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Cloning, pharmacological characterization and expression analysis of Atlantic cod (*Gadus morhua*, L.) nuclear progesterone receptor

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ABSTRACT

To better understand the role(s) of progesterone in fish spermatogenesis, we cloned the nuclear progesterone receptor (Pgr) of Atlantic cod. The open-reading frame of the cod *pgr* consists of 2076 bp, coding for a 691-amino acids-long protein that shows the highest similarity with other piscine Pgr proteins. Functional characterization of the receptor expressed in mammalian cells revealed that the cod Pgr exhibited progesterone-specific, dose-dependent induction of reporter gene expression, with 17α,20β-dihydroxy-4-pregnen-3-one (DHP), a typical piscine progesterone, showing the highest potency in activating the receptor. During ontogenesis, the *pgr* mRNA was undetectable in embryo's 24 h after fertilization, but became detectable 4 days after fertilization. During the larval stage, the expression levels increased steadily with the development of the larvae. In adult fish, *pgr* was predominantly expressed in gonads of both sexes. During the onset of puberty, testicular *pgr* transcript levels started to increase during rapid spermatogonial proliferation, and peaked when spermiation started. *In situ* hybridization studies using testis tissue during the rapid growth phase containing all germ cell stages indicated that in cod, *pgr* mRNA is predominantly located in Sertoli cells that are in contact with proliferating spermatogonia. Taken together, our data suggests that the Pgr is involved in mediating progestagen stimulation of the mitotic expansion of spermatogonia, and in processes associated with the spermiation/spawning period in Atlantic cod.

1. Introduction

In mammals, the important role of progesterone signaling in a variety of reproductive processes like menstruation, mammary gland development, or establishment and maintenance of pregnancy is well established in females [8]. Moreover, data is accumulating that progesterone also has a role in male reproductive events [29]. In teleost fish, for example, sex steroids of the progestin family, such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), are involved in sperm maturation [20,21], in addition to their roles during resumption of meiosis in final oocyte maturation [22]. Recent evidence indicates that progestins exert regulatory functions at early stages of fish spermatogenesis. For example, in Japanese eel (*Anguilla japonica*) DHP stimulated spermatogonial DNA synthesis and the initiation of meiosis, by inducing expression of the short form of 11β-hydroxysteroid dehydrogenase (Hsd11b) in Leydig cells and of trypsinogen in Sertoli cells [18,19].

Many of the biological actions of progestins are mediated through the progesterone receptor (PGR), a member of the nuclear receptor superfamily of ligand-activated transcription factors [11]. The gene encoding this receptor arose early in vertebrate evolution via duplication of an ancestral estrogen receptor [23]. The reproductive functions of the PGR have been well studied in mammalian models [10]. However, less information is available as regards the specific biological functions of Pgr in teleost fish, the largest and most diverse group of vertebrates. Our previous research indicated that also in zebrafish (*Danio rerio*), Pgr mediates DHP induction of Hsd11b activity in adult testis tissue [7]. In addition, our work in Atlantic salmon (*Salmo salar*, L.) suggests a role of Pgr in mediating DHP-stimulated, early steps of spermatogenesis, such as the increase in the number of spermatogonial cysts [6].

The Atlantic cod (*Gadus morhua*, L.), a member of the order of Gadiformes, is an economically important marine fish in the Northern hemisphere. Recent research on spermatogenesis in this species revealed a novel mode of cystic germ cell development that

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occurs in a specific spatio-temporal organization: testis tissue is composed of several lobes, in which undifferentiated spermatogonia are located in the periphery of each lobe, while increasingly advanced stages of germ cell development are found in a maturational gradient towards the common collecting duct. Spermatogenesis in cod therefore represents an intermediate form between restricted and unrestricted spermatogonial distribution [1].

To study the role of Pgr during spermatogenesis in teleost fish from another order than Anguilliformes, Cypriniformes and Salmoniformes, we set out to clone the cod *pgr* cDNA. After pharmacological characterization of the cod Pgr, we examined the expression profile of *pgr* mRNA during ontogenesis as well as in different adult tissues. Thereafter, we determined the cellular localization of the *pgr* mRNA in cod testis during the rapid growth phase by *in situ* hybridization. Finally, we analyzed changes in testicular *pgr* expression during the onset of puberty in samples where the stage of spermatogenesis had been analyzed histologically.

2. Materials and methods

2.1. Cod Pgr cDNA cloning

To isolate the cod *pgr* cDNA, we used a set of degenerate primers (primers 2197 and 2198; Supplementary Table S1) in a PCR on random-primed cod brain cDNA, and obtained a ~730 bp PCR product that was gel purified and cloned. Four clones were sequenced, all providing the same partial *pgr* cDNA sequence, while no evidence for the presence of a second cod *pgr* cDNA sequence was found with this primer set.

To isolate the 5′- and 3′-ends of the cod *pgr* cDNA, gene-specific primers (primers 2228 and 2235; Supplementary Table S1) based on the consensus nucleotide sequence of the 4 clones were used in combination with a universal primer mix (UPM) for 5′- and 3′-RACE, respectively. These initial 5′- and 3′-RACE products were then used for nested PCR amplifications using gene-specific nested primers (primers 2229 and 2236; see Supplementary Table S1), respectively, in combination with a nested universal primer (NUP). Both the UPM and NUP were supplied with the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). All RACE reactions were carried out according to the manufacturer's instructions in a Perkin-Elmer 2400 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using Advantage 2 polymerase (Clontech). RACE products were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Breda, The Netherlands).

