Estrogen treatment up-regulates female genes but does not suppress all early testicular markers during rainbow trout male-to-female gonadal transdifferentiation

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Abstract

In non-mammalian vertebrates, estrogens are key players in ovarian differentiation, but the mechanisms by which they act remain poorly understood. The present study on rainbow trout was designed to investigate whether estrogens trigger the female pathway by activating a group of early female genes (i.e. cyp19a1, foxl2a, foxl2b, fst, bmp4, and fshb) and by repressing early testicular markers (i.e. dmr1, nr0b1, sox9a1 and sox9a2). Feminization was induced in genetically all-male populations using 17α-ethynylestradiol (EE2, 20 mg/kg of food during 2 months). The expression profiles of 100 candidate genes were obtained by real-time RT-PCR and 45 expression profiles displayed a significant differential expression between control populations (males and females) and EE2-treated populations. These expression profiles were grouped in five temporally correlated expression clusters. The estrogen treatment induced most of the early ovarian differentiation genes (foxl2a, foxl2b, fst, bmp4, and fshb) and in particular foxl2a, which was strongly and quickly up-regulated. Simultaneously, Leydig cell genes, involved in androgen synthesis, as well as some Sertoli cell markers (amh, sox9a2) were strongly repressed. However, in contrast to our initial hypothesis, some genes considered as essential for mammalian and fish testis differentiation were not suppressed during the early process of estrogen-induced feminization (dmr1, nr0b1, sox9a1 and pax2a) and some were even strongly up-regulated (nr0b1, sox9a1and pax2a). In conclusion, estrogens trigger male-to-female transdifferentiation by up-regulating most ovarian specific genes and this up-regulation appears to be crucial for an effective feminization, but estrogens do not concomitantly down-regulate all the testicular differentiation markers.

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Introduction

The molecular mechanisms underlying early ovarian development in vertebrates still remain poorly understood (Loffler & Koopman 2002, Brennan & Capel 2004, Yao 2005), however, modulation of both estrogen synthesis and estrogen receptivity has been shown to affect ovarian differentiation in most vertebrate species. In contrast to the important effects of estrogens on ovarian differentiation in non-mammalian vertebrates, the gonads in eutherian mammals appear quite insensitive to direct estrogen treatments (Yao 2005). However, in mice knockout models (KO) either for aromatase (Cyp19a) enzyme (ArKO) or for estrogen receptors α and β (ERαβKO), females are unable to synthesize estrogens, or to respond to the estrogenic signal, and develop testicular features within their ovaries (Britt & Findlay 2002, Britt et al. 2002). In non mammalian species, increasing evidence suggests that estrogens play a central role during ovarian differentiation, based on studies carried out in fish (Guiguen et al. 1999, Fenske & Segner 2004), amphibians (Chardard & Dournon 1999, Miyata & Kubo 2000), reptiles (Wibbels & Crews 1994, Richard-Mercier et al. 1995), and birds (Wartenberg et al. 1992, Vaillant et al. 2003). Estrogen treatments applied to male embryos during the critical period of development induce a phenotypic gonadal sex change from a testis to an ovary (Loffler & Koopman 2002). In addition, the inhibition of estrogen synthesis in female embryos leads to testicular development (Wartenberg et al. 1992, Wibbels & Crews 1994, Richard-Mercier et al. 1995, Chardard & Dournon 1999, Guiguen et al. 1999, Miyata & Kubo 2000, Fenske & Segner 2004). The resulting hypothesis is that estrogen synthesis is required to trigger the development of the embryonic gonad towards the female phenotype, and its absence (through an inhibition of cyp19a1 expression) is sufficient to trigger the testicular development. In fish, there are two cyp19 genes (Tchoudakova & Callard 1998), an ovarian cyp19 gene (cyp19a1) and a brain cyp19 gene (cyp19a2). The fact that
the gonadal cyp19a1 is the first unambiguous marker of ovarian differentiation (Vizziano et al. 2007), supports the hypothesis that estrogen synthesis plays a major role in triggering the gonadal sex fate. However, the mechanisms of action of estrogens, with regards to their feminizing effects on the gonads, are still not well understood.

