2001). The use of this reference gene has been validated previously (Redon *et al.* 2010). Significant statistical groups (a, b, c, and d; $P < 0.05$ between each) were created using the Mann–Whitney *U* test, and relative expression results were shown by mean $\pm$ S.E.M.

In situ hybridization

Dig-conjugated riboprobes were synthesized from cDNA clones. cDNA synthesis was carried out through a standard PCR procedure with specific primers (Table 1) and then cloning was performed using the T/A Cloning kit (Clontech) and electro-competent TOP10 *Escherichia coli*. Plasmids were purified with the DNA Miniprep purification kit (Promega), and riboprobe cDNA matrix was generated by M13 PCR on 100 ng plasmid with 1 mM MgCl₂, 0.2 mM of each dNTPs, 0.4 µM M13 primer, and 0.625 U Go Taq DNA Polymerase. The cycling parameters were as follows: 1×(95 °C, 2 min), 35×((95 °C, 45 s) and (50 °C, 1 min) (72 °C, 2 min)), and 1× (72 °C, 5 min). PCR products were subsequently purified through precipitation. Their size was checked by gel migration and *in vitro* transcription was carried out for 1 h at 37 °C on 2 µg purified PCR product with 40 U RNasin, 25 U T7 or SP6 polymerase, 10 mM dithiothreitol, 1 mM rATP/rCTP and rGTP, 0.65 mM rUTP, and 0.35 mM digoxigenin-UTP (Roche). The riboprobes were purified by precipitation, ethanol washed, quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and their quality checked by migration (Bioanalyzer 2100, Agilent, Waldbronn, Germany).

Testicular slices, 2 mm thick, were fixed in ice-cold 4% paraformaldehyde (w/v in PBS) for 24 h, then progressively dehydrated in a series of PBS/methanol mixtures at staggered concentration levels (100%/0%; 75%/25%; 50%/50%; 25%/75%; and 0%/100%), and stored at –20 °C. Before mounting, the testis slices were progressively rehydrated in PBS and cryoprotected for 12 h at 4 °C in a 30%/70% sucrose/PBS solution. Testis slices were embedded in OCT compound (VWR), frozen on carbonic ice for 20 min, and stored at –80 °C. Eight to 12 µm-thick cryosections were cut at –20 °C with a cryotome (cryocut 1800, Leica), mounted on Superfrost slides (Thermo Scientific), and directly subjected to hybridization as follows.

Hybridizations with sense and antisense RNA probes were performed in parallel. Sections were rinsed in PBS, fixed for 10 min in 4% PFA solution, washed in PBS, and treated at room temperature for 4 min with 5 µg/ml proteinase K (Roche) in 0.05 M Tris and 0.01 M EDTA, pH 8. Sections were then washed in PBS; fixed for 10 min with 4% PFA; and washed and acetylated for 10 min in 100 mM tri-ethanolamine, 0.25% anhydride acid solution, followed by 5 min in 100 mM Tris, pH 8, 100 mM glycine solution. After one PBS wash, sections were washed for 5 min with 150 mM NaCl and directly incubated overnight at 65 °C with 500 ng of probes (previously denatured for 5 min at 75 °C) in the hybridization mixture (50% de-ionized formamide, 2× SSC, 5 mM EDTA, pH 8, 0.1% Tween 20, Denhardt's solution, 10% dextran sulfate, 0.1 mg/ml heparin, 0.1% Chaps, 0.5 mg/ml tRNA, and complemented with DEPC-treated water). After hybridization, sections were washed at 65 °C in 1× SSC and 1.5× SSC, for 10 min each, followed by two 20-min washes at 37 °C with 2× SSC. Sections were then incubated for 30 min with 0.2 µg/ml RNase A in 2× SSC at 37 °C before another 2× SSC wash, followed by two 30-min incubations at 60 °C with 0.2× SSC. After two 15-min MABT washes (0.1 M maleic acid, 150 mM NaCl, and 0.1% Tween 20 in DEPC-treated water, pH 7.5) at room temperature, sections were incubated for 3 h at room temperature with the blocking solution (2% blocking reagent, 20% decomplexed sheep serum, and MABT in DEPC-treated water). Immediately after this step, anti-DIG antibody coupled with alkaline phosphatase (Roche) diluted at a 1:2000 ratio in the blocking solution was added to the slides and incubated overnight at 4 °C. Unbound antibodies were removed through eight washes with MABT for 5–30 min at room temperature. Pre-revelation treatment was performed in an NTMT solution (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCl₂, and 0.1% Tween 20 in DEPC-treated water) and revealing was done by addition of NBT-BCIP (Roche) on the sections for a few minutes or a few hours until staining could be visually detected. Sections were finally washed in PBS and mounted with Mowiol medium (1.5 µM Mowiol 4-88, 1.3 mM glycerol, 0.15 M Tris-HCl, pH 8.5, and 33% H₂O).

