Maintenance of potential spermatogonial stem cells in vitro by GDNF treatment in a chondrichthyan model (*Scyliorhinus canicula* L.)

**Running title:** In vitro maintenance of dogfish spermatogonia

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1Supported by the “Conseil Régional de Basse-Normandie” and “Fonds Européen de Développement Régional” (PEPTISAN project certified by the “Pole Mer Bretagne” cluster). A.B. PhD studies are supported by ANRT and Kelia. The dogfish artemin cDNA sequence has been submitted to GenBank under accession number KJ877674.

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**ABSTRACT**

Previous work on dogfish *Scyliorhinus canicula* has identified the testicular germinative area as the spermatogonial stem cell niche. In the present study, an in vitro co-culture system of spermatogonia and somatic cells from the germinative area has been developed. Long term maintenance of spermatogonia has been successful and addition of GDNF has promoted the development of clones of spermatogonia expressing stem-cells characteristics such as alkaline phosphatase activity, and has allowed to maintain self-renewal of spermatogonia for at least five months under culture conditions, notably by decreasing cell apoptosis. Furthermore, clones of spermatogonia expressed the receptor of GDNF, GFRalpha1, which is consistent with the effect of GDNF on cells despite the lack of identification of a GDNF sequence in the dogfish’s transcriptome. However, a sequence homologous to artemin has been identified and in silico analysis supports the hypothesis that artemin could replace GDNF in the germinative area in dogfish. This study, as the first report on long-term in vitro maintenance of spermatogonia in a chondrichthyan species, suggests that the GFRalpha1 signaling function in self-renewal of spermatogonial stem cells is probably conserved in Gnathostomes.

**Summary sentence:** In a chondrichthyan model, the spermatogonia contained in the testicular germinative area were maintained in culture in vitro and showed an increased colony formation, stemness marker expression and a lower apoptosis level in response to GDNF.

**Keywords:** Spermatogonial stem cells, culture, self-renewal, dogfish, GDNF

**INTRODUCTION**

Spermatogonial stem cells (SSCs) are crucial for the maintenance of a continuous spermatogenesis and for the transmission of the genetic information to the next generation. SSCs are the origin of the different lineages which are engaged in successive differentiation pathways resulting in spermatozoa production. They are specifically regulated in a close micro-
environment where they are in close proximity with Sertoli cells. In Invertebrates as well as in Vertebrates, the SSCs pool is maintained by symmetric divisions, asymmetric divisions and the reversion which consists of the return of engaged spermatogonia to the stem state after testicular injuries. Those mechanisms were well described in drosophila [1] and rodents, for which this reversion is called fragmentation [2].

In order to identify the various spermatogonial generations, morphologic characteristics were first defined in various species such as rodents [3], Teleosts such as zebrafish [4] or Nile tilapia [5], and Chondrichthyes such as dogfish [6]. Next, the search for molecular markers to improve the identification of spermatogonial subpopulations and potential SSCs has been considerably increased these last years. For example, the GFRα1 (GDNF Family Receptor α1) has been described as a marker of undifferentiated spermatogonia in mouse [7], equids [8], buffalo [9] or collared peccary [10] and more recently in fishes such as the Nile tilapia [5]. Other undifferentiated spermatogonial markers were identified in various species such as the cell surface marker Thy1 (Thymus cell antigen 1) in mouse [11], bull [12] and carp [13] or the following transcriptional factors: PLZF (Promyelocytic Leukaemia Zinc Finger protein) in mouse [7], equids [8] and zebrafish [14], POU2/POU5F1 in mouse [15], buffalo [9] and carp [13] and ID4 (Inhibitor of DNA binding 4), a more selective marker of SSCs than Thy1, in the mouse [16]. Some of these defined markers were used to improve in vitro culture systems by selecting undifferentiated spermatogonia from the testicular cell population, including potential SSCs, using the MACS technology (Magnetic-Activated Cell Sorting) in mammals as well as in Teleosts. For example, Thy1 has been largely used in various species such as bull where it allowed the selection of spermatogonia containing 80% of PLZF+ spermatogonia [12] and carp where 60% of selected cells corresponded to Pou2/Pou5f1+ spermatogonia [13]. The development of in vitro culture systems allowed a finer characterization of SSCs and highlighted their regulation. In rodents, SSCs were maintained for several weeks on an inactive feeder cells layer (STO) with a culture media containing 10% of FBS (Fetal Bovine Serum) and a cocktail of various growth factors like GDNF (Glial cell Derived Neurotrophic Factor), LIF (Leukemia Inhibitory Factor), EGF (Epidermal Growth Factor), FGF-2 (Fibroblast Growth Factor 2), IGF-1 (Insulin-like Growth Factor 1) and SCF (Stem Cell Factor) [17,18]. More recently, a culture system without feeder cells, with only 1% of FBS and containing less growth factors (GDNF, FGF-2 and EGF) was proposed for the maintenance of mouse spermatogonia proliferation during several months [19]. In a teleostean fish, the carp, spermatogonial cells were maintained in vitro for two months with serum and GDNF but without feeder cells [13]. Most culture systems were developed using GDNF because this Sertolian factor was shown to regulate the stem fate of undifferentiated spermatogonia in mammals [20].

In addition to the expression of the protein markers previously mentioned, SSCs were characterized in vitro by their ability to form colonies [21] and their stability in long-term culture. In medaka, a spermatogonial stem line (SG3) was maintained during two years [22]. The pluripotent fate of SSCs in culture was also demonstrated by their expression of phosphatase alkaline activity, like embryonic stem cells (ESC) [23], in mouse [24] and goat [25]. Finally, SSCs are the sole germ cells capable of colonization and re-establishing spermatogenesis in recipient testes. Transplantation experiments were used to detect SSCs in mouse [21] but also in fish models [5,26,27]. This way, SSCs stemness was demonstrated to be maintained after one month of culture in zebrafish [26] and Nile tilapia [5].

The dogfish, Scyliorhinus canicula, is a chondrichthyan model presenting several advantages to study spermatogenesis and SSCs. First, this cartilaginous fish is at a key phylogenetic position
for evolutionary studies concerning spermatogenesis [28]. Second, the linear spermatogenetic wave of the dogfish testis induced the polarization of the testis and so, the easy separation of different zones containing specific spermatogonial subpopulations like undifferentiated spermatogonia in the germinative zone. Third, the abundance of this species on our coast allowed the supply of necessary adult specimens, each one providing a large amount of biological material. The molecular characterization of dogfish spermatogonia was recently initiated by our research group. Dogfish differentiated spermatogonia expressed e-Kit receptor, like mammals differentiated spermatogonia [6] and mRNAs of classical undifferentiated spermatogonia markers in mammals like GFRα1, POU2/POU5F1 or PLZF were detected in dogfish undifferentiated spermatogonia in the germinative zone [29]. A culture medium has been previously defined for studying stage-related changes during spermatogenesis such as steroid production by isolated cysts during 12 hours [30] or DNA and protein syntheses by cysts and isolated Sertoli cells during a longer time culture of 15 days in another chondrichthyan, *Squalus acanthias* [31,32].

