

# Stage-specific gene expression during spermatogenesis in the dogfish (*Scyliorhinus canicula*)

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## Abstract

In the dogfish testis, the cystic arrangement and polarization of germ cell stages make it possible to observe all stages of spermatogenesis in a single transverse section. By taking advantage of the zonation of this organ, we have used suppressive subtractive libraries construction, real-time PCR, and *in situ* hybridization to identify 32 dogfish genes showing differential expressions during spermatogenesis. These include homologs of genes already known to be expressed in the vertebrate testis, but found here to be specifically expressed either in pre-meiotic and/or meiotic zones (ribosomal protein S8, high-mobility group box 3, ubiquitin carboxyl-terminal esterase L3, 20 $\beta$ -hydroxysteroid dehydrogenase, or cyclophilin B) or in post-meiotic zone (speriolin, Soggy, zinc finger protein 474, calreticulin, or phospholipase c- $\zeta$ ). We also report, for the first time, testis-specific expression patterns for dogfish genes coding for A-kinase anchor protein 5, ring finger protein 152, or F-box only protein 7. Finally, the study highlights the differential expression of new sequences whose identity remains to be assessed. This study provides the first molecular characterization of spermatogenesis in a chondrichthyan, a key species to gain insight into the evolution of this process in gnathostomes.

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## Introduction

Spermatogenesis is a complex process in which spermatogonial stem cells undergo differentiation and mitotic proliferation to form primary spermatocytes and, following two meiotic divisions, secondary spermatocytes, and round spermatids. These spermatids subsequently enter spermiogenesis to give mature spermatids. Spermatogenesis involves at least three levels of regulation, relying on cell-intrinsic genetic programs, interactions between germ and somatic cells, and extrinsic influences of peptides and steroid hormones (Eddy 2002). The genetic interactions and regulatory networks integrating these different control levels remain largely unknown. Deciphering these programs is crucial for understanding the genetic basis of male infertility and establishing conditions to obtain *in vitro* propagation of germ cells.

Analyses of the genetic control of spermatogenesis have been conducted in various model organisms, including vertebrates such as mice (Schultz *et al.* 2003, Shima *et al.* 2004, Xiao *et al.* 2008), rats (Schlecht *et al.* 2004), zebrafish (Sreenivasan *et al.* 2008), rainbow trout (Mazurais *et al.* 2005) or sea bass (Viñas & Piferrer 2008),

and invertebrates, primarily *Drosophila melanogaster* and *Caenorhabditis elegans*. These studies have shown that spermatogenesis continuity is ensured by the balance between self-renewal, differentiation, and proliferation of spermatogonial stem cells (Spradling *et al.* 2001, Dadoune 2007, Oatley & Brinster 2008). This balance appears to be tightly linked to the microenvironment of the germ stem cell, referred to as the niche, a notion first defined in *Drosophila* (Yamashita *et al.* 2005). Germ stem cells are also known to express a distinctive set of molecular markers (Ebata *et al.* 2008), including *Gfra* (He *et al.* 2007), *Pou5f1* (Pan *et al.* 2002), or *Zbtb16* (Buaas *et al.* 2004). However, our knowledge of the regulation of the initiation of spermatogenesis is far from exhaustive, and many differentiation and proliferation regulators remain unknown. The molecular and cellular mechanisms controlling the last phase of spermatogenesis, i.e. spermiogenesis have been more extensively studied. For instance, the molecular mechanism driving chromatin compaction through the replacement of histones by protamines has been extensively documented (Dadoune 2003, Tanaka & Baba 2005, Balhorn 2007), and analyses mainly conducted in mice have led to the identification of a number of genes

specifically expressed in the post-meiotic stages (Schultz *et al.* 2003, Tanaka & Baba 2005). However, many genes involved in these later steps of spermatogenesis probably also remain to be identified, as it was estimated that 4% of the mouse genome might be involved in this process (Schultz *et al.* 2003). Furthermore, evolutionary conservation of the genetic factors involved in spermiogenesis also remains to be assessed.

Suppressive subtractive hybridization (SSH) technique is a powerful tool for identifying new genes involved in specific mechanisms, tissues or cell types, through the comparison of two cDNA populations and isolation of the genes specifically expressed in each of them. Contrary to microarray techniques, this approach does not require prior extensive cDNA sequence characterization, a major advantage when dealing with nontraditional model organisms. This approach has also been shown to enable identification of rare cDNAs (Diatchenko *et al.* 1996).

The dogfish *Scyliorhinus canicula* belongs to the class of chondrichthyans which occupy a key phylogenetic position among jawed vertebrates and as such is also the sister group of osteichthyans. This outgroup position makes the dogfish an excellent reference species when comparing all traditional vertebrate model organisms, including mammals, as reported previously at molecular (Robinson-Rechavi *et al.* 2004) and mechanistic (Coolen *et al.* 2007) levels. Such comparisons are important to identify ancestral characteristics or mechanisms and gain insight into the evolution of physiological processes such as spermatogenesis, in gnathostomes. The dogfish, as well as other elasmobranchs, has a testis organization which makes it a particularly relevant model for analyzing this process (Dubois & Callard 1990, McClusky 2005). In this species, the testis is polarized, and spermatogenesis progresses within spermatocysts (cysts) in which germ cell development is synchronous (Stanley 1966). 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 generated in the germinative area at the ventro-lateral length of the testis and progressively displaced toward the opposite face during maturation, where spermiation occurs. The resulting organ zonation thus makes it possible to observe all spermatogenesis stages in a transverse section (Sourdaine & Jégou 1989). Additionally, accurate descriptions of the dogfish testis have led to a stage classification ranging from I to XVIII, initially proposed on morphological criteria by Stanley (1966) and later refined by Loir *et al.* (1995). Four main zones can be distinguished in the organ: i) zone A (stages I–VI), which includes cysts with spermatogonia from the germinative zone to the last mitotic proliferation step, ii) zone B (stages VII–X), which corresponds to the meiotic stages in which primary and secondary spermatocytes are found, iii) zone C (stages XI and XII), which groups cysts

with round spermatids and spermatids in the first steps of elongation, and iv) zone D (stages XIII–XVIII), with cysts showing the final steps of spermiogenesis and spermiation.

This testis zonation makes the dogfish a unique model for the molecular characterization of the different stages of spermatogenesis. Here, we have taken advantage of this characteristic to identify cDNAs specifically expressed in different zones of the dogfish testis using suppressive subtractive libraries construction, real-time PCR, and *in situ* hybridization. Based on these expression patterns, we discuss their possible involvement in the differentiation and proliferation of spermatogonia and in spermiogenesis in regard of the functions of their homologs, as reported in other model organisms.

## Results

### **Identification of transcripts preferentially expressed in zones A (spermatogonia) and C (spermatids)**

Taking advantage of the dogfish testes organization, we conducted SSH between zones A and C cDNA populations to construct two cDNA libraries respectively enriched in transcripts selectively expressed in zone A (spermatogonia zone: A-library) and zone C (round and young elongating spermatids zone: C-library). These two libraries were plated, and 96 clones were randomly picked from each of them for an initial screening. Thirty-nine and 82% of A- and C-clones respectively were found to contain an inserted fragment following PCR amplification of the insert. Dot blot hybridization confirmed that 28 and 70 of these clones are preferentially expressed in zone A and C respectively. These 98 clones were sequenced and produced 81 satisfactory sequences: 15 for A-library clones and 66 for C-library clones ranging from 141 to 1030 bp. After editing and elimination of redundancy, 12 and 20 independent clones were finally identified as preferentially expressed in spermatogonia (A), and round and young elongating spermatids (C) zones respectively (Table 1).