For histology, paraffin sections of formaldehyde-fixed tissues were stained with hematoxylin, eosin, and green light and mounted with roti-histol. All observations and analyses were performed using a Nikon eclipse 80i microscope and Nikon NIS-Elements D 3.0 Software. As expected, none of the sense riboprobes gave any signal (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Results

Identification of dogfish orthologs of spermatogonial expressed sequences

The candidate genes selected for this study have two origins: *gfra1*, *plzf*, and *pou2/pou5f1* encode for classical markers of undifferentiated spermatogonia based on bibliographical references, and *mcm6* and *hmgb3* were shown to be over-expressed in the spermatogonial zone of the dogfish testis in our previous suppression subtractive hybridization study. As the genome of the dogfish *S. canicula* has not yet been sequenced, TBLASTN blasts were performed on three different dogfish cDNA banks: a testicular suppressive and subtractive cDNA library, an embryonic and juvenile cDNA bank, and a testicular and ovarian RNA sequencing bank. The complete cDNA sequences of candidates were successfully identified, and the accession numbers are indicated in Fig. 1. The global homology of the translated sequences was rather high for *Plzf*, *Mcm6*, *Hmgb2*, and *Hmgb3* (>70%) but much lower for *Pou2/Pou5f1*, and *Gfra1* (30–60%). Protein sequences were then analyzed using the SMART Software in order to identify the protein domains. For all candidates, these domains showed a high conservation level with an identity between dogfish and other species sequences usually around 70–100% (Fig. 1). A phylogenetic analysis was performed to validate the orthologous relationship of dogfish sequences with the genes of interest and to establish their evolutionary relationships. In the rooted phylogenetic trees, dogfish sequences always segregated with the expected orthologous genes. Concerning *Pou2/Pou5f1*, the sequence presented a glutamic acid residue in the POU domain in spite of the presence of an aspartic acid in *Pou5f1* orthologs. It also lacked the arginine deletion specific to *Pou5f1* orthologs. Therefore, the corresponding protein segregated with *Pou2*. Globally, dogfish sequences were found, either at the root of the gnathostomata (*Hmgb3* and *Gfra1*), which is a position consistent with current hypotheses on species evolution, or at the root of the Osteichthyes (*Plzf*, *Mcm6*, and *Hmgb2*), a position that could be explained by the derivation of teleostean fish sequences after an additional genome duplication.

Figure 1 Protein domain identification and conservation and phylogenetic analysis. Dogfish sequences of interest (*gfra1*, *plzf*, *pou2*, *mcm6*, *hmgb2*, and *hmgb3*) were obtained from several cDNA banks and their identities were validated by additional *in silico* analyses. For each sequence of interest, protein domains were identified using the SMART v7 Software. The global homology (in brackets) and the homology of protein domain sequences (on top of the related boxes) were determined between dogfish and other species sequences (mice, chicken, xenopus, and zebrafish) using BioEdit Software. All protein domains present in Osteichthyes were found to be well conserved in dogfish. Phylogenetic trees were built using the MUSCLE Alignment Software and the MEGA v5.1 Software. Trees were constructed using the maximum likelihood and neighbor-joining methods but only the latter is represented. The reliability of the inferred trees was estimated using the bootstrap method over 1000 replications. Phylogenetic trees were rooted with agnatha or non-vertebrate sequences. Dogfish sequences segregated with proteins coded by their orthologs at the root of gnathostomata as expected, or between fish and tetrapods, a position that could be explained by the specific evolution of the teleostean genomes.

Table 1 Nucleotide sequences of the primers used for real-time PCR and riboprobe synthesis. Primer sequences and PCR product sizes are indicated for each gene. As referenced in the table, the cDNA library previously established by Redon *et al.* (2010) was used with M13Fwd/Rev primers to produce riboprobes for *in situ* hybridization.