In this paper, our main goal was to maintain potential SSCs in culture in a chondrichthyes. Specific protein markers were determined for germinal and somatic subpopulations present in the germinative zone in order to characterize cells in vitro afterwards. As GDNF is the main factor known to maintain SSCs in vitro in mammals, the ability of GFRα1 to link human recombinant GDNF was studied in silico and revealed the conservation of most amino acids involved in this interaction. Surprisingly, no GDNF sequence was identified in dogfish testis suggesting its potential replacement by another GFRα1 family ligand. We took advantage of the organization of the dogfish testis to isolate and cultivate cells from the testicular germinative zone. Undifferentiated spermatogonia were enriched by the differential plating technique. The formation of colonies harboring alkaline phosphatase activity suggested the successful amplification of SSCs in vitro. In order to optimize this culture and to test the conservation of undifferentiated spermatogonia regulation through Vertebrates, dogfish spermatogonial culture was treated with human recombinant GDNF. This has improved the long-term maintenance of the culture and has induced, in a dose-dependent manner, protection against apoptosis, increase of the colony number and of alkaline phosphatase activity. This result suggests that undifferentiated spermatogonia may be maintained through GFRα1 signaling in Chondrichthyes like in Osteichthyes.

**MATERIALS AND METHODS**

**Animals**

Mature male dogfish *S. canicula* were captured off Cherbourg (Manche, France) using the facilities of the Lycée Maritime et Aquacole and stored in natural seawater tanks at the Centre de Recherches en Environnement Côtière (Luc sur Mer, France). Sharks were allowed to acclimate for at least 2 weeks before tissue sampling and were euthanized by sectioning of the spinal cord and pithing. Testes were sampled and directly transferred into Gautron’s buffer [33] complemented by 58 mM TMAO. The procedures used were in agreement with the SSR’s guidelines for care and use of animals. The personnel were trained and qualified for animal experimentations.

**Phylogenetic analysis and sequence alignment**

GFRα1, GDNF and ARTN protein sequences were collected from whole genome databases.
deposited at the EMBL or NCBI institutes. Additional ARTN sequences were selected using the TBLASTN algorithm and the rat ARTN sequence as a query, from the NCBI database to identify the medaka orthologous sequence, and from unpublished dogfish transcript libraries to identify dogfish ARTN sequence. Different dogfish transcript libraries were consulted: an embryonic (stages 9-25) and juvenile transcript bank [34,35] amended with new 454 data from a panel of adult tissues and kindly shared by S. Mazan and gonadal transcripts libraries generated by Illumina (PHYLOFISH project coordinated by J. Bobe and Y. Guiguen). A phylogenetic tree was constructed using the mature part of the GFLs (GDNF Family Ligands). This tree was built using the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.1 [36] with the Neighbor-Joining and Minimum Evolution methods. The reliability of the inferred trees was estimated using the bootstrap method with 1000 replications. In addition, protein sequences were aligned using the BioEdit sequence alignment editor software version 7.1.9. The GFRα1 domain D2 and the mature part of the GFLs were annotated on the base of previous studies [37,38].

Culture conditions
After decapsulation, testes were cut transversally into 2-mm slices. Then, the germinative area (A0) was taken from each slice and pooled in dissection buffer (TMAO complemented Gautron media [33], 60 mg/ml penicillin, 100 mg/ml Streptomycin, 250 µg/ml amphoterycin). Areas were then dissociated for 3 h under agitation at RT in disassociation media (dissection buffer, 0.1% collagenase (Sigma, C9891) and 0.05% DNAse, (Sigma, DN25)). Dissociated cells were then centrifuged for 2 min at 300 g. Pellets were resuspended in fresh complete media (50% dissection buffer / 50% L-15, 20 mM Hepes, 0.5% BSA, 1% FBS, 0.25% heat decomplemented dogfish serum, 4.6 mM D(+) Glucose, 280 mM NaCl, 330 mM Urea and 58 mM TMAO) and incubated overnight at 17°C. After gentle up and down pipetting and filtration through a 100 µm nylon filter, cells were centrifuged in the same conditions and resuspended in the culture media (L-15, 20 mM Hepes, 0.5% BSA, 1% FBS, 0.25% heat decomplemented dogfish serum, 4.6 mM D(+) Glucose, 280 mM NaCl, 330 mM Urea and 58 mM TMAO). A step of enrichment was next performed in order to select spermatogonia by differential plating [39]. Thus, cells were seeded in gelatin-coated 25 cm² flasks (0.5-1 x10⁶ cells/flask T25) and incubated 24 h at 17°C. After this step, supernatant (containing a majority of spermatogonia, presenting a low capacity of adherence) was removed from the flask (which mostly contained adherent somatic cells) and seeded in gelatin-coated 25 cm² flasks and gelatin-coated 24 wells dishes (0.5-5 x10⁵ cells/well) in order to perform in vitro studies. Cell cultures in flasks were then treated with culture media or culture media containing 10 ng/ml of recombinant human GDNF (PeproTech, 450-10) and then maintained for several months with a once-weekly partial change of medium (10-20% of volume) adjusted (treated cells) or not (control cells) with GDNF. Cell cultures in 24 wells dishes were treated with culture media containing 0, 1, 10 and 100 ng/ml GDNF and were maintained for 7 or 28 days with partial change of medium once a week. Gelatin coating consisted in covering culture surfaces with a sterilized 0.5% gelatin solution (in water) and allowing complete air-drying for a few hours before cells seeding.

Immunohistochemistry (IHC)
Five µm-thick testis sections were cut from paraffin blocks and disposed on polysine slides (Thermoscientific, J2800AMNZ). The slides were deparaffinated in two baths of roti-histol (Roth, 6640.2) and rehydrated by a series of ethanol dilutions (2x 100%, 95%, and 70%). After two washes in distilled water and antigen unmasking (2x 90 s micro-waves 600 W followed by 1
h RT cooling), endogenous peroxynase activity was blocked for 30 min at RT in presence of 3% hydrogen peroxide in PBS. Non-specific labeling was blocked by 30 min incubation with PBS-T (triton 0.1%) / 1% BSA. The first antibody, diluted in the blocking solution was incubated overnight at 4°C. After three washes, sections were incubated 2 h at RT with the secondary antibody. After rinses, DAB solution (Sigma, D4293) was applied until color developed (5-30 min). After washing, sections were counterstained for 1 min in hematoxylin and washed under running water. The slices were then dehydrated by successive ethanol baths and a roti-histol wash to be mounted in roti-histokit media. Images were taken using an eclipse 80i microscope equipped with NIS-Elements D 3.0 software (Nikon Instruments).