### **Identification of vertebrate homologs of the differentially expressed sequences**

The 32 cDNA sequences characterized were submitted to systematic searches for sequence homology in two parallel steps. In the first one, searches were conducted versus GenBank with the 32 clone sequences obtained as described above as queries. In the second one, currently available databases of chondrichthyan sequences were also queried to extend the dogfish sequences or identify elephant shark homologs whenever possible. When significant hits were obtained (>95% identity), these sequences were in turn used as queries in a second independent search versus GenBank. Highly coherent results were obtained by these two strategies (Table 1)

except for A-C7 and A-D2 which showed homologies with different, but related, proteins (86% identity between laminin receptor 1 and 40S ribosomal protein Sa, and 68% identity between 20 $\beta$ -hydroxysteroid dehydrogenase (20 $\beta$ -HSD) and carbonyl reductase 1). Five sequences corresponded to previously known *S. canicula* sequences respectively encoding three protamines (C-A3, C-B7, and C-G8), one transition protein (C-A7), and fructose-bisphosphate aldolase C (C-F11). Notably, the protamine S4 cDNA was highly represented in the subtracted cDNA fractions of the C library, since 35 clones out of the 96 initially picked contained this sequence. Two additional sequences were identified in other chondrichthyan databases and showed higher homology to type I procollagen  $\alpha$ 1 chain of *Raja kenoei* (A-G10) and to fructose-bisphosphate aldolase B of *Cephaloscyllium umbratile* (C-E1). The remaining sequences gave no significant hits in the elephant shark genome and were not sequenced previously in *S. canicula* or in another chondrichthyan. Among them, 6 (A-C4, A-C7, A-D2, A-E4, A-H2, and A-H10) gave significant hits (>75% identity), and 16 (A-A2, A-B4, A-G11, A-H1, A-H3, C-A2, C-A4, C-A5, C-A6, C-B10, C-C5, C-C11, C-D11, C-D12, C-F3, and C-G9) led to the detection of weaker homologies. Among these clones, C-A6 and C-C5 displayed similarity with hypothetical proteins of unknown function. No sequence similarity was found for clones C-A9, C-G12, and C-H11.

### Stage-specific expressions of the selected cDNAs during spermatogenesis

To validate the zone specificity suggested by SSH results and have a more detailed picture of the dynamics of gene expression throughout spermatogenesis, we measured the relative expression levels of the selected sequences in the four zones of the testis using real-time PCR. Primers were designed for each of the 32 SSH clones except those encoding protamines for which a single clone (C-A3 encoding protamine S4) was retained as a control. Expression was studied in three different animals, and 5S RNA was used as a reference. Figure 1 shows results obtained with  $\Delta\Delta C_t$ , and data were expressed as relative expression to zone A. Concerning the clones retrieved from the A-library, clones A-A2, -E4, -G10, -G11, and -H1 showed higher expression levels (relative expression inferior to 1) in the spermatogonia zone (A) than in zones B, C, and D (Student's *t*-test,  $P < 0.05$ ), in line with the SSH results. Clones A-C4, -D2, -H2, and -H10 did not display significant expression variation between zones A and B, but expression in zones C and D was significantly lower ( $P < 0.05$ ), revealing a larger specificity to spermatogonia and spermatocytes zones. Incongruencies to the SSH results were only observed for clones A-B4, -C7, and -H3, for which expression was higher in zones B and/or C

(relative expression greater than 1). These three clones were not further analyzed. For all the C-clones tested, expression was significantly higher ( $P < 0.05$ ) in zone C than in zone A (relative expression greater than 1) thus confirming SSH results. Student tests performed on the expression in the four zones highlighted three distinct expression profiles for C-clones: i) the highest expression in zone C (C-A2, -A5, -A7, -B10, -C5, -C11, -E1, -F3, and -F11), ii) higher expression in zones D (C-A3 and -A9), and iii) higher expression in zones C and D (C-A4, -A6, -D11, -D12, -G9, -G12, and -H11). Noticeably, expression variations displayed by C-clones were stronger (ratios C/A between 2.4 and 95.0) than those observed for A-clones (ratios A/C from 1.5 to 11.6).

### Tissue specificity of the selected clones

In order to assess the tissue specificity of the selected transcripts, we then measured their level of expression in seven distinct tissue types in addition to the testis: spleen, liver, muscle, epigonal tissue, brain, kidney, and epididymis (Fig. 2, see summary in Table 2). As described above, this analysis was conducted using real-time PCR. Applying the  $\Delta\Delta C_t$  formalism, results were expressed as a relative expression compared with the expression found in testis in zones A and C for A- and C-clones respectively (Fig. 2). With the exception of A-H10 which was predominantly expressed in testis compared with other tissues (factor 54–3400), expression for A-clones was not found markedly higher in testis as relative expression varied from  $5 \times 10^{-2}$  to 22; the corresponding transcripts were therefore considered as ubiquitously expressed. Four main patterns of tissue expression were observed for C-clones. For eight of them, expression appeared to be specific for the testis as their relative transcript expression was lower than  $10^{-4}$  in all the tissues (C-A3, -A4, -A7, -A9, -B10, -D12, -F3, and -G9). Among these clones, C-B10 displayed marked tissue specificity as its expression was not detected in liver, muscle, kidney, brain, or epididymis. For six clones, the relative expression level ranged between  $7.2 \times 10^{-2}$  and  $1.5 \times 10^{-3}$  in the seven tissue types, indicating that the transcripts are predominantly expressed in the testis (C-A2, -A5, -A6, -D11, -G12, and -H11). Finally, for clones C-C5, -C11, and -E1, expression appeared ubiquitous while the results were more ambiguous for clone C-F11 with a predominant expression in testis and brain.

### Cell type specificity of the genes identified by SSH: in situ analysis

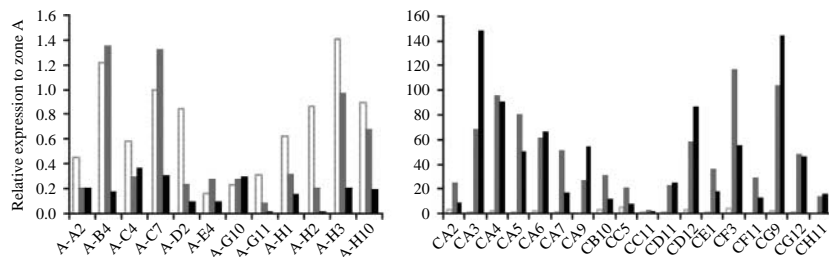
To define precisely in which stages of spermatogenesis the clones were expressed and identify whether they were expressed by germ or Sertoli cells, *in situ* hybridizations of testicular sections were undertaken for nine C-clones, shown by quantitative PCR (qPCR) as

**Table 1** Dogfish cDNA identified by suppressive subtractive hybridization and preferentially expressed in spermatogonia (A) or round and young elongating spermatids (C): homologies identified in GenBank database and on dogfish embryo cDNA library.