Experimental procedure	Gene name	GenBank accession number	Primer name	Primer sequence (5'–3')	PCR product size (bp)
RT-PCR	5S rRNA	M24954.1	Sc_5S rRNA_1q	TCGTCTGATCTCGGAAGCTA	85
			Sc_5SrRNA_1Aq	AGCCTACTGCACCTGGTATTC	
	<i>gfra1</i>	KF597022	Sc_GFR α _1q	ACCGGTGACCTGGCCTGTAGCA	131
			Sc_GFR α _1Aq	GGGCTCTGTTGGACCACCTCCA	
	<i>plzf</i>	KF597023	Sc_Plzf_Q-F1'	AGACTTTCCAGCAGATTCTCGA	155
			Sc_Plzf_Q-R1	ATCTGTTGCCTGAATTGTCT	
	<i>pou2</i>	KF548040	Sc_Pou5f_qF2	GAAGCCCAACAGTGAAGAAA	99
			Sc_Pou5f_qR2	TTCCCTTCTGCCTCTATT	
	<i>hmgb2</i>	KF548038	Sc_HMGB2_qF1	TCAAGAGCGAGTCTCCTGGA	123
			Sc_HMGB2_qR1	TCTCCTTAACCGGGATGC	
	<i>hmgb3</i>	KF548039	Sc_HMGB3_1q	AAGCCGTTCTTAGCAAAGC	102
			Sc_HMGB3_1Aq	AGGGGGCTCTTTGCACTAT	
ISH	<i>mcm6</i>	KF597024	Sc_MCM6_1q	GATTGAAGTGAAGCAGACTGG	78
			Sc_MCM6_1Aq	ACAAGGAAGGGATCCTGCTC	
	<i>gfra1</i>	KF597020	M13 Fwd31	CAGTCACGACGTTGTAAAACGACGGCCAGTG	755
			M13 Rev33	CAGGAAACAGCTATGACCATGATTACGCCAAGC	
	<i>pou2/pou5f1</i>	KF597021			804
	<i>hmgb3</i>	GT916248			529
	<i>mcm6</i>	GT916249			497

Conserved and new factors are expressed in the proliferative area of the mature dogfish testis

The expression pattern of identified transcripts was assessed by real-time PCR in a large panel of tissue samples (brain, spleen, kidney, epididymis, muscle, eye, liver, epigonal tissue, gill, and testis) to assess their tissue distribution, and in the five testicular zones corresponding to the germinative zone (A0), the differentiating spermatogonial zone (A-), the meiotic zone (B), the early spermatids zone (C), and the late spermatids zone (D), in order to assess their stage-specific expression during spermatogenesis (Fig. 2). The *gfra1* mRNA was predominantly detected in brain and testis tissue comparatively to other tissue types, with an mRNA relative abundance eight times higher in brain than in testis material (Fig. 2A). During spermatogenesis, *gfra1* was significantly more expressed in the germinative zone (A0) than in the A- zone containing differentiating spermatogonia and almost undetectable in spermatocyte- and spermatid-related zones (Fig. 2A'). The *plzf* mRNA was predominantly expressed in the liver and eye compared with other tissues (Fig. 2B). In testis, *plzf* expression was significantly higher in the germinative zone (A0) than in the differentiating zone (A-) and presented a persistent level of expression in more advanced stages (Fig. 2B'). Both *hmgb2* and *hmgb3* showed a large distribution of expression in the observed tissues (Fig. 2C and D). In the testis, *hmgb2* and *hmgb3* were more highly expressed in the germinative and spermatogonial differentiation zones than in the meiosis and spermiogenesis zones (Fig. 2C' and D'). The *mcm6* gene shows a preferential expression in testis, around tenfold higher than in the other tissues (Fig. 2E).

An equivalent expression level was detected between areas A0 and A- and the expression gradually decreased during the progress of spermatogenesis (Fig. 2E'). In summary, *gfra1* and *plzf* were preferentially expressed in the germinative area, while *hmgb2*, *hmgb3*, and *mcm6* appeared preferentially expressed throughout the whole spermatogonial zone of the mature dogfish testis.

Stages of spermatogonia in the dogfish

Spermatogonia were classified according to their number of mitoses and their progress from the germinative zone to the cyst at stage VI in order to precisely define spermatogonial stages on the basis of types described in rodents (i.e., A_s, A_p, and A_a) (Figs 3, 4A, B, C, D, E, and F and 5A, B, C, D, and E). Considering that the formation of cysts, completed at stage IIIa, coincides with the end of Sertoli cell divisions and the start of the synchronous proliferation of spermatogonia, differentiating spermatogonia can be subdivided into stages IIIa, IIIb, IV, V, and VI (the last stage corresponding to PL spermatocytes) and named A_{d1}, A_{d2}, A_{d4}, A_{d8}, and PL respectively. Consequently, undifferentiated stage I (germinative zone) and stage II (in and outside of the germinative zone) spermatogonia can be subdivided into stages A_s, A_p, A_{u4}, A_{u8}, and so on up to stage A_{u12} in order to take into account the following: i) the theoretical number of spermatoblasts per cyst; ii) the fact that spermatogonia are interconnected after their first division; and iii) the lack of division between the late A_a spermatogonium and the A₁ spermatogonium in rodents. As illustrated in Figs 3, 4, and 5, large single spermatogonia (Figs 4A and 5A) were observed in the