In fishes, the simultaneous recording of many gene expression profiles prior to the first morphological changes of the differentiating gonad, revealed many groups of genes with a clear sexually dimorphic expression both in the rainbow trout (Baron et al. 2005c, Vizziano et al. 2007) and in the Nile tilapia (Ijiri et al. 2008). Among them, early female genes including Cyp19a1, Foxl2a, Foxl2b, Fst, Fshb, and Bmp4 (Baron et al. 2005c, Vizziano et al. 2007) were shown to be repressed in trout following masculinizing treatments in females (Baron et al. 2007, 2008, Vizziano et al. 2008). Based on these results we proposed the hypothesis that some of these genes (cyp19a1, foxl2a, and fst) could act in a concerted fashion to induce the female pathway (Vizziano et al. 2007, 2008). This idea is well supported by results in fishes and birds showing that estrogens can modulate foxl2 gonadal expression (Baron et al. 2004, Hudson et al. 2005, Wang et al. 2007), which is known as one of the few important ovarian determining genes in vertebrates (Baron et al. 2004, 2005a, Govoroun et al. 2004, Ottolenghi et al. 2005, Nakamoto et al. 2006, Pannetier et al. 2006, Wang et al. 2007). This estrogen modulation of foxl2 and the recent demonstration that FOX1.2/FOX2 positively regulates Cyp19/cyp19a1 expression (Pannetier et al. 2006, Wang et al. 2007, Yamaguchi et al. 2007), also provided strong support to the hypothesis that foxl2 and estrogens act within a short positive feedback loop (Hudson et al. 2005, Vizziano et al. 2008). Apart from the early down-regulation of female genes, the masculinization process also involved some up-regulations of Sertoli cells (i.e. amh, sox9a2, dme1), Leydig cells (i.e. cyp11b2.1, cyp11b2.2, hsd3b2, cyp17a, star, nr5a1) and testicular differentiation markers (i.e. nr0b1; Vizziano et al. 2007, 2008, Baron et al. 2008). The differentiation of a bipotential gonad into a female gonad can be the consequence of the balance between female pathway activation and male pathway repression. We therefore hypothesized that the molecular mechanisms underlying feminization of the gonads include a quick up-regulation of female genes, combined with the quick repression of genes involved in early testis development. To test this hypothesis, we compared gonadal gene expression profiles of genetically all-male and all-female rainbow trout populations with gonadal gene expression profiles of an all-male population feminized by a 17α-ethynylestradiol (EE2) treatment at a dosage that has been previously shown to be effective (Govoroun et al. 2001b). This analysis was carried out on 100 genes known to be involved in natural ovarian and testicular differentiation in vertebrates, and previously described during rainbow trout differentiation (Baron et al. 2005c). Based on these 100 gene expression profiles, 45 genes with a significant differential expression pattern among the three groups of fishes were selected. The expression of some of these genes was also followed at the cellular level in ovaries, testes and gonads treated with EE2 by in situ hybridization using specific cell markers, as previously described in trout (Vizziano et al. 2007).

Methods

Animals and sampling

Research involving animal experimentation conformed to the principles for the use and care of laboratory animals, in compliance with French and European regulations on animal welfare. Genetically all-male (XY) and all-female (XX) rainbow trout larvae were obtained from the Institut National de la Recherche Agronomique experimental fish farm (Drennec, France) as previously described (Guiguen et al. 1999). Fish were maintained at 10 °C from fertilization until 24 days post fertilization (dpf) and transferred to the experimental installations i.e., 0.3 m³ tanks with a recirculating water system, at 10 ± 0.1 °C, under constant photoperiod (12h light:12h darkness). After complete yolk resorption by 53 dpf, 800 fish were divided into four batches (with or without treatments), transferred to tanks at a constant temperature of 12 °C and fed ad libitum daily with a commercial diet (dry pellet food, BiomarTM, Brande, Denmark). The groups were as follows: Female – all female fish were fed on a diet containing ethanol (control group). Male – all male fish were fed on a diet containing ethanol (control group). M-EE2 – All male fish were fed with a diet supplemented by 17α-ethynylestradiol (EE2, Sigma) in ethanol at EE2, 20 mg/kg food. Treatments were applied during 2 months, from the first feeding (53 dpf) to 116 dpf. The feminizing efficiency of this treatment has been previously investigated (Govoroun et al. 2001b) and the resulting females were fertile. Gonads were sampled for real-time PCR analysis at the following stages of development post fertilization: 53, 60, 69, 83, 116, 144, 164 dpf. For each sampling date, gene expression profiles were obtained for two independent pools (except for stage 60 dpf in the all-male control population) of gonads, each containing 20–100 gonads that were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Additional gonads were fixed in glutaraldehyde, embedded in Epon and cut at 2 μm Vizziano et al. (2007) for histological analysis, and in 4% paraformaldehyde for in situ hybridization.