Clone	Length (bp)	Number of identical clones	dbEST accession numbers	Homology putative ID	Matching sequence	% identity	Homology in embryonic library <sup>a</sup>	Homology/putative ID of embryonic cDNA	Matching sequence	% identity
A-A2	781	1	GT916240	Predicted: similar to poly-protein ( <i>Danio rerio</i> )	XP_001332599.2	41	FP885545	Predicted: similar to poly-protein ( <i>Danio rerio</i> )	XP_001333892.2	41
A-B4	974	2	GT916241	Programmed cell death 2 ( <i>Gallus gallus</i> )	NP_001026235.1	53	FP885828	Programmed cell death 2 transcript a3PDCD2 ( <i>Gallus gallus</i> )	ABL09365.1	65
A-C4	355	2	GT916242	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase) ( <i>Danio rerio</i> )	NP_001019576.1	82	FP885531	Ubiquitin carboxyl-terminal hydrolase isozyme L3 ( <i>Salmo salar</i> )	NP_001140074.1	78
A-C7	359	1	GT916243	Laminin receptor 1 (ribosomal protein SA, 67 kDa) ( <i>Xenopus laevis</i> )	NP_001080730.1	90	FP885530	40S ribosomal protein Sa ( <i>Ictalurus punctatus</i> )	AF402808_1	82
A-D2	627	2	GT916244	Carbonyl reductase-like 20 $\beta$ -hydroxysteroid dehydrogenase ( <i>Oreochromis niloticus</i> )	AF439713_1	75	FP885825	Carbonyl reductase 1 ( <i>Homo sapiens</i> )	NP_001748.1	71
A-E4	297	1	GT916245	Similar to ribosomal protein S8 ( <i>Equus caballus</i> )	XP_001496811.2	92	FP885533	Ribosomal protein S8 ( <i>Homo sapiens</i> )	NP_001003.1	94
A-G10	629	1	GT916246	Type I procollagen $\alpha 1$ chain ( <i>Raja kenjoi</i> )	BAD98524.1	96	FP885827	Type I procollagen $\alpha 1$ chain ( <i>Raja kenjoi</i> )	BAD98524.1	93
A-G11	555	1	GT916247	Adenine phosphoribosyltransferase ( <i>Danio rerio</i> )	NP_956962.1	67	FP885823	Adenine phosphoribosyltransferase ( <i>Osmerus mordax</i> )	ACO09049.1	64
A-H1	529	1	GT916248	Putative 3'-UTR of HMGB3	-	-	FP885528	High-mobility group box 3, isoform CRA_b ( <i>Homo sapiens</i> )	EAW99398.1	70
A-H2	497	1	GT916249	Minichromosome maintenance complex component 6 ( <i>Bos taurus</i> )	NP_001039699.1	70	FP885532	Minichromosome maintenance complex component 6 ( <i>Gallus gallus</i> )	NP_001006527.1	83
A-H3	470	1	GT916250	Putative 3'-UTR of cyclin I	-	-	FP885526	Cyclin I ( <i>Gallus gallus</i> )	XP_420590.1	64
A-H10	533	1	GT916251	Peptidylprolyl isomerase B (cyclophilin B) ( <i>Homo sapiens</i> )	CAG33110.1	88	FP885529	Peptidylprolyl isomerase B (cyclophilin B) ( <i>Homo sapiens</i> )	CAG33110.1	89
C-A2	476	1	GT916252	Putative 5'-UTR of soggy	-	-	FP885829	Dickkopf-like 1 (soggy) ( <i>Bos taurus</i> )	NP_001039934.1	43
C-A3	237	35	GT916253	-	-	-	-	Full = protamine S4 ( <i>Scyllorhinus canicula</i> ) <sup>b</sup>	P30259.1	100
C-A4	426	1	GT916254	Similar to ring finger protein 152 ( <i>Equus caballus</i> )	XP_001489983.1	58	-	-	-	-
C-A5	405	7	GT916255	Sulfonylurea receptor 1 variant ( <i>Homo sapiens</i> )	BAD92321.1	46	-	-	-	-
C-A6	327	1	GT916256	Hypothetical protein ( <i>Canis familiaris</i> )	XP_854628.1	56	-	-	-	-

C-A7	262	3	GT916257	Full = spermatid-specific protein S2 ( <i>Scyliorhinus canicula</i> )	P11020.1	100	-	-	-	-
C-A9	141	2	GT916258	-	-	-	-	-	-	-
C-B7	231	2	GT916259	-	-	-	-	-	Full = protamine Z2 ( <i>Scyliorhinus canicula</i> ) <sup>b</sup>	100
C-B10	576	1	GT916260	Similar to spermatogenesis and centriole associated 1 ( <i>Homo sapiens</i> )	XP_001720789.1	57	-	-	-	-
C-C5	866	1	GT916261	Similar to hCG2042943 ( <i>Monodelphis domestica</i> )	XP_001374498.1	48	-	-	-	-
C-C11	700	1	GT916262	-	-	-	FP885527	-	F-box only protein 7 isoform 1 (predicted) ( <i>Callithrix jacchus</i> )	49
C-D11	329	1	GT916263	Calreticulin ( <i>Epatretus burgeri</i> )	BAA88476.1	60	-	-	-	-
C-D12	342	1	GT916264	-	-	-	FP885824	-	PREDICTED: similar to A-kinase anchor protein 5 isoform 3 ( <i>Macaca mulatta</i> )	64
C-E1	1030	1	GT916265	Fructose-bisphosphate aldolase B ( <i>Cephaloscyllium umbratile</i> )	BAD17932.1	97	FP885525	-	Fructose-bisphosphate aldolase B ( <i>Cephaloscyllium umbratile</i> )	99
C-F3	999	1	GT916266	Zinc finger protein 474 ( <i>Bos taurus</i> )	NP_001098482.1	53-64	-	-	-	-
C-F11	241	1	GT916267	Fructose-bisphosphate aldolase C ( <i>Scyliorhinus canicula</i> )	AAM93485.1	100	FP885826	-	Fructose-bisphosphate aldolase C ( <i>Scyliorhinus canicula</i> )	100
C-G8	446	3	GT916268	Full = protamine-Z1 ( <i>Scyliorhinus canicula</i> )	P08433.2	93	-	-	-	-
C-G9	336	1	GT916269	Phospholipase c-ζ ( <i>Coturnix japonica</i> )	BAF94336.1	52	-	-	-	-
C-G12	400	1	GT916270	-	-	-	-	-	-	-
C-H11	345	1	GT916271	-	-	-	-	-	-	-

<sup>a</sup>GenBank accession numbers. <sup>b</sup>Homology was identified with BlastP queried with putative ORF protein sequence.



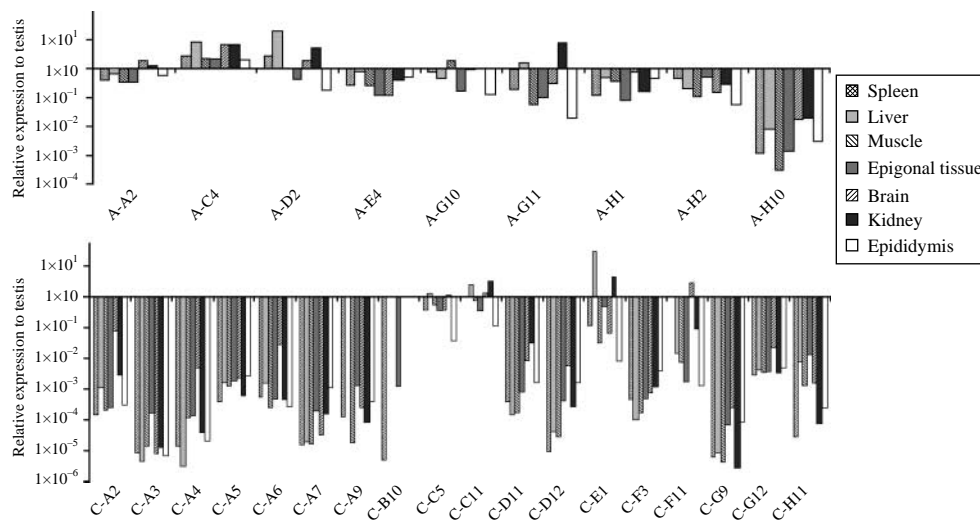
**Figure 1** Ratios of expression (determined by qPCR and normalized by 5S RNA) between zones B (spermatocytes, white bars), C (round spermatids, grey bars), or D (elongated spermatids, black bars) and zone A (spermatogonia) of dogfish testis, for clones identified in SSH libraries.