Combining the partially overlapping sequences of the 5′- and 3′-RACE products yielded a full-length cod *pgr* cDNA sequence with 2076 nucleotides in the open-reading frame. The open-reading frame was PCR amplified using primers 2259 and 2260, and subcloned in the correct orientation into the pcDNA3.1/V5-His-TOPO expression vector. Sequence analysis of several clones revealed identical *pgr* sequences as those obtained in the 5′- and 3′-RACE clones, and the sequence of this cod *pgr* open-reading frame was deposited at GenBank with accession number EU625299.

2.2. Transactivation assays for cod Pgr

Receptor activation was measured using a reporter gene assay as described previously [7]. Briefly, HEK 293T were seeded in 10 cm dishes in DMEM medium (without phenol red) supplemented with 10% v/v fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (Gibco, Breda, The Netherlands) at 37 °C in a 5% CO_2 incubator. After 24 h, the cells were co-transfected with 1 μg of the cod *pgr* expression plasmid and 7 μg of the MMTV-luciferase plasmid using a standard calcium phosphate precipitation method. The next day, the medium was replaced by transactivation assay medium (DMEM without phenol red, supplemented with 0.2% v/v charcoal-stripped FBS, glutamine, and non-essential amino acids) containing different steroids with final concentrations ranging between 1 nM and 3.16 μM. After incubation at 37 °C for 24–36 h, the cells were harvested in lysis mix (100 mM potassium phosphate buffer pH 7.7, 1% v/v Triton X-100 [Sigma–Aldrich], 15% v/v glycerol, and 2 mM dithiotreitol [DTT]) and stored at -80 °C. Luciferase activity was determined by adding an equal volume of substrate mix (100 mM potassium phosphate buffer pH 7.7, 250 mM D-luciferin [Invitrogen], 1 mM DTT, 2 mM ATP [Roche, Woerden, The Netherlands] and 15 mM magnesium sulfate [Promega, Leiden, The Netherlands]) to thawed samples and luminescence was measured in a Packard TopCount NXT luminometer (Perkin Elmer Life Sciences, Meriden, CT, USA). Each concentration of each compound was tested in quadruplicate in two independent experiments using cells from different transfections.

The following steroids were used in this study: DHP, 20β-S, progesterone (P4), 17α-hydroxy-4-pregnen-3-one (17α(OH)P4), testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E_2), cortisol, and synthetic progestin promegestone (R5020). All steroids were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands) except for R5020 that was obtained from Perkin Elmer (Waltham, MA, USA).

2.3. Cod Pgr mRNA expression during ontogenesis and in adult tissues

To examine a variation in the *pgr* mRNA expression in relation with the changes in testis growth and spermatogenic activity in cod, we measured the *pgr* mRNA levels from testis tissue of animals during pubertal development. To this end, Atlantic cod (Norwegian Coastal Cod) were reared in lightproof 7 m^3 seawater tanks (n = 175/tank) at the Institute of Marine Research, Austevoll Research Station, Norway (60°N). Maintenance and sampling of Atlantic cod were described in a previous report [2]. A fragment of testis tissue was dissected from animals exposed to natural photoperiod conditions, wrapped in aluminium foil and immediately snap-frozen by immersion in liquid nitrogen, before storage at −80 °C. Other fragments of the same testis were fixed in Bouin's solution, dehydrated, embedded in paraffin according to conventional techniques [1], and used for histological classification of the gonad and for immunohistochemistry, respectively. For morphological analysis, 4-μm-thick sections were stained with hematoxylin-eosin. The presence of running milt in the efferent duct system was noted as "spawning" during sampling when slight pressure on the abdomen induced milt release from the genital pore. All fish were treated and euthanized according to Norwegian National Legislation for Laboratory Animals.

To study *pgr* mRNA expression during ontogenesis, total RNA was isolated from a pool of the following materials: embryos 24 h and 4 days after fertilization; larvae of 5–6 mm total length, just before first feeding. Total RNA was extracted from individual larvae, after head removal, from 6.3 mm to 57 mm full-length (n = 2–21); the sex of the larvae was unknown, as a genetic sex marker is not yet available for Atlantic cod. From adult cod, total RNA was isolated from head kidney, gill, spleen, heart, intestine, liver, muscle, and the gonads of three males and three females.

All RNA extractions were performed by the acid phenol-guanidine thiocyanate method after tissue homogenization in the FastPrep tube containing Lysing Matrix D ceramic beads (MP Biomedicals, Solon, OH, USA). DNAse treatment (Turbo DNA-free, Ambion/Applied Biosystems, Oslo, Norway) was applied to 10 μg of total RNA before reverse transcription. Random-primed cDNA was synthesized from 500 ng RNA using the Reverse Transcriptase Core Kit (RT-RTCK-05, Eurogentec, Belgium) according to the manufacturer's instructions.