**Immunocytochemistry (ICC)**
The cells were washed twice with supplemented Gautron (1% FBS, 0.25% heat decomplemented dogfish serum), collected and centrifuged 2 min at 300 g and then fixed 15 min in PFA 4% and attached to slides by a cytopsin run of 1 min at 300 g. The cells were permeabilized for 5 min with 0.1% triton in Gautron with 1% serum and incubated overnight with primary antibodies at 4°C. Then, the cells were washed three times and incubated 2 h at RT with secondary antibodies. After washes, cover slides were set with antifade buffer containing DAPI. All observations and analyses were made using the eclipse 80i microscope equipped with NIS-Elements D 3.0 software (Nikon Instruments). The same primary antibodies were used for both IHC and ICC experiments. The following primary antibodies were used at the indicated dilutions: anti-PCNA (Proliferating Cell Nuclear Antigen) mouse monoclonal antibody (1:50, Invitrogen, 13-3900); anti-human α- and β-spectrins monoclonal mouse antibody (1:50, SB-SP2, Tebu-bio), anti-human GFRα1 rabbit polyclonal antibody (1:100, Abcam, ab84106), anti-human PLZF (N-21) goat polyclonal antibody (1:100, Santa Cruz Biotechnology, sc-11146), anti-human c-KIT rabbit polyclonal antibody (1:50, Santa Cruz Biotechnology, sc-168); anti-human ERα (Estrogen Receptor, H-184) rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology, sc-7207), anti-Artemin (C-18) goat polyclonal antibody (1:50-IHC, Santa Cruz Biotechnology, sc-9330). The following secondary antibodies were used at the indicated dilutions: anti-mouse IgG HRP-conjugated rabbit polyclonal antibody (1:500, DAKO, P0260); anti-rabbit IgG HRP-conjugated swine polyclonal antibody (1:100-IHC, 1:500-ICC, DAKO, P0399); anti-goat IgG HRP-conjugated rabbit polyclonal antibody (1:200-IHC, 1:500-ICC, Sigma, A5420), anti-mouse IgG AlexaFluor 488-conjugated goat antibody (1:250, Invitrogen, A11001); anti-rabbit IgG AlexaFluor 594-conjugated goat antibody (1:250, Invitrogen, A11012); anti-goat IgG PE-conjugated rabbit antibody (1:250, Santa cruz, sc-3755).

**Protein extraction and Western-blot**
Proteins were extracted from a panel of fresh dogfish tissues (epigonal tissue, epididymis, whole testis, spleen, and brain). Tissue samples were homogenized on ice using conical pestles in 1 ml of lysis buffer: HEPES 20 mM pH 7.5, EDTA 1 mM, DTT 0.5 mM and anti-protease mix (AEBSF 1 mM, E64 10µM). Samples were sonicated three times 5 s with a pause of 30 s between each and centrifuged 30 min at 14 000 g, 4°C. Proteins (50 µg) from the supernatant of each sample were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (GE Healthcare, Orsay, France). After a blocking step of 1 h in 3% BSA (PBS) at RT, membranes were incubated overnight in primary antibodies dilutions (anti-Actin 1:500, anti-GFRα1 1:400, anti-Artemin 1:50, anti-PLZF 1:200 and anti-ERα 1:200). After 5 washes of 5 min, membranes were incubated during 1 h 30 at RT with the acute
secondary antibody: anti-mouse IgG HRP-conjugated goat antibody (1:5000, ThermoFisher Scientific, 31430), anti-goat IgG HRP-conjugated rabbit polyclonal antibody (1:1000, Sigma, A5420) or anti-rabbit IgG HRP-conjugated swine polyclonal antibody (1:1000, DAKO, P0399). After washes, peroxidase reaction was developed with DAB solution (Sigma, D4293). Actin protein detection was used as loading control.

Alkaline phosphatase activity detection
The cells were washed twice with supplemented Gautron (1% FBS, 0.25% heat decomplemented dogfish serum) then fixed for 15 min in PFA 4%. Cells with alkaline phosphatase activity were directly stained by incubation for 15 min in detection buffer (100 mM tris pH9.5, 100 mM NaCl, 2% NBT-BCIP). Cells were finally washed and observations and analyses were made with a Leica DM IRB inverted microscope and the Leica Application Suite software V4.1.

Viability (trypan blue) and apoptosis (TUNEL) assays
The cells were washed twice with supplemented Gautron (1% FBS, and 0.25% heat decomplemented dogfish serum), collected and centrifuged 5 min at 300g. The cells reserved for viability assay were resuspended in 0.04% trypan blue solution and analyses were directly made with a Leica DM IRB inverted microscope and the Leica Application Suite software V4.1. The cells reserved for the TUNEL assay (Roche, 11684795910) were fixed 15 min in 4% PFA, attached to slides by a cytopsin run of 1 min at 300g, washed, permeabilized for 5 min with 0.1% Na-citrate solution containing 0.1% Triton-X100 in PBS and then incubated for 1 h with the kit label and enzyme solutions in obscurity conditions. The cells were then washed and cover slides were set with antifade buffer including DAPI. All observations and analyses were made using an eclipse 80i microscope equipped with NIS-Elements D 3.0 software (Nikon Instruments).

RESULTS

Artemin as potential ligand for GFRα1 in dogfish testis
An in silico study was preliminarily conducted in order to assess if dogfish spermatogonia could be regulated by the GDNF/GFRα1 signaling. We aligned the domain D2 of GFRα1 protein sequences from selected species and showed its conservation from shark to tetrapods (Fig. 1A). In addition, this alignment underlines the ancestral character of the chondrichthyan S. canicula among Vertebrates. Indeed, like tetrapods, dogfish GFRα1 sequence presents an aspartic acid (D) in spite of an asparagine (N) in position 5 as well as the RD residues in positions 70-71 in spite of a 5 amino acids motif for teleostean sequences. On contrary, dogfish GFRα1 sequence shows similarities with Teleosts with AS amino acids in spite of N at position 33. The ten cysteines potentially involved in disulfide bonds are conserved in dogfish as well as in the other species. Moreover, sixteen out of the 17 GFRα1 residues involved in the ligand-receptor interface in mammals are conserved in dogfish and Teleosts. The single difference consisted in a serine (S) or asparagine (N) in spite of a threonine (T) in position 25. Surprisingly, GDNF sequence was neither found in dogfish transcript libraries, nor in libraries generated from embryos, juveniles and a panel of adult tissues, nor in libraries newly generated by Illumina RNA sequencing from male and female gonads, and in particular the library generated from the testis germative area (the dogfish genome is not yet available, consequently, synteny analyses cannot be performed to demonstrate the absence of GDNF in dogfish and it is not possible to exclude its expression in other adult tissues). In contrast, the artemin (ARTN), another GDNF family ligand (GFL), was
retrieved from library generated from the testis germinative area. The orthology relationship of the only GFL found to be expressed in the germinative area with the artemin has been confirmed by a phylogenetic analysis (Fig. 2). Curiously, the ARTN protein sequences of two tetrapod species segregate with the dogfish sequence at the root of vertebrates’ ARTN sequences. The green sea turtle (*Chelonia mydas*) and anole lizard (*Anolis carolinensis*) ARTN sequences diverge from the sequence of another sauropsida species included in this tree, the chicken (*Gallus gallus*). More precisely, the mature part of GDNF and ARTN sequences were aligned and compared. They present the same conserved cysteines involved in disulfide bonds. Those are also conserved in dogfish ARTN (Fig. 1B). Three GFL residues are hypothesized to form the core binding epitopes for GFL interactions with the receptors: E24, Y88 and L81. E24 is conserved between GDNF and ARTN, GDNF Y88 is substituted by W in ARTN, and, interestingly, the GDNF L81, substituted by M in ARTN in most species presented, is conserved in ARTN in dogfish, xenopus, green sea turtle and anole lizard. Concerning the three GFL residues hypothesized to determine the specificity of the interaction with the receptors, E25 is substituted by various residues in ARTN (L, S, I, T), I27 is substituted by R in mammals but by the close residue L in dogfish, reptiles and Teleosts. Similarly, D76 is substituted by various residues in ARTN with the exception of dogfish, green sea turtle and anole lizard which present the same residue as GDNF. In conclusion, the dogfish ARTN sequence was the only GFL sequence found in the transcriptomic library produced from dogfish testicular germinative area. Additionally, the dogfish ARTN presents some amino acid substitutions, making it closely related to GDNF, at key positions for the interaction with the receptor.