being specific to zones C and/or D, and specifically or predominantly expressed in testis (Fig. 3). For clones C-A2, -A3, -A4, -A6, -A9, -D12, -F3, -G9, and -H11, sense and antisense probes were synthesized from SSH clones and hybridized on transverse testicular sections showing germinative to spermiation zones. Hybridization with sense probes did not result in specific labeling, despite weak nonspecific background being observed for clones C-A3 and C-D12, and despite a labeling for clone C-G9 in zone A but not in zone C (Supplementary Figure 1, see section on supplementary data given at the end of this article). For the nine clones tested, a purple labeling was easily discernable at cyst level in zones C and/or D (Fig. 3A). Closer observation of labeled cysts (Fig. 3B) helped to define stage-specific expression which has been detected between stages XIa (early spermatids) and XVII (just before spermiation). These expression kinetics are summarized in Fig. 4. Only two clones were expressed throughout spermiogenesis (C-A4 and -F3). Two main expression patterns could be distinguished: clones C-A2, -A6 and -A9 were predominantly expressed in round spermatid stages (XIa, XIb, or XII), while clones C-A3, -A4, -D12, -F3, -G9, and -H11 displayed a maximum of expression in elongated spermatid stages later when bundles formed (stages XIV or XV). Observations at higher magnification allowed to precise in which cellular type the clones were expressed

(Fig. 3C). Particularly, stage XIV analysis was really informative as in this stage Sertoli cells nuclei are easily distinguishable near the basement membrane of the cyst. Eight clones (C-A2, -A3, -A6, -D12, -F3, -G9, and -H11) seemed to be expressed by germ cells, while only clone C-A4 seemed to be expressed by Sertoli cells. The cellular pattern of expression of clone C-A9 was somewhat unclear but seemed to be localized in Sertoli cells when stages XI and XII were observed, although a germ cell labeling was noticed in stages XIV and XV.

**Discussion**

In this study, the use of a SSH strategy has made it possible to identify genes differentially expressed during spermatogenesis in the dogfish. This approach was used for retrieval of genes previously known to be specifically expressed in testis, such as protamines, as well as for the identification of novel candidates. Among the genes identified in this study, nine appeared to be specifically transcribed during mitotic (zone A) and/or meiotic (zone B) stages, while the expression of 20 other genes was restricted to the post-meiotic phase in the zones of round (zone C) and/or elongated (zone D) spermatid stages. A limited variation of expression was observed for A-clones, and eight out of nine were ubiquitously expressed. In contrast, C-clones exhibited a marked



**Figure 2** Ratios of expression (determined by qPCR and normalized by 5S RNA) between spleen, liver, muscle, epigonal tissue, brain, kidney or epididymis, and testis in dogfish, for clones identified in SSH libraries.

**Table 2** Zone and tissue specificities identified by quantitative PCR for cDNA isolated by suppressive subtractive hybridization in dogfish testis.

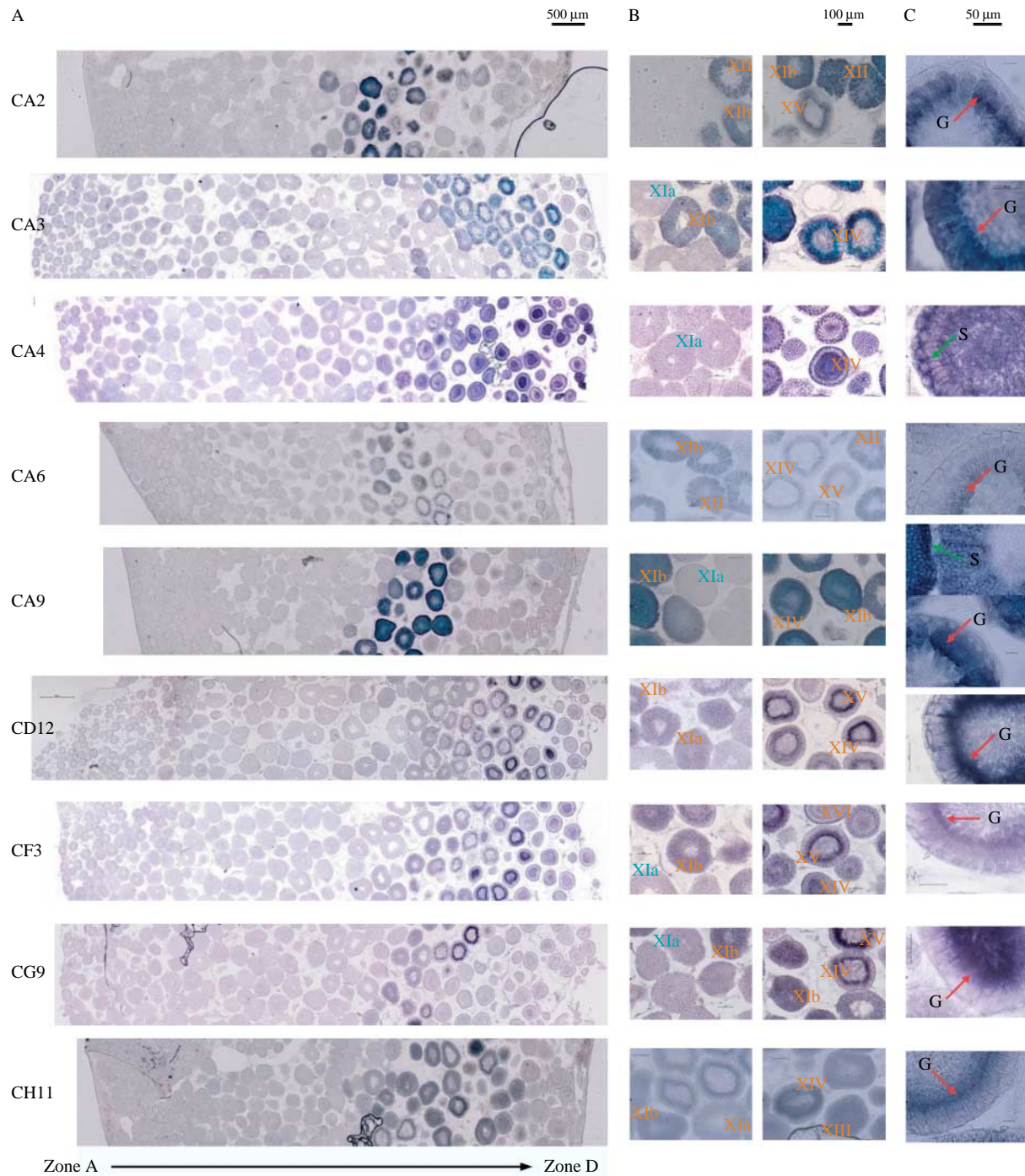
Clone	Zone specificity	Tissue specificity
A-A2	A	Ubiquitous
A-B4	Invalidated	Ubiquitous
A-C4	A-B	Ubiquitous
A-C7	Invalidated	Ubiquitous
A-D2	A-B	Ubiquitous
A-E4	A	Ubiquitous
A-G10	A	Ubiquitous
A-G11	A	Ubiquitous
A-H1	A	Ubiquitous
A-H2	A-B	Ubiquitous
A-H3	Invalidated	Not tested
A-H10	A-B	Testis predominant
C-A2	C	Testis predominant
C-A3	D	Testis specific
C-A4	C-D	Testis specific
C-A5	C	Testis predominant
C-A6	C-D	Testis predominant
C-A7	C	Testis specific
C-A9	D	Testis specific
C-B7	Not tested	Not tested
C-B10	C	Testis specific
C-C5	C	Ubiquitous
C-C11	C	Ubiquitous
C-D11	C-D	Testis predominant
C-D12	C-D	Testis specific
C-E1	C	Ubiquitous
C-F3	C	Testis specific
C-F11	C	Testis-brain predominant
C-G8	Not tested	Not tested
C-G9	C-D	Testis specific
C-G12	C-D	Testis predominant
C-H11	C-D	Testis predominant

increase in expression, starting from round spermatid stages, and 15 out of 20 of them (75%) were specifically or predominantly expressed in the testis. This high proportion of testis-specific genes in the post-meiotic cDNA was reported previously in mice by several investigators (Schultz *et al.* 2003, Shima *et al.* 2004, Hong *et al.* 2005), albeit to a lesser extent (from 11 to 20%). The enrichment of testis-specific clones in our study might result from the SSH technique used, which leads to the preferential isolation of stage-specific cDNAs and underselects genes involved in general aspects of spermatogenesis. More generally, expression profiles defined by qPCR revealed an important correlation between zones A and B on the one hand, and between zones C and D on the other hand. Such observations were also reported previously (Schlecht *et al.* 2004), and taken in its entirety the data suggests that an important expression reprogramming occurs between pre- and post-meiotic phases, in the dogfish and in osteichthyans.