RNA extraction and RT

Total RNA was extracted using TRIzol reagent (Invitrogen) as previously described (Govoroun et al. 2001a).
cDNA synthesis was carried out on 1 μg total RNA. Total RNA was denatured in the presence of random hexamers (0.5 μg) for 5 min at 70 °C, and then chilled on ice. RT was performed at 37 °C for 1 h using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) as described by the manufacturer.

Real time PCR

Real-time RT-PCR was carried out using an iCycler iQTM (BioRad). The gene list, the primer selection, and the complete procedure were as previously described (Baron et al. 2005). Reactions were performed on 20 μl samples with 300 nM of each primer, 5 μl a 1/50 dilution of the RT reaction and the SYBER-Green PCR master Mix (Eurogentec, Liège, Belgium), according to the manufacturer’s instructions. After two incubation steps (50 °C for 10 min, 95 °C for 2 min), the thermal cycling protocol was 95 °C for 10 min followed by 40 cycles of PCR (95 °C for 30 s, 60 °C or 65 °C for 1 min). For each primer set (see Table online in Baron et al. 2005a,b,c) the efficiency of the PCR (linear equation: y = slope + intercept) was measured in triplicate on serial dilutions of the same cDNA sample (pool of reverse-transcribed RNA samples). Real-time PCR efficiencies for each reaction were then calculated using the formula: Efficiency (E) = (10(1/slope))-1. Melting curve analysis was also performed for each gene to check the specificity of the RT-PCR products. The relative amount of the target RNA called the starting quantity (SQ) was then determined using the I-Cycler IQ software by comparison with the corresponding standard curve for each sample run in duplicate. SQ was calculated as follows: \( SQ = 10\left(\frac{C_i - \text{intercept}}{\text{slope}}\right) - 1 \) where \( C_i \) is the Cycle threshold of the unknown sample. Each transcript level was then normalized by division with the expression values of the constitutive elongation factor 1α (EF1A), used as an internal standard. In the current study, EF1A did not show any significant variation following EE2 treatments.

In situ hybridization

In situ hybridization was carried out as previously described (Vizziano et al. 2007), with digoxigenin-labeled anti-sense RNA probes. For each condition (control males, control females and treated males), we processed one fish for all different time points (53, 60, 69, 83, 116, 144, 164 dpf).

Data analysis

The 100 gene expression profiles obtained by real-time RT-PCR were first log-transformed, median centered (relative to the median expression across all samples) and then normalized (the sum of the square values of each gene expression profile is set to 1) using the Cluster-3 program (de Hoon et al. 2004). This datasheet was then analyzed using ‘Significance Analysis of Microarrays’ (SAM) software (Tusher et al. 2001), implemented in the TIGR MultiExperiment Viewer (Saeed et al. 2006). Multi-class SAM analysis was run to distinguish significant differential expression profiles across the three classes of samples (Females, Males, and M-EE2). The lowest delta value, producing 0% median number of falsely significant genes, was selected for further analysis. Using the significant gene expression profiles, characterized by SAM analysis, we then classified the relationship between the different biological samples, by unsupervised hierarchical clustering, using a centroid linkage clustering with Pearson’s un-centered correlation as a similarity metric. Gene clusters were distinguished using the non-hierarchical unsupervised learning k-means algorithm as implemented in the Cluster-3 program (de Hoon et al. 2004), with a maximum cycle parameter of 100. The optimal minimal ‘k’ number of clusters, corresponding to the stability of the k-means clustering, was empirically set at 5, based on previous analysis of unsupervised clustering. Results were displayed using the Java Tree-View program (Saldanha 2004).