**GFRα1, ARTN, PLZF, c-Kit and ERα as dogfish testicular cells markers**

Spermatogonial and somatic protein markers were defined in order to identify these cells in culture later. As the antibodies used were produced against mammalian targets, the homology between these antigen sequences and those of dogfish homologs were calculated using BioEdit software and BLOSUM62 matrix. Identity and similarity are respectively 74.5% and 89.2% for GFR alpha 1 sequences, 95.2% and 100% for PLZF sequences, 44.3% and 61.4% for ER alpha sequences, 50% and 77.8% for ARTN sequences. The specificity of recognition of dogfish homologs was validated by Western-blot using a panel of dogfish tissue extracts. GFR alpha 1, PLZF and ARTN proteins were detected in testis and brain at the expected molecular weights (54, 74 and 21 kDa respectively) (new Fig. 3). ER alpha (64 kDa) was slightly detected in epididymis, testis and spleen extracts (Fig. 3). The specificity of the c-Kit antibody for the dogfish protein had been checked by Western-blot in a previous work [6]. The cellular expression pattern of all these markers was determined by immunohistocchemistry on dogfish sections. GFRα1 was highly expressed in all undifferentiated spermatogonia (Fig. 4 A1-A3, stages I-II), in differentiating spermatogonia (stage III, Fig. 4 A4 and stage IV, data not shown) and it drastically decreased during the last spermatogonial divisions till to be no more expressed in primary spermatocytes (Fig. 4 A5). As expected, no staining was detected in somatic cells. ARTN was highly expressed in spermatogonia (Fig. 4 B1-B4), maintained in primary spermatocytes and not detected in secondary spermatocytes (Fig. 4 B5). PLZF was detected in undifferentiated spermatogonia in and out the germinative area (Fig. 4 C1-C3), in proliferating spermatogonia (Fig. 4 C4) and strongly decreased thereafter (Fig. 4 C5). However, undifferentiated spermatogonia appeared with a more intense perinuclear staining (Fig. 4 C2-C3) than more advanced germ cells in which the cytoplasm exhibits a dispersed and lighter staining (Fig. 4 C4). PLZF seems not expressed in all somatic cells even though a doubt persists.
concerning early Sertoli cells of the germinative area (Fig. 4 C2). C-Kit was expressed in the cytoplasm of some undifferentiated spermatogonia (Fig. 4 D1-D2), in all differentiated spermatogonia (Fig. 4 D4), and at later spermatogenic stages (Fig. 4 D5). ERα was expressed in all somatic cells except in Sertoli cells at all stages of spermatogenesis (Fig. 4 E1-E5).

**Establishment of a dogfish spermatogonial culture**

Taking into account the advantage of the polarization of the dogfish cystic testis, the germinative zone A0, including potential SSCs, undifferentiated spermatogonia and somatic cells, was easily dissociated from the whole testis (Fig. 5A). The germinative zone samples were collected, dissociated and seeded in a gelatin-coated flask for enrichment of spermatogonia by differential plating. In our co-culture system, this method allowed the enrichment of spermatogonia as expected. At day 3 post-enrichment, the enriched fraction contained fewer adherent somatic cells and more large germinal cells forming colonies (Fig. 5C) in comparison to the adherent fraction (Fig. 5B). Without enrichment step, the germinal culture has been maintained no more than 3 weeks because of the excessive expansion of the somatic layer. With an enrichment step, the culture was maintained at most 3 months and colonies were observed during this period as shown for day 7 and day 28 (Fig. 5D, 5E) but the culture declined after the third month as shown for the fourth month (Fig. 5F). GDNF treatments were applied to improve the long-term maintenance and the quality of the culture. With an enrichment step and a GDNF treatment per week (10 ng/ml), the culture could be maintained more than 5 months. For example, colonies were still observable in GDNF-treated culture (Fig. 5G) contrary to the untreated culture (Fig. 5F).

In order to illustrate the cell composition of colonies, cell markers were analyzed by ICC on cells collected by cytopsin after 90 days of culture with 10ng/ml of GDNF (Fig. 6). Round cells appeared positive or negative for GFRα1 and these two types of potential SSCs appeared also positive for PCNA (Fig. 6A). Immunocytofluorescence detection of α-spectrin, underlining the presence of a potential spectrosome, allowed to distinguish GFRα1 positive cells in spectrosome positive and negative subpopulations (Fig. 6B). We also showed that c-KIT positive cells were spectrosome-negative whereas c-KIT negative cells were spectrosome-positive (Fig. 6C). PLZF-positive germinative cells were also detected (Fig. 6D). Finally, the detection of ERα allowed to discriminate germinal somatic cells from germ cells (Fig. 6E).

**GDNF induced dose-dependent protection against apoptosis of cultured dogfish spermatogonial cells.**

Cell apoptosis and viability were investigated in vitro at day 7 and 28 post-enrichment in control culture (GDNF: 0 ng/ml) and in GDNF-treated cultures (1, 10 and 100 ng/ml) using the TUNEL and blue trypan methods respectively. At day 7, there was no significant difference between the untreated control and the lowest dose of GDNF applied (1 ng/ml of GDNF) concerning cell apoptosis (67% and 65%, Fig. 7E) and viability (21% and 27%, Fig. 7F). In contrast, higher doses of GDNF (10 and 100 ng/ml) generated a significant decrease of apoptosis (39% and 27% respectively) and increase of viability (40% and 54% respectively) in a dose-dependent manner (Fig. 7E, 7F). At day 28, these differences between each conditions were amplified as the 100 ng/ml GDNF-treated culture presented a cell apoptosis level corresponding to the third of the untreated control level (24% versus 70%) and viability was increased by three (27% versus 79%). The positive impact of GDNF on cell viability and apoptosis was significant from the lowest dose 1 ng/ml (Fig. 7G-7L). In conclusion, GDNF improved the viability of dogfish’s
spermatogonia by reducing their apoptosis, notably during the long-term culture.