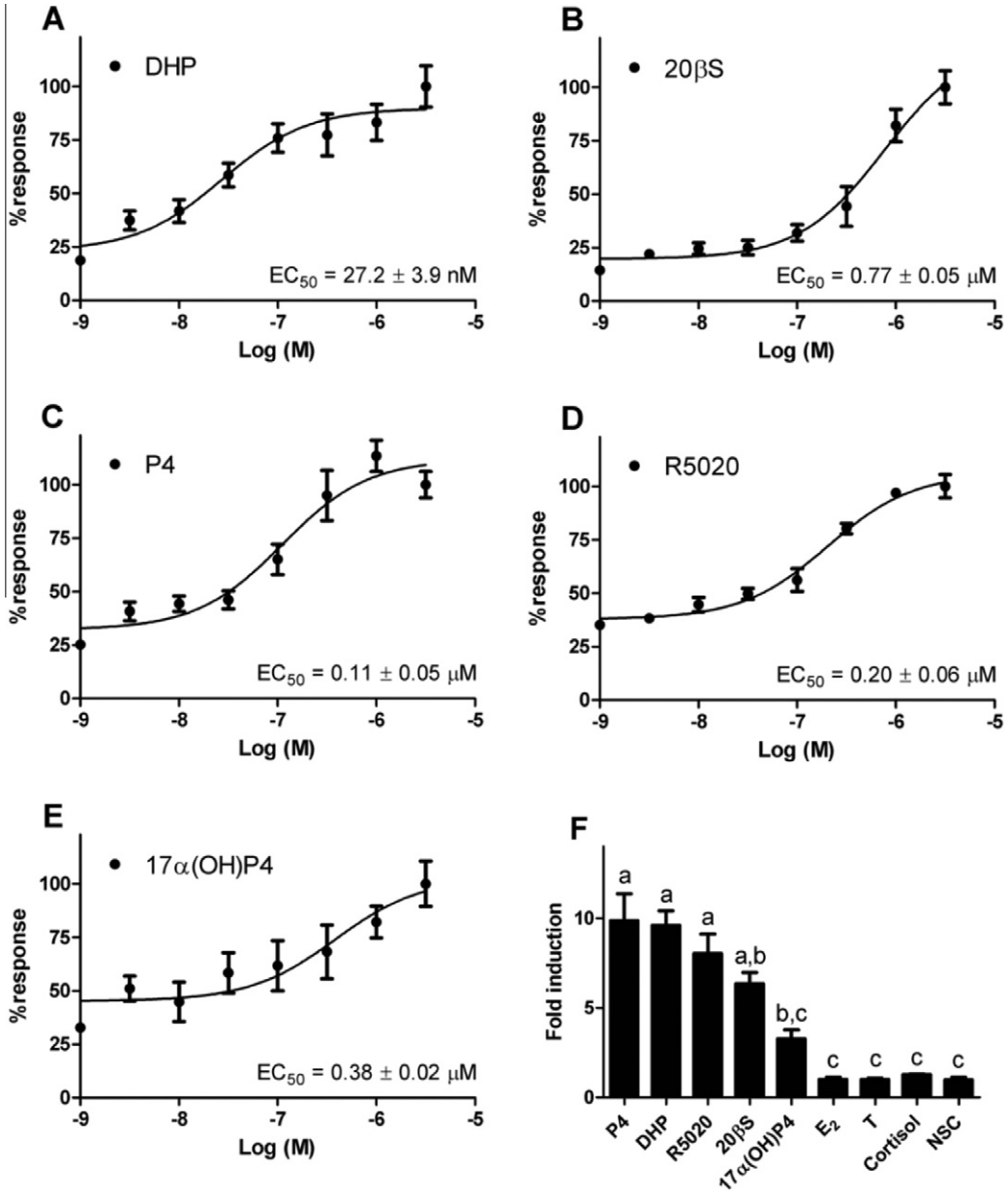

Fig. 1. Ligand-induced transactivation properties of the Atlantic cod Pgr, after transient co-transfection of HEK 293T cells with the cod *pgr* expression vector construct and the MMTV-luciferase reporter construct. (A–E) Transfected cells were incubated with increasing concentrations (from 1 nM to 3.16 μM) of DHP (A), 20β-S (B), P4 (C), R5020 (D) and 17α(OH)P4 (E). Percentage (%) response: values are given relative to the maximal amount of luciferase activity for each steroid. Each point represents the mean ± SEM of two independent experiments, with quadruplicates for each steroid concentration. Curves were generated using non-linear regression (GraphPad Prism 4.0). (F) Transfected cells were incubated with or without 1 μM of the steroids indicated. Data are expressed as the ratio of steroid versus NSC (no steroid control). Each column represents the mean of two independent experiments, with the vertical bar representing the SEM.

To detect cod *pgr* using quantitative real-time PCR (qPCR), primers 3068 and 3069 (Supplementary Table S1) were designed using Primer Express software (Applied Biosystems, Foster City). Atlantic cod *elongation factor 1α* (*ef1α*) was used as endogenous control; no significant differences in *ef1α* expression levels were found in the testes samples analyzed (Supplemental Fig. S3). All qPCRs were performed in 20 μl reactions and C_q values were determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Differences in gene expression were calculated with the $\Delta\Delta_{Ct}$ method, as described in Bogerd et al. [3].

The samples, in which cod *pgr* expression during ontogenesis were measured, normalized to *ef1α* and calibrated with the mean *pgr* mRNA expression in embryos (4 dpf), which was set as 1 (see Fig. 2A). Expression levels in the different cod tissues were normalized to *ef1α* and calibrated with the mean *pgr* mRNA expression in immature testis, which was then set as 1 (see Fig. 2B). Expression levels in testis tissue are presented in two ways: normalized to *ef1α* mRNA and calibrated with the mean *pgr* mRNA expression in immature testis tissue, which was set to 1 (see Fig. 2D), and normalized to *ef1α* mRNA, but also corrected for RNA yield (μg RNA recovered per mg tissue extracted), testis mass and body weight as described by Kusakabe et al. [16], and then calibrated with the mean *pgr* mRNA expression in immature testis tissue, which was set to 1 (see Fig. 2C).

2.4. Cellular localization of Pgr expression in cod testis

Localization of receptor expression by *in situ* hybridization was done exactly as described previously [12], using 10 μm cryo-sections prepared from paraformaldehyde-fixed testis tissue from fish

sampled during the onset of spermatogenesis. Specific primers (3197 and 3130; Supplementary Table 1) were designed to PCR amplify a cod *pgr* cDNA fragment for sense and antisense digoxigenin-labeled cRNA probe synthesis.