Five cDNAs identified in the dogfish testis as specific to germinative and spermatogonia zone (zone A) were found to encode the ribosomal protein S8 (*Rps8*, A-E4), the adenine phosphoribosyltransferase (*Aprt*, A-G11), a putative untranslated region of *Hmgb3* (A-H1), the  $\alpha 1$  chain of procollagen (*Col1a1*, A-G10), and a polyprotein (A-A2). In mice, expression of *Rps8* mRNA was observed

previously in the pre-meiotic stages (Chan *et al.* 2006), but its role during the first steps of spermatogenesis remains to be clarified. The high temporal and spatial specificity of expression observed in the dogfish supports the hypothesis of an extraribosomal function in the spermatogonia zone, as suggested previously (Wool 1996, Naora 1999). In mice, *Aprt* showed high levels of expression in pachytene spermatocytes (Singer-Sam *et al.* 1990). By contrast, results presented here show expression restricted to the spermatogonia zone. *APRT* is usually considered to be ubiquitous, but its involvement in a purine salvage pathway might account for a higher expression in spermatogonial stages in which proliferative activity increases. Clone A-H1 was identified as *hmgb3*, based on strong similarity with the 3'-untranslated region of the gene known to be highly conserved in mice, humans, and chickens (Wilke *et al.* 1997). The HMG box subfamily is thought to play a fundamental role in DNA replication, nucleosome assembly, and transcription (Wilke *et al.* 1997), and HMGB2 is required for normal spermatogenesis and fertility, in mice at least (Ronfani *et al.* 2001). In addition, a role has been suggested for HMGB3 in the regulation between self-renewal and differentiation of hematopoietic stem cells in mice (Nemeth *et al.* 2006). In the dogfish, the high level of expression of *hmgb3* mRNA during the proliferative steps of spermatogenesis (zone A) suggests that HMGB3 is evolutionary conserved. Further analysis of the germinative zone could assess its potential function in the self-renewal of previously described spermatogonial stem cells (Loppion *et al.* 2008). Previous studies on the *Col1A1* gene, encoding the  $\alpha 1$  chain of procollagen I, showed a higher level of expression in immature than in adult mice testes, and the protein was immunolocalized in spermatogonia and preleptotene spermatocytes, but was not immunolocalized in Sertoli cells (He *et al.* 2005). It was therefore suggested that COL1A1 could play a major role in the regulation of the attachment of germ cells to the basal membrane and thus in their differentiation (He *et al.* 2005). The high expression observed for the clone A-G10, a putative homolog of COL1A1, in the dogfish testicular zone A is consistent with these observations and hypotheses. Finally, this approach also highlighted the pre-meiotic expression of a cDNA encoding a putative homolog of *Danio rerio* polyprotein. Currently, little is known about its expression and function, and its possible involvement during the first steps of spermatogenesis remains to be established.

Four additional clones displayed specificity for the mitotic and meiotic zones: A-C4, -D2, -H2, and -H10, putatively encoding ubiquitin carboxyl-terminal esterase L3 (*Uchl3*), 20 $\beta$ -HSD, minichromosome maintenance complex component 6 (*Mcm6*), and cyclophilin B (*Ppib*) respectively. UCHL3 belongs to the ubiquitin C-terminal hydrolases family, which are deubiquitinating enzymes. In the mouse, four UCH isoforms were

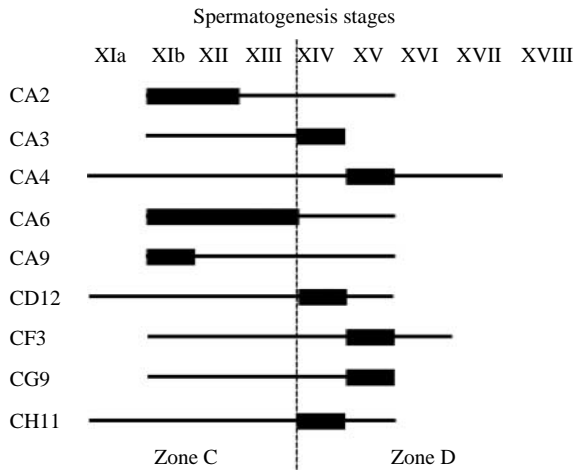


**Figure 3** Sections of dogfish testis after ISH with antisense RNA probes directed against cDNAs characterized following the SSH procedure. Clone numbers are indicated on the left. (A) Cysts with round and/or elongated spermatids were stained in purple on transverse sections from zones A to D. (B) Cysts were labeled (orange legend) or unlabeled (blue legend) between stages XIa and XVI (roman numerals) defined by Loir *et al.* (1995). (C) Germinal (G) or Sertolian (S) staining were observed on higher magnification views of labeled spermatocysts.

identified, with L1 and L3 being the prevailing forms. *Uchl1* expression is specific to the reproductive and neuronal systems, with expression in the testis restricted to spermatogonia, while *Uchl3* is expressed nearly in all tissues, including spermatocytes and spermatids (Kwon *et al.* 2004a). It has been proposed that UCHL1 and

UCHL3 were reciprocal modulators of apoptosis during spermatogenesis, and that UCHL3 may be involved in the differentiation of spermatocytes (Kwon *et al.* 2004a, 2004b, Kwon 2007). In the dogfish, *uchl3* seems to encompass the pattern of expression of both mouse isoforms, suggesting that in *S. canicula*, a single isoform,





**Figure 4** Representation of the range of expression of SSH clones during spermatogenesis between stages XIa and XVIII (Loir *et al.* 1995), defined following ISH with antisense RNA probes. Thin lines indicate expression, and thick lines represent strongest expression.

probably reflecting its ancestral state, may fulfill the different functions of the UCHL1 and -L3 in the mouse (Kwon 2007). A comprehensive analysis of the different UCH isoforms in the dogfish is needed to confirm this hypothesis. In actinopterygians, 20 $\beta$ -HSD is a major steroidogenic enzyme involved in the synthesis of 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP), a major progestin related to the meiosis resumption of oocytes (Nagahama & Yamashita 2008) and sperm maturation (Miura *et al.* 1991). Similarly, the highest DHP concentrations were found previously in isolated cysts with late spermatids in *S. canicula* (Sourdaine & Garnier 1993). However, in our present study, 20 $\beta$ -HSD cDNA was expressed in spermatogonia and spermatocytes zones. This is consistent with the reported 20 $\beta$ -HSD activity at an early stage of spermatogenesis in rainbow trout testis (Vizziano *et al.* 1996) and DHP's involvement in the initiation of meiosis in the Japanese eel (Miura *et al.* 2006). From these data, a lack of correlation between hormone levels and the level of expression of genes encoding steroidogenic enzymes is observed. Recently, in the sea bass, it was also shown that a higher expression of genes was involved in the mode of action or the synthesis of steroids during the early stages of spermatogenesis (Viñas & Piferrer 2008). MCM6 belongs to the highly conserved minichromosome maintenance complex involved in the initiation of replication (Maiorano *et al.* 2006, Costa & Onesti 2008). In the testis, specific expression of *Mcm2* in spermatogonia was reported in the mouse (Wu *et al.* 2004), and the MCM7 protein was detected in spermatogonia and primary spermatocytes in adult rats (Com *et al.* 2006). Likewise, the expression of a putative dogfish *mcm6* messenger prevails in the highly proliferative spermatogonia zone. Persistent expression observed in the spermatocyte zone suggests that MCM6, and more

generally the MCM complex, is also involved in the pre-meiotic DNA replication cycle, as reported previously in yeast (Lindner *et al.* 2002). Finally, clone A-H10, putatively encoding cyclophilin B, was specifically expressed in the pre-meiotic and meiotic stages. This is the first reported incidence of tissue- and stage-specific expression for *PPIB*, since cyclophilins are generally considered to be ubiquitous (Wang & Heitman 2005). Cyclophilins are the host cell receptors for the immunosuppressive drug cyclosporine A (Bukrinsky 2002), but a chaperone function has also been suggested (Barik 2006). In addition, cyclophilins belong to the immunophilins family, which are suggested to have roles in nervous system regeneration (Avramut & Achim 2003) and during meiosis in mice, rats, and chickens (Sananes *et al.* 1998, Crackower *et al.* 2003). The testis- and stage-specific expression observed for clone A-H10 suggests that *ppib* may be similarly involved in the differentiation of spermatogonia and/or meiotic stages.