**GDNF promoted the formation of colonies of undifferentiated spermatogonia**

After 3 days of culture, cells started to form colonies. In order to study their formation and cell-content, morphometric parameters and alkaline phosphatase (AP) activity were investigated in vitro in control and GDNF-treated cultures (1, 10 and 100 ng/ml) at day 7 and 28 post-enrichment (Fig. 8). At day 7, the number of colonies per mm² doubled in 10 ng/ml GDNF-treated cells and tripled in 100 ng/ml GDNF-treated cells comparatively to control (respectively 4.5, 6.2 and 1.9, Fig. 8A). At day 28, the number of colonies significantly increased in all conditions when compared to day 7 results and GDNF treatment significantly increased the number of colonies at 10 and 100 ng/ml (x1.7 and x2.3 respectively). Colony size also showed differences between culture conditions and duration (Fig. 8B). In culture without GDNF, 100% and 95% of the colonies were smaller than 100 µm at day 7 and 28, respectively (Fig. 8B). In culture supplemented with GDNF, the sizes of colonies significantly increased according to the concentration of GDNF. Indeed, when cells were treated with 1 ng/ml of GDNF 15% of colonies were larger than 100 µm at days 7 and 28, whereas they represented more than 50% of colonies in culture with the highest GDNF concentration (100 ng/ml) (Fig. 8B). In culture with 10 or 100 ng/ml of GDNF, the presence of large colonies (more than 250 µm) was also reported for 10-25% of these colonies (Fig. 8B).

Differences in cell AP activity were observed between the control and GDNF-treated cultures (Fig. 8C-8E). Untreated culture presented 74% and 54% of colonies without AP activity at day 7 and 28, respectively (Fig. 8C-8E). In GDNF-treated cells, unstained colony decreased and represented 50%, 33% and 25% of colony population in 1, 10 and 100 ng/ml GDNF-treated cultures, respectively, at day 7. Additionally, the number of AP-positive cells per colony increased in dose-dependent and time-dependent manners. Indeed, at day 7, the proportion of colonies containing more than 5 AP-positive cells were respectively 0%, 10%, 18% and 33% in 0, 1, 10 and 100 ng/ml GDNF-treated cultures. Similarly, at day 28, the proportion of colonies containing more than 5 AP-positive cells were respectively 3%, 9%, 40% and 54% in 0, 1, 10 and 100 ng/ml GDNF-treated cultures (Fig. 8C-8E). The study of isolated single cells and paired cells also showed more AP-positive cells in GDNF treated cultures (Fig. 8F, 8G). In control, 16% of single cells presented a positive AP activity at day 7 and 25% at day 28. By comparison, in GDNF-treated culture, percentages of single cells presenting a positive AP activity were 30%, 32% and 43% at day 7 and 32%, 43% and 48% at day 28, in 1, 10 and 100 ng/ml GDNF-treated cells, respectively (Fig. 8F). Concerning paired cells, the number of unstained paired cells decreased with time and GDNF treatment, whereas the number of stained paired cells increased (Fig. 8G). Stained paired cells were subdivided in two groups in order to identify paired cells which were both stained and paired cells presenting differential staining. Differentially stained paired cells were stable during the time but were increase twofold in all GDNF-treated cultures. The proportion of paired cells which were both stained increased with culture duration in controls but not in GDNF-treated cultures. GDNF treatment multiplied their proportion by 3 at day 7 and by 1.5 to 2 at day 28 (Fig. 8G). To conclude, the GDNF treatment was responsible for the increase in density, size, and content of AP-positive potential SSCs of colonies. Moreover, GDNF was also responsible for the increase of potential SSCs among isolated single and paired cells.
DISCUSSION
Several studies have recently initiated SSC characterization in teleostean fish. These studies included SSC specific marker identification, SSC isolation and, eventually, their transplantation and were conducted in a classical experimental model, zebrafish [40] and in farmed fish: trout, carp and Nile tilapia [5,13,41]. The high potential of these SSCs could benefit fish reproduction biotechnologies. SSC transplantation could be used to produce farmed or endangered fish species from surrogate parents or transgenic lines for functional studies [27]. Despite the crucial phylogenetic position of Chondrichthyans at the root of Vertebrates and the number of threatened shark species, chondrichthyan SSCs have still not been studied. As SSC molecular markers and regulators are relatively well conserved between teleosts and mammals, it was interesting to generate an in vitro model of undifferentiated spermatogonia in a widespread chondrichthyan species to test their conservation through Vertebrates. The germinative area containing potential SSCs was easily identified, as Chondrichthyes harbor a polarized cystic testis, and different spermatogonial subpopulations were distinguished on the basis of morphological criteria [6] and transcripts expressions [29]. In the present study, we first confirm at a protein level that the GDNF receptor GFRα1 was expressed in undifferentiated spermatogonia in the dogfish testis until the first differentiating stages, in accordance with the mRNA expression pattern previously described [29]. An expression pattern in undifferentiated spermatogonia was also reported in many mammalian species such as mouse [7], primates [42], equids [8], domestic cat [43], collared peccary [10] or in fish such as the Nile tilapia [5] and a similar expression pattern extended to spermatocytes, but at a much lower level, was also observed in trout [44]. PLZF expression pattern appeared less restrictive than GFRα1 with an expression extended from undifferentiated spermatogonia to primary spermatocytes, which is consistent with the mRNA localization in germ cells of the dogfish [29]. This extended expression pattern differs from the specific expression observed in undifferentiated spermatogonia of mammals such as mouse [7], primates [45] or equids [8]. However, the perinuclear or nuclear localization of PLZF only observed in undifferentiated spermatogonia suggested that it was transcriptionally active at these stages rather than in further germ cells, which is consistent with its function in mammals during spermatogenesis [46,47]. In fish, PLZF was poorly described but it was shown to be expressed until type B spermatogonia in zebrafish testis [14], suggesting that, like in dogfish, it had a less restrictive expression pattern than in mammals. Those results suggest that GFRα1 is strongly conserved through the evolution, whereas PLZF presents a restriction of its expression pattern in mammals, suggesting that its function and/or regulation have evolved as well. Interestingly, c-KIT also presented an expression pattern less restrictive with a proportion of c-KIT+ undifferentiated spermatogonia as previously reported in the dogfish [6] but not in mammals [48]. However, the c-KIT expression pattern in dogfish differentiated spermatogonia and spermatocytes was consistent with its function during proliferation and differentiation in mammals [49]. Moreover, PLZF was described as a repressor of c-KIT [47] and the expression pattern of those factors in the dogfish suggested that a balance of their activities could control the transition between SSCs, progenitors and differentiated spermatogonia. Although rodent data describe GDNF expression in Sertoli cells [50,51], a recent work on a teleostean model, the rainbow trout, shows GDNF protein expression in spermatogonia [44]. In trout, gdnf transcripts are strongly expressed in A spermatogonia and decrease until primary spermatocytes, gdnf is no more detected in spermatids [44]. This expression pattern is similar to the Artemin protein expression observed in the present study in the dogfish. Therefore, a potential role of Artemin instead of GDNF in the autocrine regulation of spermatogonial stem cell fate in the dogfish
germinal niche cannot be excluded. Concerning ERα, we reported that it was a marker of testicular somatic cells, with exception of Sertoli cells, in the dogfish testis as it has been shown in adult rodents [52]. Tissue distributions of proteins observed by Western-blot were consistent with the literature. Thus, GFRα1 protein was expressed in testis and brain in accordance to its mRNA distribution in this species [29]. PLZF protein was also detected in testis and brain, tissues showing a weak mRNA expression [29], in accordance with data in mouse [46]. ERα seemed widely expressed but at a low expression level. ARTN showed an expression in testis and brain like in zebrafish [53].

