

Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens

Daniel Baron^{a,b}, Rémi Houlgatte^{b,c}, Alexis Fostier^a, Yann Guiguen^{a,*}

^a INRA, UR1037 SCRIBE, IFR140, Ouest-Genopole, Rennes F-35000, France

^b Inserm, U533, Nantes F-44000, France

^c Université de Nantes, Nantes Atlantique Universités, IFR26, l'institut du thorax, Ouest-Génopole, Nantes F-44000, France

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Abstract

Fish gonadal phenotype is very sensitive to sex steroid and functional masculinizations can be obtained in most species using androgen treatments. To gain insight into the molecular effects of androgen-induced masculinization we characterized, in the rainbow trout, the gonadal expression profiles of 103 candidate genes involved in sex differentiation and early gametogenesis. The androgen treatment (11 β -hydroxyandrostenedione, 10 mg/kg of food for 3 months) was administered in a genetic all-female population. Gonads were sampled at different time points in genetic all-male and all-female control populations and in the androgen-treated group. Gene expression profiles were recorded by real-time RT-PCR and biological samples and gene expressions were compared using a global clustering analysis. This analysis revealed that masculinization with androgens acts firstly by repressing granulosa cell related genes, including genes involved in ovarian differentiation (*foxl2a*, *fst*, *cyp19a1a*), and subsequently by repressing genes important for early oogenesis (*gdf9*, *bcl2lb*, *fanc1*, *gcl*, *fshb*, *lhb*, *sox23*, *sox24*, *nup62* and *vtgr*). However, this masculinizing treatment did not induce a testicular differentiation similar to what was observed in the control male population. This was especially noticeable for many Leydig cell genes encoding proteins involved in steroidogenesis or its control (*hsd3b1*, *star*, *cyp17a1*, *cyp11b2.1* and *nr5a1b*) that were down-regulated in the androgen-treated group. Concomitantly some Sertoli cells marker genes were up-regulated by the androgen treatment (*sox9a.1*, *nr0b1*, *cldn11*, *dmrt1*) whereas others were down-regulated (*amh*, *sox9a.2*), suggesting a partial differentiation of the Sertoli cell lineage. Overall, this suggests that the crucial step of this masculinization process is the de-differentiation of the granulosa cells.

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1. Introduction

The gonadal primordium differentiation into a testis or an ovary is a crucial step in gonad developmental process. In mammals, testis differentiation is initiated by a single gene localized on the Y chromosome, and designated SRY for Sex determining Region, Y chromosome. Since SRY has been identified as the master sex determining gene (Sinclair et al., 1990), numerous other genes have been

shown to be involved in the regulation of the sex differentiation gene cascade (Brennan and Capel, 2004). In teleostean fish, no sex determining gene, equivalent to SRY, has been found except the putative testis determining factor, *dmy* gene, in the medaka, *Oryzias latipes* (Matsuda et al., 2002), which seems restricted to the genus *Oryzias* (Vollf et al., 2003). Besides, sex differentiation in fish can be experimentally controlled by *in vivo* treatments with sex steroids [reviewed in Baroiller and Guiguen (2001)] like in reptiles and amphibians and to some extent in birds [reviewed in Hayes (1998), Pieau and Dorizzi (2004), Smith and Sinclair (2004)]. During the last decades, most of the research in fish sex differentiation has been focused on these steroid

* Corresponding author. Address: INRA SCRIBE, Campus de Beaulieu, 35042 Rennes Cedex, France. Fax: +33 2 23 48 50 20.

E-mail address: Yann.Guiguen@rennes.inra.fr (Y. Guiguen).

hormones highlighting the crucial role of estrogens and androgens for ovarian and testicular differentiation, respectively. Nevertheless, the molecular mechanisms of steroids action when used to feminize or masculinize the fish gonad still remain poorly understood.

Genetic sex determination of the rainbow trout, *Oncorhynchus mykiss*, has been shown to behave predominantly according to a male heterogametic XY genetic system (Chevassus et al., 1988). Using the possibility to sex-inverse trout larvae by steroid treatments, all-male and all-female genetic populations have been obtained by producing males bearing either a XX or a YY genotype, respectively (Chevassus et al., 1988). These mono-sex populations have been widely used for sex differentiation studies (Guiguen et al., 1999) because they offer the possibility to work on undifferentiated gonads for which the natural fate as a testis or an ovary is known *a priori*. Genomic resources have recently been produced in the rainbow trout including some sequencing projects of a large number of expressed sequenced tags (ESTs) (Govoroun et al., 2006). This now greatly facilitates the characterization of the molecular events underlying ovarian or testicular differentiation (Baron et al., 2005; Vizziano et al., 2007).