Among the 20 clones identified as specific to spermiogenesis stages, three clones were found to encode protamines S4 (C-A3), Z1 (C-B7), and Z2 (C-G8), and one was found to encode a transition (or spermatid-specific S2) protein (C-A7) identified previously in *S. canicula* (Gusse *et al.* 1983, Chauviere *et al.* 1989). Transition proteins and protamines are involved in chromatin compaction during spermiogenesis, as these nucleoproteins in turn replace histones from the post-meiotic stages (Dadoune 2003, Balhorn 2007). Notably, these clones represented 45% of the C-clones tested, with a high predominance of protamine S4 (encoded by 35 clones: see Table 1), a very high relative abundance consistent with their roles during spermiogenesis.

Five additional clones were identified in this study, putatively encoding *Dkk1* (C-A2), a spermatogenesis- and centriole-associated protein (C-B10), calreticulin (Calr, C-D11), zinc finger protein 474 (Zfp474, C-F3), and phospholipase C- $\zeta$  (Plcz1, C-G9), all were previously known to be expressed during spermatogenesis. *Dkk1* mRNA expression was reported previously to be testis specific and restricted to the developing acrosome in mice (Kohn *et al.* 2005), an observation which is consistent with the specific expression of the *dkk1* messenger in zone C and at the rear of spermatid heads from stages XI to XV, the stages of acrosome formation in the dogfish (Loir *et al.* 1995). *DKKL1* was also recently proposed to regulate spermatocyte apoptosis and testosterone production (Dakhova *et al.* 2009). The spermatogenesis- and centriole-associated protein, also named speriolin, was shown to be testis specific and mainly expressed in spermatids in mice (Goto & Eddy 2004), findings which support our results. The spermatid specificity observed in our study suggests its involvement in the formation of the flagella rather than in the control of meiosis as suggested previously in mouse (Goto & Eddy 2004). Calreticulin fulfills many cellular functions,

including chaperone activity and regulation of  $\text{Ca}^{2+}$  homeostasis (Michalak *et al.* 1999). While calreticulin is considered ubiquitous in mammals (Michalak *et al.* 1999), we find here a predominant expression in testis with a significant but a reduced expression in kidney, brain, and epigonal tissue. In the dogfish testis, expression was found to be restricted for spermatids, while in the rat testis, mRNA was found to be expressed in the pre- and post-meiotic spermatogenic cells (Nakamura *et al.* 1993), and protein was located in the developing acrosome of spermatids (Nakamura *et al.* 1992). The tissue and stage specificities of calreticulin expression in the dogfish suggest a possible role in spermatozoon mobility and/or egg activation. PLCZ1 is a sperm-specific protein that triggers calcium oscillation and egg activation in mammals (Swann *et al.* 2006). In chickens, expression was detected from secondary spermatocytes to sperm (Rengaraj *et al.* 2008). In the dogfish, mRNA expression appeared limited to spermatids, especially between stages XIb and XV, although a protein expression in late spermatogenesis stages and sperm cannot be excluded. ZFP474 belongs to the superfamily of zinc finger transcription factors. Although its role is still unknown, this gene was described as specifically expressed in the testis and ovary in the mouse (Zhou *et al.* 2005). Similarly, we find a testis-specific expression of its homolog in the dogfish. Zfp474 was also shown to be expressed in mouse cilia cells (McClintock *et al.* 2008). Since cilia and flagella are structurally similar, ZFP474 may play a regulatory role in flagella formation or more largely in mobility function, a notion consistent with the predominant expression observed by ISH at stage XV, during which the head posterior periplagellum forms (Mellinger 1965).

Two clones encoding fructose-bisphosphate aldolases B (Aldob, C-E1) and C (Aldoc, C-F11) were also identified as being specific to zone C of dogfish spermatogenesis. In mice and rat, glycolytic isozymes specific to germ cell were reported in testes (Eddy 2002, Schultz *et al.* 2003, Wu *et al.* 2004), for example the spermatid-specific aldolase A isozymes (Vemuganti *et al.* 2007). In our study, expression for Aldob was ubiquitous, and the expression for Aldoc was specific to brain and testes. Their expression in the late stages of spermatogenesis in the dogfish is consistent with their involvement in the glycolytic activity of sperm, which is required for sperm mobility and successful fertilization in rodents (Eddy 2002).

A new finding was the identification of three clones in the testis never described before. Sequence annotations suggest that these clones respectively encode the ring finger protein 152 (Rnf152, C-A4), the F-box only protein 7 (Fbxo7, C-C11), and the A-kinase anchor protein 5 (Akap5, C-D12). However, the identification of clone C-D12 as an *akap5* encoding cDNA should be taken cautiously since it relies on a relatively low identity level over a short sequence. AKAP5, also named

AKAP79/150 coordinates the subcellular localization of second-messenger-regulated enzymes (Hoshi *et al.* 2005, Beene & Scott 2007), and it has been shown that it controls the uterine contraction in the rat (Dodge & Scott 2000) or the  $\text{Ca}^{2+}$  channels in the *Xenopus* oocytes (Plata *et al.* 2004). While expression of AKAP5 was never described during spermatogenesis, other AKAP proteins (AKAP4 and AKAP110) were identified previously as testis and spermatid specific (Vijayaraghavan *et al.* 1999, Schultz *et al.* 2003, Schlecht *et al.* 2004) and involved in sperm quality (Miki *et al.* 2002, Hu *et al.* 2009). The expression of the messenger identified here was testis specific and restricted to the spermatids zone. Its involvement in germ cell quality requires further analysis. RNF152 belongs to the large family of ring finger proteins that simultaneously possess a zinc finger domain and an ubiquitin ligase activity, and expression of its dogfish homolog in Sertoli cells (stages XIa–XVII) would suggest a role in regulating the Sertoli cell function during spermiogenesis. Finally, a putative Fbxo7 encoding clone was identified as expressed in round spermatids. F-box proteins are part of the SCF (Skp1-Cul1-Fbox) complexes, a large class of E3 ubiquitin ligases involved in phosphorylation-directed proteolysis and in many different processes including cell cycle, apoptosis, and signal transduction (Craig & Tyers 1999, Chang *et al.* 2006). More generally, the identification of the two clones, C-A4 and C-C11, putatively encoding proteins linked to E3 ubiquitin ligase activity stresses the importance of protein degradation by the 26S proteasome during spermiogenesis (Sutovsky 2003) and suggests finely tuned regulatory mechanisms involving at least two distinct classes of ubiquitin ligases.

One clone putatively encoding a sulfonylurea receptor (C-A5) was also identified, and found preferentially expressed in testes and round spermatids. Because *S. canicula*, like other elasmobranchs, accumulates important amounts of urea (Smith 1936), this receptor could possibly be involved in the regulation of osmolarity.

Finally, no clear annotation or function could be proposed for five transcripts (C-A6, -A9, -C5, -G12, and -H11). Four of them were found specifically or predominantly expressed in the testis, and their range of expression was precisely defined by qPCR and *in situ* hybridization (ISH) experiments. Although no function could be proposed, the tissue and stage specificities suggested that these genes may play a role in spermiogenesis. Further explorations are needed to better characterize these clones.