The long term SSC in vitro culture, which is a powerful tool for understanding SSCs regulation, is mainly limited considering the difficulty to isolate those cells, which are the rarest spermatogonial subpopulation, and also considering the difficulty to maintain their self-renewal in vitro. In the present paper, we reported that without GDNF supplement, the dogfish spermatogonial co-culture from the germinative zone has been maintained at most 3 months with persistent colonies and a light alkaline phosphatase activity, suggesting that the undifferentiated state was also maintained. In comparison, spermatogonial culture has been maintained at most 2 weeks without GDNF in mouse [17,18,21], bull [54] or pig [55]. In Teleosts, some studies reported the formation and maintenance of colonies in cultures of spermatogonial cells such as enriched type A spermatogonia in the Nile tilapia [5], Thy1+ enriched cells in carp [13] and SSCs in zebrafish [26]. The growth factors added in culture media were different: no GDNF treatment (but FGF-2/EGF), a moderated GDNF treatment (40 ng/ml) or a strong GDNF treatment (100 ng/ml, and a cocktail of FGF-2, IGF-1 and EGF) respectively. In presence of GDNF, we reported the maintenance of the dogfish spermatogonial culture for at least 5 months. In mammals, the addition of GDNF was also reported to be responsible for the duration increase from two weeks to several months, of in vitro maintenance of spermatogonia [17,18,21,54,55]. It can be also noticed than in mammals, those long maintenance durations were obtained using culture media enriched in additional growth factors, like FGF-2, LIF or EGF. So, we hypothesize that in dogfish, the maintenance of undifferentiated spermatogonia in GDNF-free culture media was probably allowed by: (i) the addition of 1% FBS and 0.25% dogfish serum containing endogenous growth factors which could limit the somatic cell number and increase the spermatogonial proliferation as previously reported in rainbow trout [56]; (ii) the presence of somatic cells originating from the germinative area in the co-culture, including Sertolian precursors, which probably contributed to the maintenance of spermatogonia, notably by the secretion of paracrine factors. Unfortunately, the stem state of the spermatogonia present in the niche or forming colonies in culture could not be demonstrate by a transplantation assay due to the long time of generation of this species and to the complexity of its genital tract which not allows an injection through the urogenital pore. It urges to unlock this technical challenge for Chondrichthyes reproduction research.

A functional analysis of human recombinant GDNF on the dogfish spermatogonial culture was conducted in order to assess the conservation of SSCs regulation in Vertebrates and to optimize our culture conditions. GDNF treatment decreased apoptosis in a dose-dependent manner, in accordance with its positive effect on mouse SSCs survival through the stimulation of the anti-apoptotic factor bcl6b expression [57]. The level of apoptosis in control (without GDNF) was high when compared with apoptosis in the culture of spermatogonial cysts in the spiny dogfish (10-30% in presence of 1% FBS) [58]. In McClusky’s experiment, intact cysts were maintained
in culture and they probably produced endogenous growth factors which contribute to a moderate apoptosis level [58] whereas in the cultured described here, the presence of somatic supporting cells was drastically limited by an initial differential plating step. In mammals, such high frequency of apoptosis (75%) has been observed in culture of isolated primordial germ cells in absence of feeding cells or growth factors [59]. The decrease of cell apoptosis observed in our present study in presence of GDNF is in accordance with the fact that GDNF is essential for long term maintenance of SSC in vitro in several species [18,60]. Otherwise, the increase of the number and size of colonies in GDNF-treated dogfish spermatogonial cultures was also in agreement with in vitro studies on mouse [17,21,24]. Furthermore, the GDNF-dose dependent increase of alkaline phosphatase activity within the colonies in the dogfish spermatogonial culture strongly suggests that the GDNF promoted the pluripotent state of the undifferentiated spermatogonia, in addition to their self-renewal. The AP activity is indeed used as a marker of stem cells such as embryonic stem (ES) cells in human [23] or in carp [61], as well as of SSCs in mouse [18,24] or in medaka [22] although this activity has been reported once in testicular myoid cells in rat [62]. Our immunocytofluorescence experiments have shown that dogfish spermatogonia maintained in vitro with GDNF expressed GFRα1, PLZF and α-spectrin for some of them, which are markers of undifferentiated spermatogonia and that these spermatogonia also expressed in part the proliferation marker PCNA. PLZF and GFRα1 have been used to identify potential SSCs in mammals [63,64] and GFRα1 has been similarly used in Nile tilapia [5]. The fact that the two markers of SSCs, GFRα1 [65] and the spherical spectrosome [66] were colocalized in some spermatogonia in our culture system strongly suggests that these spermatogonia could correspond to SSCs. Some spermatogonia also expressed c-KIT in vitro which was shown to be a spermatogonial marker in dogfish [6]. Finally, the majority of cultivated cells were germinal but, on the basis of ERα detection, adherent somatic cells were also present in contact with spermatogonia. Our results also showed that isolated single and paired cells both presented an increase of AP activity with GDNF treatment. Concerning paired cells, the increase of alkaline phosphatase activity was detected either in both cells or in one of the paired cells only, which could respectively correspond to symmetrical and asymmetrical divisions of pluripotent SSCs, suggesting that the GDNF would promote the self-renewal of potential SSCs by these two ways in culture.

GFRα1 domain D2 is well conserved among Vertebrates (Osteichthyes and Chondrichthyes). In particular, most amino acid residues appearing to be at the interface with the GDNF [37,38] are conserved from mammals to dogfish. However, GDNF sequence was not found in dogfish transcript libraries, in particular in libraries generated from testis tissue, contrary to another GDNF family ligand, ARTN. When GDNF and ARTN mature parts were aligned, dogfish ARTN sequence showed similarities with GDNF consensus at key positions for the interaction and specific-binding to the receptor. Consequently, we propose the ARTN as the GFRα1 ligand in dogfish testis. Supporting this possibility, previous studies reported that GDNF, neurturin (NRTN) and ARTN showed crosstalk in vitro [67]. Especially, ARTN was shown to activate Ret through GFRα1, to a lesser degree than GDNF, in cell lines so that the possible use of GFR α1-Ret complex to mediate ARTN effects in vivo was already mentioned. GFRα1-Ret complex could bind and mediate the effects of NRTN and ARTN in addition to its “more specific” ligand GDNF [68]. Concerning the potential loss of GDNF in dogfish, genomic data would be of high interest to test this hypothesis. Other examples of loss of a GFL were demonstrated by synteny analysis: NRTN in frog and persephin (PSPN) in chicken. Moreover, GDNF would be GFRα2
ligand in frog [38]. We notice that the cross-talk between the ARTN, a GFL, and GFRα1, another GFRα than its specific receptor, would not be an exception in Vertebrates.