Results

Effects of the treatments on gonadal morphology

Comparison of morphological changes observed in gonads undergoing natural development in genetically all-female (FEMALE) and all-male (MALE) trout populations, with those subjected to feminizing treatments (MALE-EE2), is shown in Fig. 1. Seven days after the beginning of the EE2 treatment (at 60 dpf or 60 dpf), gonads of male and female control fish remained histologically undifferentiated and no difference, was detected in males treated with EE2 at this stage when compared with the control groups. At 69 dpf, ovaries in control females started to be clearly recognizable with a specific spatial distribution of the connective tissue around the future ovarian lamellae. The control male gonads at this stage were only recognizable by the absence of the ovarian lamella formation (Fig. 1). Compared with the control groups (males and females) the gonads of the EE2-treated fish at 69 dpf appeared very thin in cross section and contained only a few scattered germ cells. No features of ovarian development were observed at that time in the EE2 treated group. At 116 dpf, gonads of control males were recognized by the presence of testis cords containing spermatogonia organized as clusters of germ cells surrounded by Sertoli cells. At this stage, EE2 treatment
in male gonads prevented the formation of the testis cord. However, clear features of ovarian development in EE2 treated male gonads could be observed, but only around 144 dpf when previtellogenic oocytes and groups of oogonias were detected within these gonads. These oocytes were characterized by a large size and a nucleus with multiple nucleoli, and were similar to those observed in ovaries of control females (Fig. 1). However, at this stage the transdifferentiated ovary was still not completely structured since no ovarian lamellae was observed (Fig. 1). In control females, these ovarian lamellae lined the lumen and contained oogonias and previtellogenic oocytes. In the feminized gonads at 144 dpf, this was displayed by a special distribution of the connective tissue that began to delimit the future ovarian lamellae. In comparison, during natural gonad female development these features were observed at around 69 dpf.

Comparison of the biological samples based on gene expression clustering

The full gene expression profile dataset is available online as hierarchical clustering files, which are downloadable and browsable on the Sigenae website (http://www.sigenae.org/) in the citations link. Following multi-class SAM analysis, 45 genes were retained with differentially significant expression between the three groups (control males, control females, M-EE2). The unsupervised classification of the biological samples, using these 45 gene expression profiles, clusters these samples into two major branches (Fig. 2A). Branch A clusters all the male samples (correlation coefficient $R=0.63$); branch B, all the females and males treated with estrogen showing a negative low correlation coefficient (M-EE2, $R=−0.15$).

Effects of the treatments on global gene expression profiling

For a better characterization of gene clusters with similar expression profiles, the 45 significant expression profiles were grouped in 5 clusters of correlated temporal expression, using a $k$ means clustering. The $k$ number of clusters ($k=5$) was chosen after visual analysis of supervised hierarchical clustering of these 45 expression profiles. Among these, 5 $k$-means clusters (Fig. 2B), cluster 1 (C1) is characterized by a specific and sustained high gene expression level in control females when compared with control male expressions. This cluster includes the following genes: cyp19a1, fst, foxl2a, foxl2b, bmp4 and fshb, (Fig. 2B), which are well known in the rainbow trout as early specific ovarian differentiation genes (Baron et al. 2005c, Vizziano et al. 2007). Following the EE2 treatment in males three genes were strongly
up-regulated in this cluster: foxl2a, foxl2b and fst. Among them, only foxl2a was quickly up-regulated as it reached expression levels similar to those observed in control females at 60 dpf or more i.e., 7 days post EE2 treatment (Fig. 3). By contrast, foxl2b and fst reached similar, or even higher expression levels, when compared with control females, but only at around 116 dpf. Similar trends were also recorded for bmp4 and fshb (Fig. 2B).

The cyp19a1 (aromatase) was not induced at all following the estrogen treatment and EE2 treated gonads exhibited the same cyp19a1 expression level as in normal control male gonads throughout all the duration of the EE2 treatment (Fig. 3).

cyp19a1 expression was only slightly induced in feminized gonads post EE2 treatment (i.e. 144 and 164 dpf, see Fig. 3).