Figure 2 Classical and new factors are preferentially expressed in the spermatogonial area of the dogfish testis. Relative abundance of mRNAs was measured by RT-PCR and normalized with 5S rRNA (*gfra1*, *plzf*, *hmgb2*, *hmgb3*, and *mcm6*) in a large panel of tissue types (A, B, C, D, and E) and in the five testicular zones (A', B', C', D', and E'). Tissue samples were brain, spleen, kidney, epididymis, muscle, eye, liver, epigonal tissue, gill, and the testicular zone A. Testicular zones were as follows: A0, germinative zone; A-, spermatogonial proliferative and differentiation zone; B, meiotic zone; C, spermatids zone; and D, spermiogenesis, spermiation, and cyst resorption zone. Statistical analysis was performed using Mann-Whitney *U* test with *P* value < 0.05 between each statistical groups a, b, c, and d. A, B, C, D, and E: from three animals in duplicates (*N*=3; *n*=6); A', B', C', D', and E': from six animals in duplicates (*N*=6; *n*=12).

germinative zone as well as paired spermatogonia and four-spermatogonia clusters. Upon leaving the germinative zone along a defined path (Fig. 3B and C), both spermatogonia and Sertoli cells proliferate and associate with each other to form clusters (stage IIa, Fig. 4B) and, progressively, spherical cysts without lumen (stage IIb, Fig. 4C). In stage IIc, spermatogonia and Sertoli cells are arranged in a single layer around the lumen (Fig. 4D). Stage III cysts correspond to cysts that have a single layer of Sertoli cell nuclei around the central lumen (stage IIIa, Figs 4E and 5C) and one or two layers of differentiating spermatogonia towards the basal lamina (stage IIIb, Fig. 5D). At stage IIIa, one spermatogonium is associated with one Sertoli cell per spermatoblast. At stage IIIb, two spermatogonia are associated with one Sertoli cell per spermatoblast. Stage IV shows four layers of spermatogonia corresponding to four spermatogonia per spermatoblast, and Sertoli cell nuclei remains at the adluminal position (Fig. 4F). Stage V presents five layers of spermatogonia, corresponding to eight spermatogonia per spermatoblast, and Sertoli cell nuclei are in an adluminal (majority) or intermediate position (Fig. 5E).

Stage VI exhibits about six layers of germ cells (corresponding to 16 PL spermatocytes per spermatoblast) and Sertoli cell nuclei are in an intermediate (majority) or basal position. In cysts containing primary spermatocytes, Sertoli cell nuclei were in basal position (Fig. 5F).

***Pou2* and *gfra1* are preferentially expressed in the germinative zone of the mature dogfish testis**

In situ hybridization was performed on slices of adult testicular tissue, and representative results for genes, preferentially expressed in the germinative zone, were shown from stage I to IV to detail gene expression pattern from the SSC niche (stage I; Fig. 4A), the progenitors transition (stages IIa–IIc; Fig. 4B, C, and D), and the spermatogonial differentiation way (stages IIIa–IV; Fig. 4E and F). The *pou2* transcript was detected in the nucleus and cytoplasm of single spermatogonia, of one-paired spermatogonia (Fig. 4A'), and of undifferentiated spermatogonia in stage IIa (Fig. 4B'). In stages IIb and IIc, the staining seems to be mostly cytoplasmic and of variable intensity depending on the spermatogonia (Fig. 4C' and D').

Figure 3 Spermatogonial classification and stem cell niche in the dogfish testis. Comparison of the spermatogonial classification between rodents and dogfish. (A) The different spermatogonial subpopulations in rodents are single (A_s), paired (A_p), aligned (A_{al4-32}), and differentiated (A_{1-4} , A_{int} , and B) (Oatley & Brinster 2008). The spermatogonial subpopulations between stages I and VII in the dogfish are single (A_s), paired (A_p), undifferentiated (A_{u4-512} corresponding to rodent A_{al}), and differentiated spermatogonia (A_{d1-8}), and PL correspond to preleptotene spermatocytes. Blue numbers represent the estimated number of germ cells and each mitosis step, defined by Holstein (1969), was represented by gray numbers. Histology and schemes of the testicular zone A of the dogfish (B) include distinct parts: the germinative niche (black), the connective tissue (light gray), and the differentiation compartment (white). Serial histological study of the stem cell compartment showed that germinal cells leave the niche on either side the main blood vessel (B and C) along spans of connective tissue (C). A_p , A_{u4+} , and A_{u8+} spermatogonia represented paired, 4, 8 (or more) undifferentiated spermatogonia respectively. ct, connective tissue; bv, blood vessel. Scale bars: 100 μ m (B), 20 μ m (C).

Pou2 was no longer expressed in stage IIIa (Fig. 4E'), in more advanced spermatogonial stages (Fig. 4F') and in later stages of spermatogenesis (Supplementary Figure 1B). Somatic cells have shown no expression of *pou2* except the adluminal edge of the Sertoli cells in stages IIc and IIIa (Fig. 4D' and E'). *Gfra1* mRNA was detected in the cytoplasm of single and paired spermatogonia (Fig. 4A''), of cluster of progenitors in stages IIa, IIb, and IIc (Fig. 4B'', C'', and D'' respectively) and of spermatogonia in stage IIIa (Fig. 4E''). No expression was detected in more advanced spermatogonia (Fig. 4F'') or in later spermatogenesis stages (Supplementary Figure 1C). Sense riboprobes showed no labeling (Supplementary Figure 1). In summary, ISH experiments confirmed that *pou2* and *gfra1* were preferentially expressed in undifferentiated spermatogonia.