To identify Sertoli cell cytoplasm and to differentiate it from germ cells within a cyst, we used an antibody against vimentin, an intermediate filament of the Sertoli cell cytoskeleton (reviewed in [28]). The immunocytochemistry protocol was modified from a previous report [17]. Briefly, sections of 4% paraformaldehyde-fixed and paraffin-embedded cod testis were dried overnight at 37 °C. After deparaffinization and hydration, the sections were subjected to antigen retrieval by boiling for 10 min in 10 mM citrate buffer (pH 6.0) and peroxidase blocking (immersion in 0.3% H_2O_2 in PBS for 10 min) followed by incubation with mouse IgM anti-human vimentin (non-hematopoietic LN6; 1:200 dilution, Biogenex, San Ramon, CA, USA) at 4 °C overnight. Thereafter, the sections were incubated with biotinylated goat anti-mouse IgM (1:200 dilution) for 1 h at room temperature, and with avidin–biotin complex (ABC Kit-Vector Laboratories, Burlingame, CA, USA) for another hour. DAB (3,3′-diaminobenzidine tetrahydrochloride, Dako, Glostrup, Denmark) substrate development was done for approximately 2 min. Nuclei were counterstained with 5% Mayer hematoxylin for 45 s. As positive control, the procedure was applied to rat testis sections, and as negative control the primary antibody was replaced by the same concentration of normal mouse IgM, using both cod and rat testis.

2.5. Statistical analyses

Data are presented as mean ± SEM. Significant differences between relative *pgr* mRNA expression in larvae of different lengths and between testis samples according to histological analysis of testis development, were identified using a Tukey unequal N HSD test. Significance was accepted at $p < 0.05$. Statistical differences of pEC_{50} values (Fig. 1A–E) and fold induction (Fig. 1F) of luciferase activity were analyzed by one way ANOVA followed by the Bonferroni test. A significance of $p < 0.05$ was considered to be significant. Columns labeled with the same letter (Fig. 1F) do not differ significantly ($p > 0.05$).

3. Results

3.1. Isolation and sequence analysis of cod Pgr cDNA

The open-reading frame of the cod *pgr* consisted of 2076 nucleotides (GenBank accession number: EU625299), encoding a protein of 691 amino acids (Supplemental Fig. S1). Comparison of the deduced amino acid sequence of the cod Pgr with receptors from other species is shown in Supplementary Table S2. The overall homology analysis indicated that cod PGR is more similar to teleost (59–67%) than to tetrapod (33–52%) PGR forms. As a member of the nuclear receptors family, the cod Pgr amino acid sequence could be subdivided into 4 domains. The N-terminal transactivation domain (TAD) is a strong regulator of transcription. However, its sequence showed low homology (8–30%) with other PGRs, and is divergent in length and primary sequences among vertebrates. On the other hand, the putative DNA-binding domain (DBD) and ligand-binding domain (LBD) are highly conserved (DBD, 84–100%; LBD, 67–88%) among all vertebrate PGRs studied to date.

A phylogenetic tree, constructed from the aligned amino acid sequences using the neighbor-joining method, revealed that the known PGRs are divided into three major clades (Supplemental Fig. S2). One consisted of fish Pgrs, a second clade contained avian, reptilian, and amphibian PGRs, and the last grouped mammalian PGRs. Within the teleost Pgr group, 99% bootstrap support distinguished a zebrafish lineage from the eel group.

3.2. Steroid-specific transactivation of the cod Pgr

To determine the steroid-dependent transactivation properties of the cod Pgr HEK 293T cells, which do not display endogenous PGR activity [7], were transfected with the MMTV-luciferase reporter construct alone or together with the cod *pgr* expression vector construct. Next, transfected cells were stimulated with increasing concentrations of different steroid hormones. Dose-dependent, Pgr-mediated activation of the MMTV promoter was clearly shown for DHP, 20β-S, P4, R5020 and 17α(OH)P4 (Fig. 1A-E). At a fixed concentration of 1 μM (Fig. 1F), DHP, P4, R5020 and 20β-S were the more potent inducers of MMTV promoter activity compared with the other progesterone-related hormone tested (17α(OH)P4). The other steroid hormones assayed (E_2, T and cortisol) were ineffective at 1 μM (Fig. 1F) or at 10 μM (not shown).

3.3. Expression profiles of cod Pgr mRNA during ontogenic development and in adult tissues

Real-time, quantitative PCR analysis of several organs from male and female cod showed that *pgr* mRNA was predominantly expressed in the gonad of both sexes. Low levels of *pgr* mRNA were found in the liver of female and the intestine of both sexes, while *pgr* mRNA levels were very low/undetectable in muscle, spleen, heart, gill and head kidney (Fig. 2A). While not tested in the tissue screen, brain tissue of course was *pgr* mRNA positive, viz. cloning of the cDNA from the brain.

Analysis of cod embryos showed that the *pgr* mRNA was undetectable in embryos 24 h after fertilization, and became detectable in embryos 4 days after fertilization. During the larval stage, the lowest *pgr* expression was found in larvae of <10 mm length, and increased steadily with the development of the larvae up to 57 mm (Fig. 2B).

As described in a previous report [2], pre-pubertal males kept under normal light conditions are progressively recruited into maturation from August and September and then undergo a testicular growth phase until February, during which spermatogenesis takes place, and the spermatogenic tubules progressively fill with spermatozoa. Testis weight increased 41-fold and reached almost 10% of the body weight.