The C2 cluster contained genes up-regulated in ovaries during the previtellogenesis period (from 116 to 164 dpf), including the following genes: chbl1, tial1, nup62, birc5a, akri1b1, sox24, casp3b, figla, and vim. The EE2 treatment applied to male gonads induced a slight expression of all these genes, which did not reach the expression levels observed in control females (i.e. figla, vim, Fig. 3).

The C3 cluster is characterized by genes that are highly sensitive to estrogens, being both strongly and quickly up-regulated by the EE2 treatment, i.e. as soon as 7 days post EE2 treatment (Fig. 2). This cluster contains many genes known as female markers (tspo, 5lox, sp3, and, zfpm2, lhcg in Fig. 4) but also genes previously described (Baron et al. 2005) as being more specific to testicular differentiation (i.e. nr0b1, erb, cav1, sox9a1, igf2). Surprisingly, sox9a1 and nr0b1, previously described as early testicular markers (Vizziano et al. 2007), were also strongly stimulated by estrogen treatments (Fig. 4). After EE2 treatment, sox9a1 showed sustained expression and nr0b1 a strong decrease.

Figure 2 (A) Unsupervised hierarchical clustering classification of the biological samples. Gonad samples are labeled according to the sample date (reported in days post fertilization) and sex i.e. red for control females, blue for control males, and black for estrogen-treated males. Correlation coefficients (R) are provided for the main branches of the cluster. (B) K-means analysis of the 45 significant gene expressions profiles during rainbow trout male and female gonadal development, and during feminization induced by estrogen (M-EE2). Each row represents a gene, and each column represents a sample. Sampling date in days post fertilization references each column. The 45 genes are referenced on the right according to the zebrafish nomenclature.
Clusters 4 and 5 contained genes that were overexpressed during natural testis development, when compared with ovarian development. Cluster 4 is only slightly sensitive to the EE2 treatment, and includes the following genes: *cdh1*, *madh7*, *fshr*, *igf1*, *tef21*, *dmrt1*, *pax2a* (Fig. 2). This cluster gathers genes that are slowly down-regulated after the beginning of the treatment (i.e. *dmrt1*, Fig. 3); while others remain unchanged after the EE2 treatment when compared with control males (i.e. *pax2a*, Fig. 3). By contrast, cluster 5 contains genes that are strongly and quickly suppressed by the estrogenic treatment compared with control male gonads (Figs 2 and 4). Cluster 5 includes many genes encoding steroid enzymes (*cyp11a1*, *hsd3b2*, *cyp17a*, *foxl2a* (forkhead box L2 a), *foxl2b* (forkhead box L2 b), *fst* (follistatin)); C2 (*figla* (factor in the germline α), *vim* (vimentin)), and C4 (*dmrt1* (doublesex and Mab-3-related Transcription factor 1), *pax2* (paired box 2)).

Figure 3 Expression profiles of some representative female genes from gene cluster C1 (aromatase), *foxl2a* (forkhead box L2 a), *foxl2b* (forkhead box L2 b), *fst* (follistatin)); C2 (*figla* (factor in the germline α), *vim* (vimentin)), and C4 (*dmrt1* (doublesex and Mab-3-related Transcription factor 1), *pax2* (paired box 2)). Open bars represent control female samples; black bars the control male samples; and squared bars the estrogen treated male samples. Results are represented as the ratio between the expression of the specific gene and elongation factor 1 (*ef1a*). Each bar represents the mean of two different measurements from different biological samples.
Figure 4 Expression profiles of some representative masculine and steroidogenesis-related genes from gene cluster C3 (sox9a1 (Sry-type HMG box protein 9 α; sry (sex determining region Y)-box 9), nr0b1 (nuclear receptor subfamily 0, group B, member 1), zfpm2 (zinc finger protein multitype 2), lhcgr (luteinizing hormone/choriogonadotropin receptor)) and C5 (amh (anti-Müllerian Hormone), sox9a2 (SRY-type HMG box protein 9 β; sry (sex determining region Y)-box 9), cyp11a1 (cytochrome P450 side-chain cleavage; cytochrome P450, family 11, subfamily A, polypeptide 1), hsd3b2 (3 β-hydroxysteroid dehydrogenase), cyp17a1 (steroid 17-α-monooxygenase; cytochrome P450, family 17, subfamily A, polypeptide 1), cyp11b2,1 (11-β-hydroxylase type 2; cytochrome P450, family 11, subfamily B, polypeptide 1)). Open bars represent control female samples; black bars represent the control male samples; and squared bars represent the estrogen-treated male samples. Results are represented as the ratio between the expression of the specific gene and elongation factor 1 (ef1a). Each bar represents the mean of two different measurements from different biological samples.
cypr11b2.1, cypr11b2.2), or known regulators of steroidogenesis, (nr5a1 or sf1, star) along with genes known for their implication in gonadal development in vertebrates (lhx9, sox9a2, amh). Within this cluster, only the cypr11a1 expression is not quickly inhibited as its down-regulation is detected rather late (around 83 dpf) during the process of testis-to-ovary trans-differentiation (Fig. 4).