Sex-inversion of genetic females into phenotypic males using androgen treatments is largely exploited for the production of all-female populations in salmonids aquaculture (Johnstone et al., 1978). In that regards, 17 α -methyltestosterone has been frequently used for that purpose (Pandian and Sheela, 1995). However, we used 11 β -hydroxyandrostenedione (11 β OH Δ 4) in our study because 11 β OH Δ 4 is a naturally synthesized 11-oxygenated androgen in the rainbow trout differentiating testis (van den Hurk et al., 1982), and this steroid has also been found to be very efficient even at very low dosages for gonad masculinization in fish (van den Hurk and van Oordt, 1985; van den Hurk et al., 1989; Govoroun et al., 2001a; Desprez et al., 2003). Previous studies have been focused, in our laboratory, on the effects of a masculinizing treatment with 11 β OH Δ 4, either on candidate gene expression profiles of a limited number of gonadal steroidogenic enzymes (Govoroun et al., 2001a), or on global changes induced at the genome-wide level (Baron et al., 2007). The present study focus on the expression profiles of 103 candidate genes involved in vertebrate gonadal sex differentiation surveyed during the androgen-induced gonadal sex-inversion. These genes have been analyzed by real-time reverse transcription polymerase-chain-reaction (RT-PCR) and have been chosen from various families (Baron et al., 2005), including: transcription factors, specific germ cells proteins, steroidogenic enzymes, hormones and growth factors, hormones and growth factors receptors and apoptosis regulators. Expression profiles were analyzed using standard classification methods developed for microarray datasets (Eisen et al., 1998) and biologically meaningful groups were identified by gene clustering.

2. Materials and methods

2.1. Animals and samplings

Research involving animal experimentation has been approved by the authors' institution (authorization no. 35–14). It conforms to principles for the use and care of laboratory animals and is in compliance with French and European regulations on animal welfare (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, ETS no. 123, January 1991). Genetic all-male and all-female rainbow trout populations were obtained at the INRA experimental fish farm (Sizun, France) as previously described (Guiguen et al., 1999) using breeders from a winter-spawning strain ('Mirwart' strain). In our experiment, the all-male population was obtained by mating 22 genetic females with 5 YY males and the all-female population by mating 22 genetic females with 6 XX-males. At 55 days post-fertilization (55 dpf), three batches of 1500 fry each, corresponding to the three experimental groups described below, were transferred indoors in a recirculated water system in 0.3 m³ tanks at a constant 12 °C temperature. They were fed *ad libitum* with a commercial diet (dry pellet food, BiomarTM, Brande, Denmark). The two first experimental groups (all-female and all-male control populations) have been used to follow the natural differentiation and they were fed with an ethanol-treated commercial diet (10 ml/kg of food). The third group (females-11 β) was an all-female population sex-inversed by an 11 β -hydroxyandrostenedione (11 β OH Δ 4) treatment (Govoroun et al., 2001a). 11 β OH Δ 4, (Sigma, St. Louis, MO, USA) was administered along with the food (10 mg/kg of food) for 3 months starting from the first feeding (day 0 = d0). This treatment has been previously shown to produce 100% sex-inversions (Govoroun et al., 2001a). An ethanol solution of 11 β OH Δ 4 was added into the commercial food (10 ml/kg of food) that was subsequently dried at room temperature under a fume hood. In each group, 20–100 gonads, depending to the age of the fish, were sampled and pooled in duplicates at different stages of development: the onset of the free swimming period after complete yolk resorption (day 0 = d0), d0 + 7 days (d7), occurrence of oocyte meiosis (d12), beginning of ovarian lamellar structures development (d27), occurrence of previtellogenic oocytes (d60), d90 and d110 (Baron et al., 2005). They were immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction. Additional gonads were also sampled at the same dates for histological analysis performed as previously described (Baron et al., 2005).

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Cergy Pontoise, France) as previously described (Govoroun et al., 2001b). The total RNA concentration was determined with an Agilent 2100 Bioanalyzer and the RNA 6000 LabChip[®] kit (Agilent Technologies, Stockport, UK) according to the manufacturers' instructions. For cDNA synthesis, 1 μ g of RNA was denaturated in the presence of random hexamers (0.5 μ g) for 5 min at 70 °C, and then chilled on ice. Reverse transcription (RT) was performed at 37 °C for 1 h using M-MLV reverse transcriptase (Promega, Madison, WI, USA) as described by the manufacturer.

2.3. Primer design

Candidate genes were chosen according to a bibliographic analysis indicating their direct or indirect involvement in the sex differentiation cascade in vertebrates and have been previously described (Baron et al., 2005). All the primers were purchased from Eurogentec (Eurogentec, Seraing, Belgium). These primers were manually designed respecting whenever possible the following restriction parameters: 21–23 bp length, no more than 4 successive identical nucleotides, 30–70% GC content, a maximum of 2 G or C among the five 3'-end bases, no potential primer-dimer structures and a short amplicon size (70–150 bp). Secondary structures were searched for using the DNA mfold program (<http://bio-info.math.rpi.edu/~mfold/DNA/form1.cgi>). Whenever possible, each pair was chosen with at least one primer flanking an intron–exon boundary, in

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