***plzf* is expressed from the SSCs niche to the early spermiogenesis area of the mature dogfish testis**

In situ hybridization of *plzf* was illustrated with wider pictures in order to highlight its large expression pattern. Indeed, *plzf* was detected in germinal cells from stage I

(undifferentiated spermatogonia) to stage XI (young spermatids) (Fig. 6A', B', C', and D'). Besides, this staining seems to gradually decrease from undifferentiated spermatogonia to spermatids. *Plzf* was found in the nucleus and cytoplasm of spermatogonia (Fig. 6A' and B'), and in the nucleus of spermatocytes and spermatids (Fig. 6B', C', and D'). The somatic cells composing the connective tissue of the niche were also stained at a lower intensity (Fig. 6A'). Finally, Sertoli cells from stage I to stage III (Fig. 6A' and B') seem to be stained as well as spermatogonia.

***hmgb3* and *mcm6* are preferentially expressed in differentiated spermatogonia of the mature dogfish testis**

Histology and ISH were illustrated at the early stages I and II (Fig. 5A and B) and during the whole period of active proliferation of spermatogonia from stage IIIa (Fig. 5C) to primary spermatocytes in stage VII (Fig. 5F). *hmgb3* mRNA was not detected in single and paired spermatogonia but clusters of A_{u4} spermatogonia

Figure 4 *gfra1* and *pou2* showed early expression, in the germinative area and during progenitor transition. Histology of spermatogonial stages in *S. canicula* (A, B, C, D, E, and F): spermatogonia (black circle), Sertoli cell nuclei (orange circle), pericyclic cell nuclei (yellow circle), and cyst lumen (asterisks), see Results section for description of stages. *In situ* hybridization on testicular sections with antisense digoxigenin-conjugated riboprobes directed against *pou2* transcript (A', B', C', D', E', and F') or *gfra1* transcript (A'', B'', C'', D'', E'', and F''). Black arrowheads, stained spermatogonia; white arrowheads, unstained spermatogonia. Scale bars: 10 µm.

showed cytoplasmic labeling (Fig. 5A'). In stages IIc, IIIa, IIIb, and through to stage V, all spermatogonia presented *hmgb3* mRNA in their cytoplasm (Fig. 5B', C', D', and E' respectively). At stage VII, all primary spermatocytes were comparatively unlabeled (Fig. 5F') like later stages (Supplementary Figure 1E). The *mcm6* mRNA was not detected in single and paired spermatogonia (Fig. 5A'') but was present in the cytoplasm of spermatogonial progenitors (stage IIc, Fig. 5B'') and of differentiating spermatogonia (stage IIIa, Fig. 5C''; stage IIIb, Fig. 5D'') up to stage V (Fig. 5E''). At stage VII, no primary spermatocytes expressed *hmgb3* (Fig. 5F'') like more advanced stages of spermatogenesis (Supplementary Figure 1F). In summary, the ISH results showed that *hmgb3* and *mcm6* were preferentially expressed in proliferating and differentiating spermatogonia.

Discussion

POU2/POU5F1 and *GFRα1* are highly conserved SSCs markers

The duplication event that gave rise to the *pou2* and *pou5f1* genes was first positioned early in the tetrapod's evolution (Frankenberg *et al.* 2010), but new genomic data now available for the spiny dogfish and the little skate seem to position this duplication at least in the gnathostomes ancestor (Frankenberg & Renfree 2013).

In the dogfish, the identification of a single-class V POU family member, which segregates with Pou2 rather than with Pou5f1 on the basis of two previously described Pou2-specific sequence signatures (Frankenberg & Renfree 2013), was consistent with the first hypothesis. A synteny analysis would confirm this orthology relationship, but unfortunately the genome of the dogfish is not available yet. In the new Pou2/Pou5f1 hypothesis, Pou5f1 would be extinct in the lesser spotted dogfish, as it is assumed to be in the elephant fish lineage but retained in some rays and sharks. Both Pou2 and Pou5f1 (Oct4) show the feature of induction of pluripotency (Tapia *et al.* 2012). In testis, Pou2/Pou5f1 is an important marker of pluripotency for the spermatogonial lineage and was involved in the stem cell fate maintenance (Tenenhaus Dann *et al.* 2008). Pou2/Pou5f1 expression was reported in primordial germ cells during development and in undifferentiated spermatogonia in adults from medaka to mammals, although some differences in the expression patterns were noticed between species (Bhartiya *et al.* 2010, Sanchez-Sanchez *et al.* 2010, Wu *et al.* 2010, Encinas *et al.* 2012). In our study, *pou2/pou5f1* transcript was detected in both cytoplasm and nuclei of single and paired spermatogonia, in the cytoplasm of undifferentiated spermatogonia until the cystic formation coinciding with the onset of spermatogonial differentiation and their synchronous