In conclusion, this study provides the first molecular characterization of spermatogenesis in a chondrichthyan with a special focus on the pre-meiotic stages and spermiogenesis. Some of the factors identified, for instance those coding for protamines, Uchl3, Plcz1, or Dkk1 correspond to genes known to play key roles in spermatogenesis in other vertebrates, which validates our SSH-based experimental strategy. In other cases, our

work helps to refine the expression pattern of genes occurring during spermatogenesis that is previously shown to be involved in this process, such as *aprt*, *calr*, or *zfp474*, thereby raising novel hypotheses on their respective roles. Finally, this analysis leads to the identification of new candidate genes, whose expression characteristics suggest an involvement in spermatogenesis. We show, for the first time, testis-specific expression patterns for a number of genes, such as *mcm6*, *akap5*, *rnf152*, or *fbxo7*, which are known to play important molecular and cellular functions in other vertebrates, but whose involvement in spermatogenesis has not been thus far reported. Combined analyses of their functional characterization in model organisms and of their expression patterns in the dogfish testis suggest possible roles during spermatogenesis, providing accurate working hypotheses for further functional approaches.

Finally, it is worth noting that two dogfish characteristics were definite advantages in this study. The first one is related to the polarized organization of its testis, which allows precise definition of the stage-specific expression patterns of genes during spermatogenesis. The second one is related to its phylogenetic position which, through comparisons with all traditional vertebrate model organisms, makes it possible to identify conserved mechanisms likely to be ancestral in jawed vertebrates (Forest *et al.* 2007, Venkatesh *et al.* 2007). The combination of these two characteristics and the increasing accessibility of large-scale transcriptomic tools should renew the interest of this thus far largely neglected model in order to gain insight into the evolution of spermatogenesis in gnathostomes.

## Materials and Methods

### Animals

Mature male dogfish *S. canicula* ( $686 \pm 131$  g and  $59.5 \pm 3.1$  cm) were captured off Cherbourg (Manche, France), 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). The animals were killed by sectioning of the spinal cord and pithing (except when brain was to be collected). Testes, brain, kidneys, spleen, epididymis and pieces of liver, muscle, and epigonal tissue were removed and stored in ice-cold Gautron buffer (Loir & Sourdain 1994). Testicular slices, 2 mm thick, were further staged into zones A, B, C, and D under a stereomicroscope as described previously (Loir & Sourdain 1994). All organs and tissue zones were then snap frozen and stored at  $-80^{\circ}\text{C}$ . For *in situ* hybridization experiments, pieces of testes were directly collected in ice-cold 4% paraformaldehyde (w/v in  $1 \times$  PBS).

### RNA extraction and mRNA purification

For library construction, total RNA was extracted from 2 g of zones A and C of testes collected from six animals, according to Chomscynski & Sacchi (1987). Poly(A) RNA was prepared from

500  $\mu\text{g}$  of total RNA using Dynabeads mRNA purification kit as recommended (Invitrogen). For real-time PCR, performed independently for three animals, total RNA was extracted from 1 g of the four zones of testes as described by Chomscynski & Sacchi (1987). For the other tissues, RNA was extracted from 100 mg of tissue using TRI Reagent (Sigma) and purified with the NucleoSpin RNA II kit (Macherey-Nagel, Hoerd, France) according to the manufacturer instructions.

### Suppressive subtractive hybridization

A- and C-libraries were constructed from mRNA isolated from zone A and zone C of dogfish testes. SSH was carried out using the PCR-select cDNA subtraction kit (Clontech). Forward subtraction (A-cDNA as a tester and C-cDNA as a driver) was run to isolate genes whose expression was higher in the spermatogonia zone. The reverse subtraction was performed to disclose genes preferentially expressed in spermatids. To test the ligation efficiency, primers specific to *S. canicula* elongation factor were used (Supplementary Table 1, see section on supplementary data given at the end of this article) instead of the G3PDH primers provided by the manufacturer. After two rounds of PCR amplification to enrich for differentially expressed genes, forward and reverse subtracted cDNA were purified using the Wizard SV Gel and PCR Clean-up system (Promega), cloned into the T/A cloning pCR II TOPO vector (Invitrogen), and introduced into TOP10 *Escherichia coli* cells to constitute A-specific and C-specific libraries respectively. Colonies were grown on LB Agar plates containing 50  $\mu\text{g}/\text{ml}$  ampicillin, and complemented with X-Gal and IPTG.

For each library, 96 clones were randomly picked and grown overnight at  $37^{\circ}\text{C}$  and at 250 r.p.m. on 96-well culture plates in 100  $\mu\text{l}$  of  $\text{LB}^{\text{Amp}^+}$  medium. Clones were named according to their position on 96-well plates during these initial cultures. To amplify cDNA sequences, nested PCR was performed on 1  $\mu\text{l}$  of each clone culture using nested primer 1 and 2R provided by the manufacturer (Clontech) and GoTaq DNA polymerase (Promega). The following parameters were used: 1 min at  $95^{\circ}\text{C}$  and 23 cycles at  $95^{\circ}\text{C}$  for 30 s,  $68^{\circ}\text{C}$  for 1 min and  $71^{\circ}\text{C}$  for 2 min. After being checked on agarose gels, each PCR product was denatured in 0.3 M NaOH and spotted on duplicate on charged nylon membranes (GE Healthcare, Orsay, France). The filters were baked for 2 h at  $80^{\circ}\text{C}$ . Four dots were spotted for each of the 96 clones from the A-specific library, and four additional dots were spotted for each of the 96 clones from the C-specific library.

Probes were prepared from native and subtracted cDNA from zones A and C. Digoxigenin (DIG) labeling was performed by using the DIG DNA labeling kit (Roche), and probes were purified with MicroSpin G25 columns (GE Healthcare) as recommended by the manufacturer. After denaturation, 100 ng of each probe (subtracted or unsubtracted A- or C-cDNA) were hybridized on the dot blots of A- or C-specific clones. Hybridization was carried out overnight at  $68^{\circ}\text{C}$  in 5 ml of the following buffer:  $6 \times$  SSC,  $5 \times$  Denhardt's, 10% SDS w/v, 100  $\mu\text{g}/\text{ml}$  torula RNA and 0.6  $\mu\text{M}$  nested primer 1 and 2R to avoid nonspecific hybridization. After three thorough washes at  $68^{\circ}\text{C}$  with  $2 \times$  SSC–0.5% SDS and  $2 \times$  SSC–0.2% SDS solutions, an immunological detection

was performed using an anti-DIG antibody coupled to the alkaline phosphatase (DIG Nucleic acid detection kit, Roche). Membranes were then rinsed, and images were captured with a transilluminator apparatus (GelDoc2000, Bio-Rad). A-specific clones displaying a strong signal with subtracted A-cDNA probe and possibly unsubtracted A-cDNA probe, but not with subtracted and unsubtracted C-cDNA probes, were selected. C-specific clones were similarly screened. Glycerol stocks of positive clones were conserved at  $-20^{\circ}\text{C}$ .

The selected positive clones were sequenced by means of the service of Cogenics Genome Express (Meylan, France) using universal forward and reverse primers. The datasets obtained were edited with Vector NTI (Invitrogen) to resolve sequencing ambiguities and remove vector and primer sequences. After elimination of redundancy, the sequences of cDNA identified in this work were deposited in the database of expressed sequence tags (NCBI), and their accession numbers are listed in Table 1. The edited sequences were annotated by comparisons with the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), with the elephant shark genomic database (<http://blast.fugu-sg.org/>) as well as with an annotated database of embryonic and juvenile cDNAs generated through systematic high-throughput sequencing (Coolen *et al.* 2007). All comparisons were conducted using the BLASTX algorithm.