In the present study, testes were isolated from males exposed to natural photoperiod conditions and grouped according to the stage of germ cell development. The lowest mean of testicular *pgr* expression was observed in immature testis, when RNA yield, testis mass and body weight were used to correct the expression level (Fig. 2C). The levels started to increase significantly when testis started full spermatogenesis – when the number of spermatogonial cysts in rapid mitotic expansion was continuously increasing and when meiosis had started – and again later when the tubules were filled with free spermatozoa (Fig. 2C). When RNA yield, testis mass and body weight were not used to correct the expression level (Fig. 2D), the *pgr* expression levels did not change until spermiation started, with a significant up-regulation in testis where the spermatogenic tubuli were filled with spermatozoa.

3.4. Cellular localization of Pgr expression in cod testis

The mRNA of cod *pgr* was detected in the cytoplasm of Sertoli cells. Not all Sertoli cells showed the same intensity of *pgr* expression; instead, the expression varied with the stage of development of the germ cells, identified based on the size and location of the cysts in the seminiferous tubules (Fig. 3A and B). The most intensive staining was observed in Sertoli cells in contact with rapidly

proliferating type B spermatogonia that occur in clones typically containing very many germ cells and close to the basal membrane of the tubules. Sertoli cells associated with more developed germ cells, such as primary spermatocytes or spermatids (Fig. 3B), presented a weak or no staining, while those in contact with type A spermatogonia were mostly negative. Immunohistochemical localization of vimentin, a cytoskeletal element of Sertoli cells, not only stained the cytoplasm of Sertoli cells outlining the border of a given cyst (Fig. 3C), but also protrudes in a punctuated manner towards the lumen of spermatogenic cysts in between the germ cells of a spermatogenic cysts (Fig. 3D). The same pattern of Sertoli cell cytoplasmic staining was observed in the cysts positive for *pgr* mRNA in *in situ* hybridization, as shown in Fig. 3B. No staining was observed when sections were incubated with sense cRNA (Fig. 3A inset).

4. Discussion

Piscine Pgrs have been previously cloned and functionally tested in species belonging to the orders Anguilliformes, Cypriniformes and Salmoniformes. In the present study, we cloned the open-reading frame of a cod *pgr* cDNA, which encodes a protein of 691 amino acids. Comparison of the deduced amino acid sequence of the cod Pgr with PGRs from other species indicated that, similar to other members of the nuclear receptors family, the DBD and LBD domains are highly conserved [11]. Moreover, the two zinc finger motifs, as well as the P box (GSCKV) and D box (AGRND) sequences were highly conserved in the DBD domain, suggesting that these regions are functionally important for the Pgr to recognize the target gene sequences. Research on the human PGR indicated that a proline rich motif in the N-terminal domain is responsible for its interaction with the c-Src family of tyrosine kinases [4]. However, in cod Pgr, as well as in other fish Pgr proteins, this motif was absent, so that a Pgr-mediated Mos/MAPK activation may not occur in teleost fish [7,14,24].

Due to the teleost-specific genome duplication, ray-finned fish have two paralogous copies for many genes while only one ortholog is present in tetrapods [30]. Two distinct *pgr* genes have been reported in the eel [14,24]. However, the present study, in line with previous studies in zebrafish, medaka, Takefugu, Tetraodon, stick-