Figure 5 *hmgb3* and *mcm6* showed a late expression during progenitors' transition and spermatogonial differentiation. Histology of spermatogonial and meiotic stages in *S. canicula* (A, B, C, D, E, and F): germ cells (black circle), Sertoli cell nuclei (orange circle), migrating Sertoli cell nuclei (red circle), and cyst lumen (asterisks), see Results section for description of stages. *In situ* hybridization on testicular sections with antisense digoxigenin-conjugated riboprobes directed against *hmgb3* transcript (A', B', C', D', E', and F') or *mcm6* transcript (A'', B'', C'', D'', E'', and F''). Black arrowheads, stained spermatogonia; white arrowheads, unstained spermatogonia. Scale bars: 10 µm.

proliferation. These results suggest that the role of Pou2/Pou5f1 in SSCs' maintenance could be conserved along the vertebrates' evolution process. Additionally, the first-time observation of an asymmetrical distribution of *pou2* mRNA in one of two paired spermatogonia strongly suggests an asymmetric division of SSCs leading them to a different functional state in the dogfish, as described in *Drosophila* and mammals (Oatley & Brinster 2008, Sheng & Matunis 2011).

In dogfish, *gfra1* transcripts were mainly detected in testis and brain. In brain, it has been reported that GFR α 1 was involved in the self-renewal process of neural stem cells, a function conserved from zebrafish to mammals (Airaksinen *et al.* 2006). As the highest expression level of *gfra1* was found in dogfish brain, we can hypothesize a similar function in *S. canicula*. In testis cells, GFR α 1 was reported to be a marker of undifferentiated spermatogonia in many mammalian models (Suzuki *et al.* 2009, Costa *et al.* 2012). GFR α 1 and its co-receptor RET stimulate SSC self-renewal in response to the GDNF (Naughton *et al.* 2005). A recent study in Nile tilapia provides the first characterization of GFR α 1 expression pattern in testis of a teleostean fish with an expression restricted to undifferentiated spermatogonia (Lacerda *et al.* 2013). Similarly, *gfra1* mRNA was located in undifferentiated spermatogonia of the dogfish, but this expression was extended, at a lower level, to early

differentiating spermatogonia, and such extended expression pattern was also described in monkey (Hermann *et al.* 2010). Although the GFR α 1 ligand, Gdnf, has not yet been characterized in the *S. canicula* databanks, these first results suggest that GFR α 1 may control the self-renewal of SSCs in our chondrichthyan model. Furthermore, in our model, GFR α 1 + spermatogonia are not restricted to the germinative area (the potential SSC niche) and undifferentiated spermatogonia expressed GFR α 1 until completion of the cyst formation (stage IIIa) and presence of differentiated spermatogonia. Considering that GFR α 1 + undifferentiated spermatogonia are potential real stem cells, that proportions of Aal spermatogonia are GFR α 1⁺, that resultant single spermatogonia after fragmentation of syncytial spermatogonia could be used to replenish the stem cell pool in mice (Yoshida 2012), and the results obtained in this study, there appears to be a clear need to achieve a better understanding of the behavior of GFR α 1-positive cells in the SSC system.

***plzf* is a germinal marker conserved in dogfish but with a larger kinetic of expression**

plzf is a transcriptional factor involved in the maintenance of the undifferentiating statement of spermatogonia by repressing the expression of the differentiating factor

Figure 6 *Plzf* showed a large expression pattern from the niche to the spermatids area. Histology of the niche (A), spermatogonial stages (B), meiotic stages (C), and early spermiogenesis stages (D) in *S. canicula*. *In situ* hybridization on testicular sections with antisense digoxigenin-conjugated riboprobes directed against *plzf* transcript (A', B', C', and D'). Inset in A' showed stained single spermatogonia (black arrowhead) in the niche. Sertoli cells seem to be also stained from stage I to stage III (A' and B'). Black and white scale bars represented 100 and 20 μ m respectively.