In this present paper, we first demonstrated that GFRα1 was expressed in undifferentiated spermatogonia in the dogfish testis as in mammals, suggesting a strong conservation of this receptor during evolution. Then, we developed, for the first time in Chondrichthyes, a culture of undifferentiated spermatogonia on a somatic feeder layer, all originating from the dogfish testicular germinative area. This primary culture was maintained for a long time with a minimum culture media. Moreover, we demonstrated that the GDNF increased the formation, growth and survival of colonies containing potential SSCs in a chondrichthyan model, like it was observed in Osteichthyes, suggesting that the GFRα1 system is conserved through Vertebrates evolution. However, GFRα1 ligand may be different and this receptor could be also involved in the regulation of differentiated spermatogonia in dogfish accordingly to its pattern of expression which is wider than observed in rodents [8,69] but similar to than observed in trout [44]. The pluripotency of the cultured cells remains to demonstrate by functional transplantation assay. In conclusion, the culture of potential SSCs established in this study will be a valuable tool to study SSCs regulations in an evolutionary point of view.

ACKNOWLEDGEMENTS
The authors are grateful to Mr Faliguerho and students of the Lycée Maritime et Aquacole de Cherbourg (France) for fishing the dogfish used in this study and to Mr Guyon of the Centre de Recherches en Environnement Côtier (Luc-sur-mer, France) for the care given to the dogfish. The authors thank S. Mazan and the members of the PHYLOFISH project (ANR- 2010-GENM-017) coordinated by J. Bobe and Y. Guiguen for sharing the transcriptomic libraries generated from dogfish tissues. Thanks also to B. Adeline for the preparation of tissue sections.

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FIGURE LEGENDS

Figure 1: Artemin as potential ligand for GFRα1 in dogfish testis. A) The alignment of domain D2 of GFRα1 includes the following sequences: Rattus norvegicus (Rn): NP_037091.1, Homo sapiens (Hs): NP_005255.1, Ornithorhynchus anatinus (Oa): XP_001513939.2, Xenopus tropicalis (Xt): NP_001096555.1, Gallus gallus (Gg): NP_990433.1, Anolis carolinensis (Ac): XP_003223549.1, Scyliorhinus canicula (Sc): KF597022, Oreochromis niloticus (On): XP_003441983.1, Oryzias latipes (Ol): XP_004080316.1 and Danio rerio (Dr): NP_571805.1. Amino acid residues shown to be involved in GDNF-GFRα1 interaction in mammals are indicated by asterisks [37]. Notice that 16 out of these 17 residues are conserved in dogfish. B) No GDNF was found in the available dogfish transcript libraries, however ARTN sequence was found in various libraries and, in particular, in one generated from the testis germinative area. Alignment of the mature parts of GDNF and ARTN was generated using the following sequences: GDNF: Rn: NP_062012.1, Hs: NP_000505.1, Xt: ENSXETP00000010369, Gg: XP_425018.3, Ac: XP_003224305.1, Sc: EMP29646.1, Ol: XP_003453908.1, Or: XP_004074824.1, Dr: XP_004074824.1; ARTN Rn: NP_445849.1, Hs: NP_476432.2, Oa: XP_001518505.2, Xt: ENSXETP00000060269, Gg: XP_003641733.2, Ac: XP_003216810.1, Cm: EMP29646.1, Sc:
Amino acid residues proposed to form the core binding epitopes for the interaction with the receptor or to determine the specificity of binding for both GDNF family ligands are indicated by asterisks [38]. Among them, the residues found to be similar or identical between dogfish ARTN and GDNF consensus whereas different in mammalian ARTN are highlighted under the alignment of the mature parts of GDNF and ARTN. These substitutions in dogfish ARTN sequence suggest that ARTN could be GFRα1 ligand in dogfish testis. C: conserved cysteines involved in disulfide [38] are also present in dogfish protein sequences.

**Figure 2:** Artemin, the only GFL found to be expressed in dogfish testicular germinative area. Only a GFL sequence was found in a transcriptomic library established from dogfish testicular germinative area although this library was established using Illumina RNA sequencing, a powerful next generation sequencing technology. This sequence was identified to be the dogfish artemin ortholog using the TBLASTN algorithm in the GenBank nucleotid database. In order to confirm this orthology relationship, a phylogenetic analysis was conducted using both Neighbor-Joining method and Minimum Evolution method (illustrated). The reliability of the inferred trees was assessed using the bootstrap method with 1000 replications. The phylogenetic tree was build using the sequences previously detailed for GDNF and ARTN alignment and the following sequences: PSPN Rn: NP_037146.1, Hs: NP_004149.1; NRTN Rn: NP_445851.1, Hs: NP_004549.1. Only the mature part of the GFLs was used, so that all sequences have the same size. The dogfish GFL sequence segregated with the artemin sequences of the green sea turtle and anole lizard in all trees generated, so that its orthology with artemin was validated.

**Figure 3:** Validation by Western-blot of the antibody specificity in dogfish. Proteins were extracted from a panel of five tissues in dogfish: epigonal tissue, epididymis, testis, spleen and brain so that the antibodies used could be validated on the basis of the molecular weight and the tissue distribution. Actin protein was used as loading control. ERα was widely distributed but signal was really weak. GFRα1, PLZF and ARTN were detected in testis and brain.

**Figure 4:** Immunohistochemical localization of GFRα1, ARTN, PLZF, c-KIT and ERα in the adult dogfish testis. A1-A5) Immunolocalization of GFRα1 in the niche (A1, A2) and during the cystic formation occurring from stage II (A3) to stage III (A4) showed stained spermatogonia (black arrows) and unstained Sertoli cells (white arrowheads). GFRα1 expression progressively decreased during spermatogonial proliferation until primary spermatocytes (A5) and was no more detected in secondary spermatocytes (black star, A5). B1-B5) ARTN showed a strong expression from undifferentiated and differentiated spermatogonia (black arrow, B1-B4) to primary spermatocytes (B5) but was not detected in secondary spermatocytes (black star, B5). Sertoli cells were unstained (white arrowheads, B4). C1-C5) Immunolocalization of PLZF during the cystic formation occurring from stage II (C3) to stage III (C4) showed a strong staining in undifferentiated spermatogonia (black arrows), this staining decreased until primary spermatocytes (C5). No signal was detected in Sertoli cells (white arrowheads). D1-D5) Immunolocalization of c-Kit during the cystic formation occurring from stage II (D3) to stage III (D4) showed both stained and unstained spermatogonia (black and white arrows, respectively) and unstained Sertoli cells (white arrowheads). Kit was still detected in the germinal lineage at more advanced stages (D5). E1-E5) Immunolocalization of ERα during the cystic formation occurring from stage II (E3) to stage III (E4) showed stained somatic cells (black arrowheads).
and unstained Sertoli cells (white arrowheads) and unstained spermatogonia (stars) ERα expression pattern was maintained at more advanced stages (E5). Zones A0, A- and B correspond respectively to the germinal niche, spermatogonia containing cysts and primary or secondary spermatocytes containing cysts. Bars = 50 µm (A1-E1, A5-E5) and 20 µm (A2-E2, A3-E3, A4-E4).