**Effects of the treatments on some key gene expressions**

Four genes were selected and further investigated by *in situ* hybridization to support their temporal gene expression profiles. These selected genes were cypr19a1 as a somatic cell ovarian marker, Amh as a Sertoli cell marker and cypr17a1 and cypr11b2.1 as Leydig cell markers. In control females, the expression of cypr19a1 was restricted to scattered somatic cells lining the ovarian lamellae (Fig. 5, 83 dpf). cypr19a1 was observed later on in follicular cells surrounding previtellogenic oocytes inside the ovarian lamellae (Fig. 5, 144 dpf). No signal was detected in the control male gonads during the study period (Fig. 5), in agreement with the previously described female-specific expression of cypr19a1. In estrogen-treated animals, the expression of cypr19a1 was partially restored in the follicular cells located near the oocytes, but only after three months of EE2 treatment (Fig. 5).

In control males, a strong expression of amh was found in the somatic cells organized as testis cords around the germ cells (83 dpf, Fig. 5). In control females, amh was also slightly expressed in somatic cells (83 dpf). The estrogenic treatment clearly repressed amh in male gonads (Fig. 5). The expression of steroidogenic enzymes involved in androgen production was restricted to scattered differentiating steroidogenic cells or pre-Leydig cells in male control gonads (see the expression of cypr11b2.1 in Fig. 5 that is representative of the expression pattern of other steroid cell markers, such as cypr17a1). At this stage, we did not detect any expression of cypr17a1 and cypr11b2.1 in the ovaries.

**Discussion**

Firstly, our results show that the feminization induced by EE2 produced a female-like expression pattern, as demonstrated by the unsupervised clustering of the biological samples grouping all female gonadal samples along with those of estrogen-treated males. This molecular feminization process included the induction of most
of the genes implicated in early ovarian differentiation and oogenesis (clusters 1 and 2), as well as the repression of some testicular differentiation genes (cluster 5).

Among these early ovarian genes, foxl2a is of special interest as it is considered to be a conserved ovarian differentiation gene in mammals (Baron et al. 2005a, Pailhoux et al. 2005), birds (Goverouin et al. 2004, Hudson et al. 2005), and fishes (Baron et al. 2004, Nakamoto et al. 2006, Wang et al. 2007). In trout, we previously showed that estrogen strongly induced this gene and that it was (Baron et al. 2005a) temporally co-expressed with cyp19a1 during the initial steps of ovarian differentiation (Vizziano et al. 2007). The fact that Foxl2/foxl2 is able to up-regulate Cyp19a/cyp19a1 in both mammals and fish (Pannetier et al. 2005, Wang et al. 2007), and that estrogens up-regulate foxl2 in fish (Baron et al. 2004, Wang et al. 2007), suggests a positive feedback loop regulating these two genes. This loop would explain the significant expression of sexually dimorphic genes, as observed for cyp19a1 and foxl2a during natural early gonad differentiation (Vizziano et al. 2007). However, our results also clearly show that even if estrogens are able to induce a strong and quick up-regulation of foxl2a, this sustained high expression by itself is not able to restore cyp19a1 expression. This suggests that foxl2a is not able to counterbalance the estrogen inhibition of the cyp19a1 gene and/or that foxl2a needs an additional partner to be able to induce cyp19a1 expression. Such a co-activation has been strongly suggested in the Nile tilapia as for this species it is hypothesized that the interaction of nr5a1 (Sfi) with foxl2 regulates cyp19a1 in a sex-specific manner (Wang et al. 2007). The fact that nr5a1 (Sfi) is clearly down-regulated by EE2 in our experiment fits in well with this hypothesis. However, even if the regulatory loop between foxl2a and cyp19a1 was broken by the EE2 treatment, the estrogenic signal provided by the exogenous estrogen treatment was sufficient to induce the female pathway. In this context, it is interesting to note that at the end of the treatment when the gonads were feminized, cyp19a1 was slowly restored, supporting the idea that the restoration of cyp19a1 expression was blocked by the exogenous estrogen treatment.