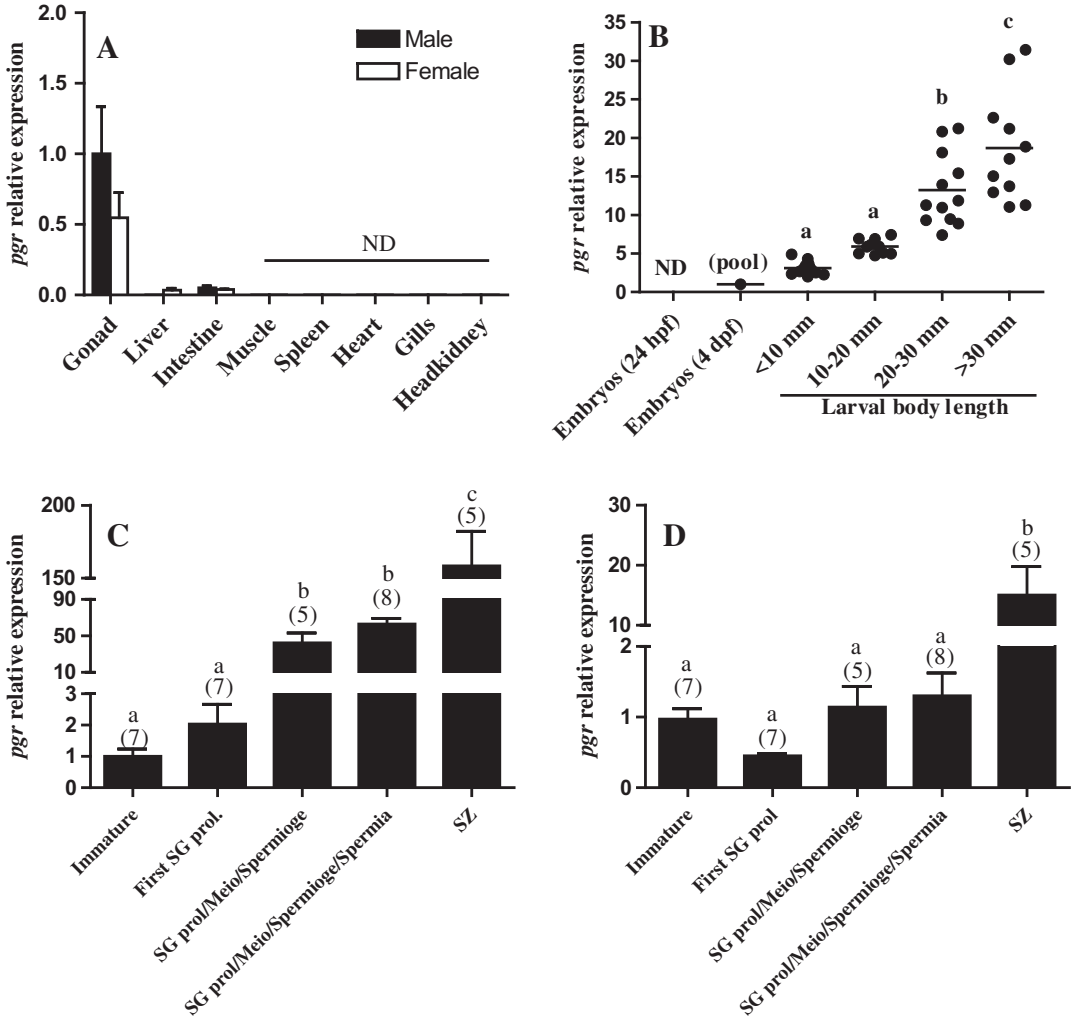

Fig. 2. Expression of cod *pgr* mRNA. (A) Relative expression of *pgr* mRNA levels in adult organs of male and female cod ($n = 3$). Values represent mean ± SEM relative to *pgr* mRNA levels in testis. ND, not detectable. (B) Relative expression of *pgr* mRNA levels in cod embryos throughout early development. Values represent mean ± SEM relative to *pgr* mRNA levels in embryos 4 days post fertilization (4 dpf). ND, not detectable; 24 hpf, 24 h post fertilization. (C and D) Relative expression of *pgr* mRNA levels in testis during one cycle of spermatogenesis, with (C) or without (D) taking into account RNA yield, testis mass and body weight [16] to correct for differences in proliferation activity between the various testicular cell types during early spermatogenesis [1]. Values represent mean ± SEM relative to *pgr* mRNA levels in immature testis. Males were assigned to groups based on the testis histology, with abbreviations indicating: SG Prol, spermatogonia proliferation; Meio, meiosis; Spermioge, spermiogenesis; Spermia, spermiation; SZ, spermatozoa. The number of animals per group is indicated between brackets above each column. Bars marked with different letters are significantly different from each other ($P < 0.05$). ND, not detectable.

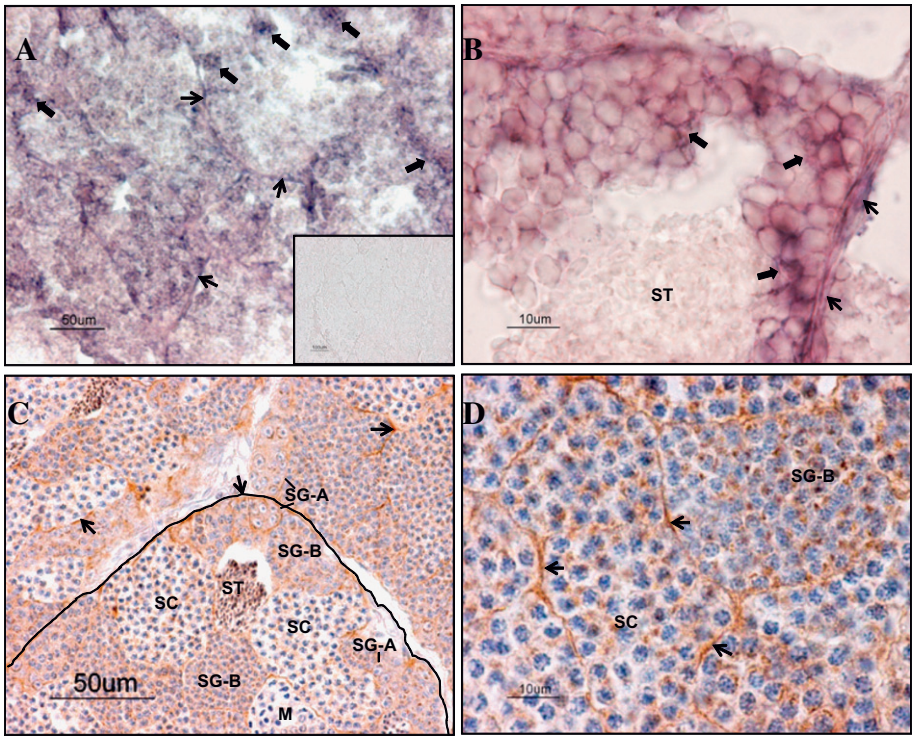

Fig. 3. *In situ* hybridization analyses of *pgr* mRNA and immunohistochemistry of vimentin in Atlantic cod. Low (A) and high (B) magnification of a cryosection of cod testis from an animal during the onset of spermatogenesis, hybridized with antisense *Pgr* cRNA probe, showing signal in spermatogonial cysts at different developmental stages. The insert shows the sense *pgr* cRNA probe did not result in specific staining. Immunostaining of vimentin in paraffin sections of cod testis from an animal during the onset of spermatogenesis at low (C) and high (D) magnification. Thick arrows indicate positive cysts, while thin arrows indicate the cytoplasm of Sertoli cells at the borders of spermatogenic cysts. The black line borders a spermatogenic tubule, with different cysts inside. SG-A, type A spermatogonia; SG-B, type B spermatogonia; SC, spermatocytes; ST, spermatids.

leback, and salmon [6,13], did not provide evidence for the existence of additional *pgr*-like genes in the Atlantic cod. Two isoforms (forms A and B) encoded by the same gene but originating from different translational initiation codons, have been reported for chicken and human PGR homologues [9,15]. However, studies using *PGR* knockout mice indicated that only PGR-A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses necessary for female fertility, while PGR-B is required to elicit normal proliferative responses of the mammary gland to progesterone [10]. Still, the present study does not exclude the possible existence of other isoforms. Considering the predominant expression of the cod *pgr* form in the gonads, we assume that the cloned cod *pgr* has important function(s) in the reproductive physiology of cod.