### RT and real-time quantitative PCR

To prevent genomic DNA contamination, a 30-min DNase I (Promega) treatment was carried out at  $37^{\circ}\text{C}$  with  $2\text{ U}/\mu\text{g}$  RNA. Total RNA was denatured for 10 min at  $70^{\circ}\text{C}$  in the presence of random hexamers ( $500\text{ ng}/\mu\text{g}$  RNA, Promega), and then RT was carried out for 1 h at  $37^{\circ}\text{C}$  with M-MLV RT ( $300\text{ U}/\mu\text{g}$  RNA, Promega), RNasin ( $50\text{ U}/\mu\text{l}$ , Promega), and  $500\text{ nM}$  dNTPs (Promega). RT was stopped by incubation at  $70^{\circ}\text{C}$  for 15 min. cDNA was prepared from  $3\text{ }\mu\text{g}$  of testis RNA extracts (zones A, B, C, and D) and from 4 to  $6\text{ }\mu\text{g}$  of the other tissues' RNA extracts.

Real-time PCR was performed on an iCycler apparatus (Bio-Rad) in 96-well plates. qPCR experiments were performed in triplicate on serial cDNA dilutions and repeated using cDNA from three different animals. qPCR consisted of  $5\text{ }\mu\text{l}$  of diluted cDNA,  $12.5\text{ }\mu\text{l}$  of Absolute QPCR SYBR Green Fluorescein Mix (AB Gene, Courtaboeuf, France) or MESA GREEN qPCR MasterMix (Eurogentec, Angers, France), and  $100\text{ nM}$  of specific forward and reverse primers in a final volume of  $25\text{ }\mu\text{l}$ . Cycling parameters were: 13 min at  $95^{\circ}\text{C}$ , and then 45 cycles at  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 45 s. Accurate amplifications of the target amplicons were checked by plotting melting curves at the end of PCR program (repeats with temperature decreasing by  $0.5^{\circ}\text{C}$  steps between  $55$  and  $95^{\circ}\text{C}$ ). Efficiencies of PCR were assessed with serial dilutions of cDNA. The threshold cycle values ( $C_t$ ) were determined with a baseline set manually at 160 relative fluorescence units. To adjust for variation in input cDNA, results were normalized against 5S RNA using the  $\Delta C_t$  method (Scheffe *et al.* 2006). The use of this reference gene was further validated by repeating the normalization with the house-keeping gene actin, which gave similar results (data not shown). Expression variations were calculated with the  $\Delta\Delta C_t$

method (Scheffe *et al.* 2006), relative to the normalized expression in zone A for the testes expression study and relative to zone A or zones C + D for the tissue expression study.

Primers for qPCR, specific to SSH clones, were designed to fulfill the following criteria: length of 20–22 bp, GC content over 50%,  $T_m$  close to  $60^{\circ}\text{C}$  and generation of a 75–150 bp amplicon. These primers, listed in Supplementary Table 1, were designed using the Primer3 web interface (<http://frodo.wi.mit.edu/>) and purchased from Eurogentec.

### In situ hybridization

Probes were synthesized from nine SSH clones, termed C-A2, C-A3, C-A4, C-A6, C-A9, C-D12, C-F3, C-G9.1, and C-H11. In each case, the cloned cDNA was amplified from  $1\text{ }\mu\text{l}$  of overnight LB-Amp cultures of SSH clones by PCR carried out in standard conditions with the universal primers M13 Fwd31 and Rev33 (Supplementary Table 1), and with GoTaq DNA polymerase (Promega). The amplification product was purified with the Wizard SV Gel and PCR Clean-up system (Promega) following the manufacturer instructions. Sense and antisense DIG-labeled RNA probes were synthesized from the amplified template by *in vitro* transcription with SP6 and T7 RNA polymerases (Promega) performed for 3 h at  $37^{\circ}\text{C}$  with  $3\text{ }\mu\text{l}$  purified PCR product,  $25\text{ U}$  RNA polymerase,  $25\text{ U}$  RNasin (Promega),  $1\text{ mM}$  ATP, CTP, GTP,  $0.35\text{ mM}$  UTP, and  $0.65\text{ mM}$  DIG-UTP (Roche). After DNase treatment for 30 min at  $37^{\circ}\text{C}$  with  $2\text{ U}$  DNase I (Promega), probes were purified with Probequant G50 columns (GE Healthcare) as recommended. To check labeling and quantify probes, serial dilutions were prepared from 1:1 to 1:10 000 in  $6\times\text{SSC}-20\%$  v/v formaldehyde 37% and spotted on positively charged nylon membranes (GE Healthcare). After baking (30 min,  $120^{\circ}\text{C}$ ), anti-DIG immunodetection was conducted as described below. Spot intensities were compared to results obtained for the labeled control RNA (Roche).

Testicular sections of dogfish were fixed at  $4^{\circ}\text{C}$  in 4% paraformaldehyde (w/v in  $1\times\text{PBS}$ ) for 24 h. Sections were then dehydrated in 100% methanol and conserved for several months at  $-80^{\circ}\text{C}$ . Before mounting, testis sections were rehydrated in PBS, cryoprotected for 12 h at  $4^{\circ}\text{C}$  in 30% sucrose (w/v in  $1\times\text{PBS}$ ). Testis sections were embedded in OCT compound (VWR, Fontenay sous Bois, France) and snap frozen in isopentane pre-cooled in liquid nitrogen. Tissue blocks were stored at  $-80^{\circ}\text{C}$  for several weeks. Cryosections,  $12\text{ }\mu\text{m}$  thick, were cut at  $-20^{\circ}\text{C}$ , collected on Superfrost Plus slides (VWR), and submitted to *in situ* hybridization as follows.

Hybridizations with sense and antisense RNA probes were performed on two slides in parallel. Sections were rinsed in  $1\times\text{PBS}$  for 2 min, post-fixed in 4% PFA (w/v) for 15 min, and rinsed thoroughly in  $1\times\text{PBS}$  ( $2\times 5\text{ min}$ ) and  $150\text{ mM}$  NaCl (5 min). Hybridization frames (ThermoScientific, Courtaboeuf, France) were fixed on the slides and pre-hybridization was performed with hybridization solution (50% v/v deionized formamide,  $2\times\text{SSC}$ ,  $5\text{ mM}$  EDTA pH 8, 0.1% v/v Tween 20,  $1\times$  Denhardt's,  $0.1\text{ mg/ml}$  heparin, 0.1% w/v CHAPS, and  $0.5\text{ mg/ml}$  tRNA) for 1 h at  $70^{\circ}\text{C}$  in a humid chamber. Two microlitres of probe were denatured for 5 min at  $80^{\circ}\text{C}$ . Slides were hybridized overnight with  $150\text{ }\mu\text{l}$  hybridization solution

containing denatured probes at 70 °C in a humid chamber. Slides were washed in 1×SSC (2×10 min, 70 °C), 0.2×SSC (2×10 min, 70 °C), and 2×SSC (2×20 min, 37 °C). After RNase A treatment (0.2 mg/ml, Roche) for 30 min at 37 °C, washes were performed in 2×SSC (10 min, RT) and 0.2×SSC (2×25 min, 55 °C).

Slides were incubated for 2×15 min in 1×MABT (0.1 M maleic acid, 0.3 M NaCl, and 0.5% v/v Tween 20), and were then blocked for 3 h with blocking solution (2×blocking reagent, Roche; 20% v/v fetal bovine serum, Sigma; and 1×MABT). Anti-DIG antibody coupled with alkaline phosphatase (Roche, 1:5000) was added to the slides and incubated overnight at 4 °C. Unbound antibodies were removed by washing for 2×5 min and 5×20 min in 1×MABT. Sections were equilibrated for 2×15 min in NTMT buffer (0.1 M NaCl, 0.1 M Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>, and 0.1% v/v Tween 20) and incubated with alkaline phosphatase substrate, i.e. BM-purple solution (Roche), until purple staining was satisfactory (from 3 to 21 h). Slides were observed, and images were acquired through Nikon Eclipse 80i microscope connected to Nikon NIS Elements D software (Nikon, Champigny-sur-Marne, France).

## Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0021>.

## 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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