After foxl2a activation by estrogens, other female genes including bst, foxl2b, bmp4 and fsbh were up-regulated. bst is one of the early genes expressed in a sexually dimorphic fashion during gonad differentiation of female trout (Vizziano et al. 2007). In the mouse, Fst has been proposed as an early ovarian differentiation gene (Yao et al. 2004, Yao 2005), acting downstream of Wnt4 Yao et al. (2004, 2006) to antagonize the testis-specific formation of the coelomic vessels. The fact that fst expression reached a maximum at around 116 dpf, corresponding to the period of testis cord regression in these estrogen-treated transdifferentiating gonads, supports the idea that fst may also be involved in the inhibition of testis-specific structures. foxl2b is a divergent paralog of foxl2 that has been suggested to be involved in the regulation of the onset of oocyte meiosis (Baron et al. 2004). Its rather late overexpression following EE2 treatment supports its implication in early folliculogenesis, as for the bone morphogenetic protein BMP4, which has been characterized in mammals as a positive regulator of the primordial-to-primary follicle transition (Knight & Glister 2006). In the gilthead seabream (Sparus aurata), FSHB is expressed in primary- and secondary-growth oocytes and this pituitary hormone may be involved in communication between oocytes and ovarian follicle cells (Wong & Zohar 2004). Interestingly, in the Japanese flounder (Paralichthys olivaceus), both FSH signaling and FOXL2 have been shown to be involved in the transcriptional regulation of CYP19A1 during gonadal differentiation (Yamaguchi et al. 2007).

After the induction of these early ovarian genes, and following the active feminization of the gonad, late ovarian markers related to early previtellogenic follicle formation (Baron et al. 2005c) were up-regulated (i.e. ebn1, tial1, np62, birc5a, akr1b1, sox24, casp3b, figla, and vim). However, some early (lhcg) and late (zp3) ovarian markers were part of another cluster (C3) of genes displaying quick and high overexpression following the application of the EE2 treatment, suggesting that these genes are direct estrogen-sensitive targets. This cluster not only contained ovarian markers but also gathered genes previously described (Baron et al. 2005b) as being overexpressed in the male (esr2, nrdb1, sox9a1 and igf2). In fish, zp3 genes are highly sensitive to estrogens and are expressed both in the liver and in the ovary (Modig et al. 2006). In the rat ovary, estrogens act in a synergizing fashion with follicle-stimulating hormone to differentiate granulosa cells by up-regulating both cyp19a1 and lhcg (Ikeda et al. 2008).