C-KIT and by repressing the mTORC1 inhibition of GDNF signaling (Buaas *et al.* 2004, Filipponi *et al.* 2007, Hobbs *et al.* 2010). Sequence analyses have shown that PLZF is highly conserved, from dogfish to mammals. Tissue expression studies have shown that PLZF is expressed in testis of different species such as the carp (Mohapatra *et al.* 2010) and the mouse (Kokkinaki *et al.* 2010), as observed in the dogfish, even if its expression was higher in the liver and eye than in the testis. In dogfish, *plzf* was detected not only in undifferentiated spermatogonia, in agreement with observations in various mammals and in zebrafish (Hermann *et al.* 2009, Kokkinaki *et al.* 2010, Ozaki *et al.* 2011, Costa *et al.* 2012), but also in differentiated spermatogonia, a larger expression pattern also described in humans and monkeys (Hermann *et al.* 2009). Surprisingly, *plzf* expression in dogfish meiotic germ cells and young spermatids was a unique case in the studied species. Moreover, *plzf* transcripts were detected in dogfish Sertoli cells from stage I to stage III. This may be explained by the fact that in dogfish, those cells actively proliferate until complete cyst formation, which occurs at stage IIIa (Stanley 1966, Holstein 1969, Loppion *et al.* 2008). These data suggest that PLZF may have a main conserved function in stem cell fate regulation in vertebrates. However, the significance of its expression in differentiating spermatogonia in some species and in meiotic germ cells and proliferating Sertoli cells in dogfish remains to be explored.

hmgb2, hmgb3, and mcm6 are new factors expressed in the spermatogonial compartment of the mature dogfish

Members of the Hmgb family are highly conserved DNA-binding proteins capable of interacting with various proteins that regulate transcription, chromatin dynamics, immune response, development, and other cellular processes in several species (Moleri *et al.* 2010, Malarkey & Churchill 2012). Hmgb1 and 2 were detected in fathead minnow mature testes (Martyniuka & Alvarez 2013). In addition to this, HMGB4 was found in adult mice testes. In mice, HMGB1 is ubiquitously expressed at high levels in lymphoid tissues and in testes where its expression in Sertoli cells and germ cells is associated with a potential antibacterial role (Zetterstrom *et al.* 2006). HMGB2 expression is mostly restricted to lymphoid organs and testes, especially in spermatocytes and *Hmgb2*^{-/-} mice, have defective spermatogenesis (Ronfani *et al.* 2001). HMGB3 was defined as a proliferative marker of hematopoietic stem cells (Nemeth *et al.* 2006). HMGB4 is preferentially expressed in adult mouse testis and is located in spermatocytes and spermatids (Catena *et al.* 2009). In dogfish, *hmgb2* and *hmgb3* were detected in tissue from several organs, contrasting with the tissue specificity observed in adult mice. Both genes are expressed in testis tissue, which is in agreement with the results observed in mice for *Hmgb2* but not for *Hmgb3*. A preferential expression was observed in spermatogonia rather than in spermatocytes. On the other hand, *hmgb3* transcripts

were not observed by ISH in the dogfish epigonal tissue, which is the chondrichthyan hematopoietic tissue. These results suggest a diverging evolution of HMGB functions between chondrichthyans and mammals. However, *hmgb2* and *hmgb3* expression patterns in dogfish testis may favor a better definition of the role of HMGB proteins during spermatogenesis and, more precisely, during spermatogonial proliferation.

MCM members are highly conserved DNA helicases involved in the S phase of cell cycle for many cell types in many species (Edwards *et al.* 2002, Sakwe *et al.* 2007). Those proteins are expressed by proliferative cells in response to the activation of ERK/MAPK signaling (Bruemmer *et al.* 2003). From zebrafish to mouse, MCM members are involved in the differentiation and proliferation of various tissue types during embryogenesis and juvenile development (Ryu & Driever 2006, Swiech *et al.* 2007). In gonads, MCM proteins 2, 6, and 7 seem to be engaged in the regulation of transcription and/or chromatin remodeling during mouse oogenesis (Swiech *et al.* 2007), and MCM5 may be involved in the meiotic recombination pathway in *Drosophila* (Lake *et al.* 2007). A testis-specific expression pattern has been reported for MCM7 in the rat (Com *et al.* 2006) and fathead minnow, with MCM 2–7 proteins being detected in mature testis (Martyniuka & Alvarez 2013). In our

study, *mcm6* had a testis-specific expression and transcripts were observed in proliferating and differentiating spermatogonia, which is comparable with MCM7 protein expression in rat spermatogonia (Com *et al.* 2006). However, ISH did not evidence transcripts in meiotic cells, even if the results of PCR showed expression in the meiotic zone. Further studies on MCM members in *S. canicula* are needed to better understand the evolution of their function during spermatogenesis. It is interesting to note that our study showed that transcripts of these two types of factors involved in replication, HMGB and MCM, were not observed in spermatogonia A_s and A_p . This could be related to their previously reported low replication activity (Loppion *et al.* 2008).