We demonstrated that the cod *pgr* cDNA codes for a functional Pgr, which is able to activate the transcription of a luciferase gene under control of the MMTV-LTR promoter [25]. Moreover, as in other teleost species, DHP was one of the most effective steroids in activating the Pgr. However, the EC_{50} value of DHP for cod Pgr (27 nM) was higher than for Pgrs from other fishes (0.12 nM for eel Pgr2 [14]; 8 nM for zebrafish Pgr [7]).

In mammals, birds, and amphibians, P4 is considered to be the main ligand for their PGRs. Comparison of the LBD of the cod Pgr with that of PGRs from other species indicated that cod Pgr has more similarity to the teleost forms (81–88%) than to tetrapods *PGR*s (67–69%), which suggest a greater likelihood of differences in ligand specificity between the teleost and tetrapod.

The major aim of this study was to reveal evidence for possible function(s) of Pgr in mediating DHP-stimulated spermatogenic process of a gadoid species, the Atlantic cod, by quantifying and localizing the testicular expression of *pgr* transcripts during a reproductive cycle. In Salmonidae and Cyprinidae high levels of circulating progestins were observed during the spermiation process, when progestins have physiological functions such as to induce spermiation [27], to increase seminal fluid production [5,31], and to stimulate spermatozoa motility [21]. However, the mechanisms by which progestin exerts these effects are still unclear. Recent results suggest that membrane-associated progestin receptor α (mPRα), which is expressed in germ cells, mediates the non-genomic actions of progestins to induce sperm hypermotility in Atlantic croaker and seatrout [26]. In the present study, although we have no information on circulating or intratesticular progestin levels, the *pgr* mRNA expression reached peak levels in spawning testes, which suggests that Pgr may be functionally related to the induction of spermiation, sperm maturation, and perhaps seminal fluid composition in the cod. Unfortunately, detection of *pgr* mRNA by *in situ* hybridization required the use of cryosections, which failed due to technical problems in fully mature testes filled with spermatozoa.

A previous report described high spermatogonial proliferation activity and formation of new spermatogonial cysts in the periphery of the lobes during the period of rapid testis growth [1], which suggests that both the cellular composition and RNA yield of the testis changes considerably during early spermatogenesis. Therefore, in order to consider other possible Pgr-mediated effects during earlier stages of testis maturation, RNA yield, testis mass and body weight were introduced to correct the relative *pgr* gene expression levels (Fig. 2C). The results indicated that entering the period of rapid testicular growth, characterized by massive spermatogonial proliferation and also accompanied by the appearance of the first meiotic cysts, a first up-regulation of testicular *pgr* mRNA levels was observed. This suggests that a possible role of *Pgr* may be related to the rapid mitotic expansion of spermatogonial cysts, i.e. relatively early steps in spermatogenesis. This is sup-

ported by our *in situ* hybridization data showing that cod *pgr* mRNA is located predominantly in Sertoli cells in contact with these rapidly proliferating spermatogonia.

The known mechanisms of action of progestagens on fish spermatogenesis (such as induction of trypsinogen expression by Sertoli cells [18], or induction of 11β-hydroxylase expression by Leydig cells [19]) suggest that Pgr/*pgr* is expressed by somatic cells in the testis. In the present study, we show a similar localization of cod pgr mRNA in testis as we have shown before in Atlantic salmon [6] and zebrafish [7], indicating that elevated expression of *pgr* mRNA in piscine Sertoli cells that contact rapidly proliferating spermatogonia might a common phenomenon. We previously found evidence for *pgr* expression in zebrafish Leydig cells, but we could not detect *pgr* mRNA in Leydig cells of Atlantic salmon [6] or cod (this study). So far, no indications exist for Pgr-mediated mechanisms operating in germ cells, except for the immunological Pgr detection described by Hanna et al. [13]. Apparently, *pgr* mRNA expression levels are lower in Leydig cells (and perhaps even lower in germ cells) compared with levels in Sertoli cells, since the biological activity and our *in situ* data jointly argue for a Sertoli cell localization.

In summary, we have cloned a single cDNA coding for a Pgr in the Atlantic cod. Pharmacological characterization indicated that a well know piscine progestin, DHP, is its most potent ligand, next to P4. The expression pattern of *pgr* mRNA during early stages of ontogenesis implies a potential role for sex differentiation towards female. Cellular localization and quantification of *pgr* mRNA in cod testis during a reproductive cycle suggest that the cod Pgr, which is expressed in Sertoli cells, may be involved at an early stage of spermatogenesis, the rapid, mitotic expansion of spermatogonia, and may also be involved in a late developmental step, the induction of spermiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012.07.022.