**Figure 5: Sampling, enrichment and maintenance of dogfish undifferentiated spermatogonia in vitro.** A) Cross sections of the dogfish testis can be dissected in 5 zones: A0, A-, B, C and D corresponding to the germinative area including potential SSCs, the pre-meiotic area containing proliferating spermatogonia, the meiotic area, the early and late spermiogenesis areas, respectively. Cysts are represented by grey circles distributed along the spermatogenetic wave represented by the red arrow. In order to cultivate cells from the germinative area A0, sections were performed on both sides of the main testicular blood vessel (red ellipse). After enzymatic and mechanic dissociation steps, most adherent somatic cells were eliminated by differential plating in gelatin-coated flasks. This method prevents rapid somatic domination in the culture. Enrichment in spermatogonia in the co-culture was controlled by the observation of both fractions at day 3 post-enrichment. B) The adherent fraction contained somatic cells (green arrow heads), but also some isolated spermatogonia (blue arrowheads) and clusters of spermatogonia (red arrowheads). C) In contrast, the supernatant fraction gave a culture enriched in germinal cells. D, E) Spermatogonia progressively formed colonies (red arrowheads) on a somatic layer in formation, as illustrated at days 7 (D) and 28 (E). F, G) At month 4, only the cultures treated with GDNF (10 ng/ml) still presented colonies of spermatogonia. Bars = 100 µm.

**Figure 6: Identification of different spermatogonial subpopulations and of somatic cells in the primary culture.** A-E) Immunocytofluorescence was performed on GDNF-treated cells (10 ng/ml) after 90 of culture. As experimental protocol includes a fixation step using methanol and a cytopspin step to attach cells on a slide, cell morphology may be truncated. A) The double immuno-labeling of GFRα1 and PCNA showed GFRα1 positive cells (red arrowheads) and negative cells (white arrowheads) which both presented PCNA positive staining. B) The double immuno-labeling of GFRα1 and α-spectrin showed that GFRα1+ cell population could be separated into GFRα1+/α-spec− which exhibit a classical spherical spectrosome (yellow arrows) and GFRα1+/α-spec−. C) Immunofluorescent detection of c-KIT and α-spectrin showed a cluster of c-KIT+/α-spectrin− (red arrowheads) and a single cell c-KIT+ /α-spectrin+ (white arrowheads) which also exhibit characteristic spectrosome form (yellow arrows). D) The detection of PLZF revealed stained spermatogonia (red arrowhead) with nuclear expression (white arrows) and unstained cells (white arrowheads). E) Somatic cells were distinguish from germinal cells (unstained; white arrowheads) by immune-labeling of ERα. They were either in suspension (red arrowhead) or adherent and spread (yellow arrowheads). Bars = 20 µm.

**Figure 7: Effect of GDNF on apoptosis and viability of cultured dogfish spermatogonial cells.** Cell apoptosis was detected using the TUNEL assay on co-cultures treated or not by the GDNF (0, 1, 10 and 100 ng/ml), as illustrated at day 7 (A-D) and day 28 (G-J). On merges, apoptotic cells appear in green or blue cyan (orange arrows) whereas non-apoptotic cells appear in blue (white arrows). Bars = 50 µm. Those events were quantified on 10 observations for each replicates of two independent experiments and were represented by means ± SD at day 7 (E) and
day 28 (K). The total number of counted events was also reported. F, L) At the same time, cell viability was determined using the blue trypan method (also represented by means ± SD). For both methods, statistical analysis were performed using the Mann and Whitney test and allowed the formation of significant statistical groups with p<0.05 (a, b, c and d).

Figure 8: Effect of GDNF on colony formation and alkaline phosphatase (AP) activity. A) The number of colonies per mm² was studied at day 7 and day 28 for control and GDNF-treated cultures (1, 10 and 100 ng/ml). Quantifications were based on 20 observations for each replicates of two independent experiments. B) The colony sizes were distributed in three groups, 0-100 µm; 101-250 µm and >250 µm, and quantified. C) Colonies were also subdivided in 4 groups according to the number of AP positive cells per colony (1; 2-4; 5-20 and more than 20). D) Representative observations of the day 28 post-enrichment showing the effect of GDNF treatment on colony formation and alkaline phosphatase activity (bar = 200 µm). Black arrowheads represented 1 AP positive cell per colony; grey arrowheads: 2-4 AP positive cells per colony; red arrowheads: 5-20 AP positive cells per colony; yellow arrowheads: more than 20 AP positive cells per colony. E) Insets illustrating colonies with 1, 4 or 5-20 AP positive cells (white arrows), numbers 1, 2, 3, respectively (bar = 20 µm). F) Proportion of AP positive cells among isolated single cells at day 7 and day 28 post-enrichment in control and GDNF-treated cultures. Stained (black arrowhead) and unstained spermatogonia (white arrowhead) are illustrated under the graph. G) Proportion of AP positive cells among isolated paired cells at day 7 and day 28 post-enrichment in control and GDNF-treated cultures. Paired cells were subdivided in two groups: both cells were AP positive; one of the two cells was AP positive (differential staining). They were quantified and illustrated (bar = 20 µm). A, G) All quantifications were represented by mean ± SEM. Statistical analysis were realized with the Mann and Whitney test and revealed significant statistical groups with p<0.05 (a, b, c and d).
Figure 1
Figure 2
Figure 3

- **GFRα1** (54 kDa)
- **ARTN** (21 kDa)
- **ERα** (64 kDa)
- **PLZF** (74 kDa)
- **Actin** (42 kDa)
Figure 4
Figure 5

**A**

- Elongated spermatids
- Young spermatids
- Spermatocytes I/II
- Proliferating spermatogonia

**B**

- Adherent somatic cells elimination

**C**

- Enzymatic and mechanic dissociation

**DIFFERENTIAL PLATING**

- Spermatogonial enrichment on gelatin-coated flask
- Co-culture enriched in undifferentiated spermatogonia

**D**

- D7

**E**

- D28

**F**

- - GDNF

**G**

- + 10 ng/ml GDNF

**D120**

-...
Figure 6
Figure 7
Figure 8