Spermatogonial markers seem well conserved among vertebrates

Spermatogonia subpopulations were well described in various species on the basis of morphological criteria (De Rooij & Russell 2000, Leal *et al.* 2009) and their molecular characterization is continuously improving (Costa *et al.* 2012, for review, Nagano & Yeh (2013)). However, the identification of spermatogonial markers in teleostean fish lags behind that of mammals and is generally limited to one or two markers per studied

Figure 7 Evolution of the expression pattern of spermatogonial markers in Osteichthyes and Chondrichthyes. The protein expression patterns (bars) reported in mice for GFR α 1, PLZF (Suzuki *et al.* 2009), C-KIT (Tokuda *et al.* 2006), and POU5F1 (Wu *et al.* 2010), in medaka for Pou2 (Sanchez-Sanchez *et al.* 2010), in zebrafish for Plzf (Ozaki *et al.* 2011) and in tilapia for Gfra1 (Lacerda *et al.* 2013) were compared with transcript expression in dogfish (*pou2*, *gfra1*, *plzf*, *hmgb3*, and *mcm6*) and C-kit protein expression pattern (Loppion *et al.* 2008). The expression patterns of genes preferentially expressed in undifferentiated or differentiating spermatogonia are indicated with black and gray bars respectively. Similarly, spermatogonial subpopulations were subdivided into undifferentiated spermatogonia (und.) including spermatogonia A_s , A_p , and A_{u4-512} in dogfish and A_s , A_p , and A_{a1} in mice (dark gray boxes), and differentiating spermatogonia (diff.) including spermatogonia A_{d1-4} and PL in dogfish and A_{1-4} , Int and B in mice (light gray boxes). Electron micrographs illustrate different spermatogonial populations in dogfish: large spermatogonia with mottled chromatin located in the germinative zone (stage I), A_u included in a single cell layer with Sertoli cells (stage IIc), A_d arranged in two layers (stage IIIb), and A_d with increasing amounts of heterochromatin (stage IV). SPC, spermatocyte.

species. Only three recent studies of teleosts have described the expression pattern of the classical molecular markers we chose in this study (Sanchez-Sanchez *et al.* 2010, Ozaki *et al.* 2011, Lacerda *et al.* 2013). Pou2, known for its role in the induction of pluripotency (Tapia *et al.* 2012), and Gfr α 1, involved in SSC self-renewal in response to GDNF (Naughton *et al.* 2005), seem well conserved as markers of undifferentiated spermatogonia from Osteichthyes (mammals and teleosts) to Chondrichthyes. Plzf, responsible for the maintenance of their undifferentiated condition (Buaas *et al.* 2004, Costoya *et al.* 2004), always shows an expression in undifferentiated spermatogonia extending to differentiating spermatogonia in zebrafish (Ozaki *et al.* 2011) and even further in dogfish. On the other hand, *hmgb2* and 3 seem preferentially expressed in differentiating spermatogonia in dogfish like c-Kit, a classical marker of differentiating spermatogonia in mammals.

Considering that a flexible SSC system exists at least in mice (Yoshida 2012), the cellular and molecular characterizations of the microenvironments, including the SSC niche, which controls spermatogonia behavior, remain a challenge. Therefore, species with polarized testis where the SSC niche is easy to locate are of particular interest. Recent data show potential germinal stem cells at the periphery of the gonad in teleostean fish and could present SSC niche structures distributed along a strip surrounding the gonad, like in zebrafish ovary (Beer & Draper 2013), or in pools scattered over a gonadal surface, called germinal cradles in medaka ovary (Nakamura *et al.* 2010). Finally, these scattered pools can be isolated within the tunica albuginea as suggested by data shown in Nile tilapia testis (Lacerda *et al.* 2013). The germinal zone seems well conserved in all these models harboring a cystic organization despite differences in term of distribution. Considering the niche conservation and other practical aspects (large size and easy isolation), the germinal zone of dogfish testis is a valuable model, which needs to be investigated further.

As a conclusion, we have shown that both GFR α 1 and POU2/POU5F1 are evolutionary conserved factors expressed in the niche of the dogfish testis. Conversely, *plzf* presents a less restrictive expression pattern than in mammals with an expression in the niche but also during the differentiation phase of the dogfish testis. Also, new spermatogonial markers such as *hmgb3* and *mcm6* were found to be expressed in all spermatogonia except in A_s and A_p (Fig. 7). On the basis of the transcript expression of those factors, four different spermatogonial sub-populations were defined in the dogfish testis: i) *pou2*⁺/*gfr* α 1⁺/*plzf*⁺/*hmgb3*[−]/*mcm6*[−] A_s and A_p (stage I); ii) *pou2*⁺/*gfr* α 1⁺/*plzf*⁺/*hmgb3*⁺/*mcm6*⁺ undifferentiated progenitor A_u (stages IIa–IIc); iii) *pou2*[−]/*gfr* α 1⁺/*plzf*⁺/*hmgb3*⁺/*mcm6*⁺ differentiating spermatogonia A_{d1} (stage IIIa); and iv) *pou2*[−]/*gfr* α 1[−]/*plzf*⁺/*hmgb3*⁺/*mcm6*⁺ differentiated spermatogonia A_{d2} to

PL (stages IIIb–VIIa). These proposed criteria now need to be confirmed at protein level, and further effort in functional studies will contribute to achieving a better understanding of the functional evolution of the SSC niche.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0316>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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