Concomitantly with estrogen induction of molecular ovarian differentiation, many testicular markers were repressed either in pre-Leydig cells (cyp11b2.1, hsd3b2, cyp17a, star, nr5a1 or sfi1) or pre-Sertoli cells (sox9a2, amh). In Leydig cells estrogens strongly repress most of the genes needed for the specific synthesis of 11-oxygenated androgens (hsd3b2, cyp17a1, cyp11b1.1), but estrogens do not affect the conversion of cholesterol to pregnenolone as the early cyp11a1 expression was not affected by the EE2 treatment. However, surprisingly some Sertoli cell markers were not repressed by the estrogen treatment, including dmrt1 and nr0b1 that are known as essential genes for testis development in mammals (Brennan & Capel 2004) and have been described as early testis differentiation markers in fish (Vizziano et al. 2007, Ijiri et al. 2008). In trout, both dmrt1 and nr0b1 genes were up-regulated during the first steps of gonad masculinization indicating their involvement in the male pathway development (Vizziano et al. 2008). In the Nile tilapia, dmrt1 has even been proposed as a crucial gene for
testicular differentiation (Ijiri et al. 2008). However, in trout, \textit{dmrt1} expression was only slowly inhibited and \textit{Nr0b1} was even up-regulated by the EE2 feminization. This indicates that, in contrast to our initial hypothesis, the down-regulation of all these testis-specific genes is not required for active ovarian differentiation. The up-regulation of \textit{Nr0b1} and the simultaneous down-regulation of \textit{nr5a1} (\textit{Sf1}) following estrogen feminization and androgen and aromatase inhibitor masculinization, is highly remarkable when we consider that in the mouse, \textit{Nr0b1} inhibits \textit{Cyp19} expression by down-regulating the \textit{Nr5a1} (\textit{Sf1}) gene that would normally stimulate \textit{cyp19} expression (Wang et al. 2001). A similar regulation could also be proposed during trout gonadal differentiation as the up-regulation of \textit{Nr0b1} was always observed concomitantly with the concerted down regulation of \textit{nr5a1} (\textit{Sf1}) and \textit{cyp19a1}.

The case of \textit{pax2a} is intriguing as this gene was highly overexpressed during and after testicular differentiation in trout (Baron et al. 2005c, Vizziano et al. 2007), but totally unaffected by estrogen-induced feminization. \textit{Pax2} was recently described as a downstream target of estrogens in endometrium cancers (Wu et al. 2005). The feminization treatment using high doses of estrogens could then explain this sustained expression of \textit{pax2a} in EE2 treated animals. However, the absence of any down-regulation nearly 50 days after the end of the exogenous estrogen treatment is not in agreement with this hypothesis. The fact that \textit{pax2a} expression was slightly restored, following masculinization of genetic females by an aromatase inhibitor (Vizziano et al. 2008), rules out the hypothesis of a male genotype effect and of \textit{pax2a} being a Y sex-linked gene in trout. Another quite similar and intriguing result concerns the up-regulation of \textit{sox9a1} during estrogen-induced gonad feminization. \textit{SOX9} is one of the earliest expressed genes in pre-Sertoli cells after the activation of \textit{Sry} in mammals (Brennan & Capel 2004). In rainbow trout \textit{sox9} genes are duplicated and both duplicates are expressed in the differentiating testis with \textit{sox9a1} being slightly early sexually dimorphic (Vizziano et al. 2007). In contrast with \textit{sox9a1}, \textit{sox9a2} was repressed by estrogens together with \textit{amh} and with steroid synthesis enzymes. These very different expression patterns clearly demonstrated that \textit{sox9a1} and \textit{sox9a2} have acquired different functions in fish.

In summary, these results show that estrogens may trigger the ovarian pathway by activating some key female genes (\textit{foxl2a}, \textit{foxl2b} and \textit{fsr}), and by disrupting some testicular differentiation genes. However, some genes previously considered as essential for rainbow trout testicular differentiation (\textit{sox9a1}, \textit{nr0b1}, \textit{pax2a}, and \textit{dmrt1}) were not repressed during this testis-to-ovary transdifferentiation process induced by estrogens. Our initial hypothesis that involved a simple up-regulation of ovarian differentiation genes, along with a concomitant down-regulation of testicular differentiation genes, is therefore not supported by the gene expression profiles of this study. The differences between a normal female gene expression pattern and the female transdifferentiation induced by estrogens may be in relation with the rather non-physiological conditions of this sex inversion treatment. For instance, the doses of steroid that are commonly used for sex inversion protocols are often very high (Pandian & Sheela 1995) and the use of synthetic steroids like EE2 or 17\textalpha-methyltestosterone may also not be physiologically relevant. Furthermore, at least in trout, the start of this steroid treatment is post initiation of early gonadal sex differentiation (Vizziano et al. 2007). This may explain, at least in part, the deregulation of the expression of certain genes, as observed in different fish species following either estrogen feminization (Kobayashi et al. 2003) or masculinization with androgens (Baron et al. 2007, Vizziano et al. 2008). More work is now needed to investigate some of the gene regulation hypotheses resulting from this current analysis in order to understand how estrogens functionally induce a female pathway.

**Declaration of interest**

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

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