References

[1] F.F.L. Almeida, C. Kristoffersen, G.L. Taranger, R.W. Schulz, Spermatogenesis in Atlantic cod (*Gadus morhua*): a novel model of cystic germ cell development, Biol. Reprod. 78 (2008) 27–34.
[2] F.F.L. Almeida, G.L. Taranger, B. Norberg, O. Karlsen, J. Bogerd, R.W. Schulz, Photoperiod-modulated testis maturation in Atlantic cod (*Gadus morhua* L.), Biol. Reprod. 80 (2009) 631–640.
[3] J. Bogerd, M. Blomenröhr, E. Andersson, H.H. van der Putten, C.P. Tensen, H.F. Vischer, J.C. Granneman, C. Janssen-Dommerholt, H.J. Goos, R.W. Schulz, Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*), Biol. Reprod. 64 (2001) 1633–1643.
[4] V. Boonyaratanakornkit, M.P. Scott, V. Ribon, L. Sherman, S.M. Anderson, J.L. Maller, W.T. Miller, D.P. Edwards, Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family of tyrosine kinases, Mol. Cell 8 (2001) 269–280.
[5] S.M. Baynes, A.P. Scott, Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*), Gen. Comp. Endocrinol. 57 (1985) 150–160.
[6] S.X. Chen, J. Bogerd, E. Andersson, F.F.L. Almeida, G.L. Taranger, R.W. Schulz, Cloning, pharmacological characterization, and expression analysis of Atlantic salmon (*Salmo salar* L.) nuclear progesterone receptor, Reproduction 141 (2011) 491–500.
[7] S.X. Chen, J. Bogerd, A. García-López, H. de Jonge, P.P. de Waal, W.S. Hong, R.W. Schulz, Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor, Biol. Reprod. 82 (2010) 171–181.
[8] C.L. Clarke, R.L. Sutherland, Progestin regulation of cellular proliferation, Endocr. Rev. 11 (1990) 266–301.
[9] O.M. Conneely, B.L. Maxwell, D.O. Toft, W.T. Schrader, B.W. O'Malley, The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA, Biochem. Biophy. Res. Commun. 149 (1987) 493–501.
[10] O.M. Conneely, B. Mulac-Jericevic, F. DeMayo, J.P. Lydon, B.W. O'Malley, Reproductive functions of progesterone receptors, Recent Prog. Horm. Res. 57 (2002) 339–355.
[11] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[12] A. García-López, J. Bogerd, J.C.M. Granneman, W. van Dijk, J.M. Trant, G.L. Taranger, R.W. Schulz, Leydig cells express FSH receptors in African catfish, *Clarias gariepinus*, Endocrinology 150 (2009) 357–365.
[13] R.N. Hanna, S.C. Daly, Y. Pang, I. Anglade, O. Kah, P. Thomas, Y. Zhu, Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain, Biol. Reprod. 82 (2010) 112–122.
[14] T. Ikeuchi, T. Todo, T. Kobayashi, Y. Nagahama, A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*, FEBS Lett. 510 (2002) 77–82.
[15] P. Kastner, A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, P. Chambon, Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, EMBO J. 9 (1990) 1603–1614.
[16] M. Kusakabe, I. Nakamura, J. Evans, P. Swanson, G. Young, Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes, J. Endocrinol. 189 (2006) 541–554.
[17] M.C. Leal, P.P. de Waal, A. García-López, S.X. Chen, J. Bogerd, R.W. Schulz, Zebrafish (*Danio rerio*) primary testis tissue culture: an approach to study testis function *ex vivo*, Gen. Comp. Endocrinol. 162 (2009) 134–138.
[18] C. Miura, T. Ohta, Y. Ozaki, H. Tanaka, T. Miura, Trypsin is a multifunctional factor in spermatogenesis, Proc. Natl. Acad. Sci. USA 106 (2009) 20972–20977.
[19] T. Miura, M. Higuchi, Y. Ozaki, T. Ohta, C. Miura, Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel, Proc. Natl. Acad. Sci. USA 103 (2006) 7333–7338.
[20] T. Miura, T. Kasugai, Y. Nagahama, K. Yamauchi, Acquisition of potential for sperm motility in vitro in Japanese eel (*Anguilla japonica*), Fish. Sci. 61 (1995) 533–534.
[21] T. Miura, K. Yamauchi, H. Takahashi, Y. Nagahama, The role of hormones in the acquisition of sperm motility in salmonid fish, J. Exp. Zool. 261 (1992) 359–363.
[22] Y. Nagahama, 17α,20β-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action, Steroids 62 (1997) 190–196.
[23] J.W. Thornton, Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions, Proc. Natl. Acad. Sci. USA 98 (2001) 5671–5676.
[24] T. Todo, T. Ikeuchi, T. Kobayashi, H. Kajiura-Kobayashi, K. Suzuki, M. Yoshikuni, K. Yamauchi, Y. Nagahama, Characterization of a testicular 17α,20β-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*), FEBS Lett. 465 (2000) 12–17.
[25] M. Truss, M. Beato, Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors, Endocr. Rev. 14 (1993) 459–479.
[26] C. Tubbs, P. Thomas, Functional characteristics of membrane progestin receptor alpha (mPRα) subtypes: a review with new data showing mPRα expression in seatrout sperm and its association with sperm motility, Steroids 73 (2008) 935–941.
[27] H. Ueda, A. Kanbegawa, Y. Nagahama, Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*, Gen. Comp. Endocrinol. 59 (1985) 24–30.
[28] A.W. Vogl, K.S. Vaid, J.A. Guttman, The Sertoli cell cytoskeleton, Adv. Exp. Med. Biol. 636 (2008) 186–211.
[29] M.J. Walton, R.A. Bayne, I. Wallace, D.T. Baird, R.A. Anderson, Direct effect of progestogen on gene expression in the testis during gonadotropin withdrawal and early suppression of spermatogenesis, J. Clin. Endocrinol. Metab. 91 (2006) 2526–2533.
[30] J. Wittbrodt, A. Meyer, M. Schartl, More genes in fish?, Mol Cell. Dev. Biol. 20 (1998) 511–515.
[31] W.S. Yueh, C.F. Chang, 17α,20β,21-trihydroxy-4-pregnen-3-one and 17α,20β-dihydroxy-4-pregnen-3-one stimulated spermiation in protandrous black porgy, *Acanthopagrus schlegeli*, Fish Physiol. Biochem. 17 (1997) 187–193.