Estrogen-induced inhibition of spermatogenesis in zebrafish is largely reversed by androgen

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Abstract

The hormonal regulation of spermatogenesis involves both gonadotropins and steroid hormones. Long-term in vivo exposure of adult zebrafish to estrogen impaired spermatogenesis associated with an androgen insufficiency, possibly induced by inhibiting gonadotropin release. Using this experimental model, we investigated if androgen treatment could enhance spermatogenesis, while maintaining the inhibition of gonadotropin release through continued estrogen exposure. Moreover, we also exposed animals to androgen alone, in order to examine androgen effects in the absence of estrogen-induced gonadotropin inhibition. Estrogen exposure depleted type B spermatogonia, meiotic and postmeiotic germ cells from the adult testis, but promoted the proliferation of type A undifferentiated spermatogonia, which accumulated in the testis. This change in germ cell composition was accompanied by reduced mRNA levels of those growth factors (e.g. insl3 and igf3) expressed by testicular somatic cells and known to stimulate spermatogonial differentiation in zebrafish. Additional androgen (11-ketoandrostenedione, which is converted to 11-ketotestosterone) treatment in vivo reversed most of the effects of estrogen exposure on spermatogenesis while insl3 and igf3 transcript levels remained suppressed. When androgen treatment was given alone, it promoted the production of haploid cells at the expense of spermatogonia, and increased transcript levels of some growth factor and hormone receptor genes, but not those of insl3 or igf3. We conclude that estrogen exposure efficiently inhibits spermatogenesis because it induces androgen insufficiency and suppresses gonadotropin-regulated growth factors known to stimulate germ cell differentiation. Moreover, our results suggest that androgens and the growth factors InsL3 and Igf3 stimulate spermatogenesis via independent pathways.

Introduction

Spermatogenesis is a complex cellular development, in which spermatogonial stem cells (SSCs) give rise via mitosis to spermatogonia that enter meiosis and spermiogenesis to produce a large number of highly differentiated haploid spermatozoa. In vertebrates, the endocrine system controls spermatogenesis through...
pituitary gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) (Holdcraft & Braun 2004, Schulz et al. 2010, Schlatt & Ehmke 2014). In mammals, FSH regulates Sertoli cell activity (Sadate-Ngatchou et al. 2004), which supports germ cell survival and maturation, while LH controls Leydig cell function, notably the production of androgens that in turn regulate Sertoli cell activity and thereby germ cell development (Wang et al. 2009, de Gendt et al. 2014), as well as myoid cell functions relevant for spermatogenesis (Chen et al. 2016).

In teleost fish, Fsh also regulates Sertoli cell function, which can occur in an androgen-independent manner (Sambroni et al. 2013, Crespo et al. 2016). In addition, Fsh is a potent steroidogenic hormone in fish (Planas & Swanson 1995), since Leydig cells not only express the Lh receptor (Lhcrgr) but also the Fsh receptor (Fshr) protein (García-López et al. 2010, Chauvigné et al. 2012). On the other hand, zebrafish (Danio rerio) Sertoli cells also express the lhcrgr gene (García-López et al. 2010), altogether resulting in overlapping actions of gonadotropin signalling (Xie et al. 2017). Nonetheless, the actions of the two gonadotropins are discernible considering, for example, that Fsh but not Lh changed the expression of growth factor or steroidogenesis-related genes (García-López et al. 2010). In this situation, it is not surprising that the loss of both gonadotropin β subunits or the loss of both receptors was required to induce male infertility (Zhang et al. 2015a,b). Male fertility in the absence of Fsh is understandable since Lh can both activate the zebrafish Fshr to a certain extent (So et al. 2005) and activate Leydig cell androgen production via the Lhcrgr. After all, gonadotropin-stimulated androgen production, as well as direct androgen exposure, promoted spermatogenesis (Miura & Miura 2001, Leal et al. 2009a). Further, both blocking (Martinović-Weigelt et al. 2011) or activating (Rolland et al. 2013) androgen receptors in zebrafish or rainbow trout (Onchorhynchus mykiss) respectively, results in complex changes in testicular gene expression. Taken together, these studies confirm that gonadotropins and androgens are both potent stimulators of spermatogenesis in fish. However, there is currently no information on the relative contribution or potential interaction of these two types of hormones on spermatogenesis in fish.

In our previous work, we used an estradiol-17β (E2)-mediated negative feedback model to inhibit gonadotropin secretion. The resulting androgen insufficiency interrupted spermatogenesis, as shown by the accumulation of type A spermatogonia and depletion of type B spermatogonia, spermatocytes and spermatids (de Waal et al. 2009). However, it is not known if androgen replacement would restart spermatogenesis while exposure to estrogen continues. In addition to androgen insufficiency, estrogen-induced hypogonadotropic status is probably important for spermatogenesis in view of the relevance of gonadotropin for the activity of several growth factor signalling systems, such as insulin-like peptide 3 (Insl3), insulin-like growth factor 3 (Igf3) and anti-Müllerian hormone (Amh) (Skaar et al. 2011, Nóbrega et al. 2015, Crespo et al. 2016).

In the present study, we first have used the estrogen-mediated negative feedback model to examine androgen effects on zebrafish spermatogenesis in vivo while maintaining the estrogen-induced low gonadotropin tone and compared this to androgen effects in males not pre-exposed to estrogen, i.e. with normal gonadotropin levels. Finally, we have evaluated the potential direct effects of estrogen on the testis, using an ex vivo primary tissue culture approach.

Materials and methods

Animals

Male zebrafish (AB strain) were bred and raised in the aquarium facility of the Faculty of Science, Utrecht University. Sexually mature animals (5–10 months old) were used as the experimental model. All experiments followed the Dutch National regulations for animal use in experimentation.

Exposure to steroid hormones in vivo

Steroid hormone treatment was carried out via the aquarium water as previously described (Van der Ven et al. 2003, de Waal et al. 2009). In brief, sexually mature male zebrafish were kept in four aerated tanks with 13L water under 12-h light/12-h dark photoperiod and identical feeding conditions. Stock solutions of 10µM E2 (Sigma-Aldrich) and 100µM 11-ketoandrostenedione (OA) (Sigma-Aldrich) were prepared in deionized water for further 1:1000 dilution in aquarium water. Fish from a control tank did not receive any steroids. Fish in the experimental groups were exposed to water containing 10nM E2 for five weeks or to 10nM of E2 for five weeks but also to 100nM OA during the last two weeks. The latter group served to investigate if the E2-induced androgen insufficiency and subsequent interruption of spermatogenesis (de Waal et al. 2009) can be stimulated by androgen treatment. The androgen concentration...
of 100nM has previously been shown to stimulate zebrafish spermatogenesis in in vitro (Leal et al. 2009a). The combined duration of meiosis and spermiogenesis is 5.5 days in zebrafish (Leal et al. 2009b), but there is no information yet on the length of the spermatogonial phase in zebrafish. We estimated that exposing fish for 14 days to androgens (i.e. 2.5-fold the length of the combined duration of meiosis/spermiogenesis), should allow us to observe androgen-induced stimulation of the spermatogonial phase also. As the latter comprises of eight mitotic divisions, we allowed time sufficient for, on average, one mitotic division per day. The third experimental group was exposed to 100nM OA for two weeks, after being kept for three weeks under control conditions. OA is the direct biochemical precursor for 11-ketotestosterone (11-KT), the main androgen in zebrafish, and is efficiently converted to 11-KT in different fish species (Schulz & Blüm 1991, Consten et al. 2002, de Waal et al. 2008). The water was exchanged daily.

E2 ex vivo exposure

To investigate potential direct effects of E2 on selected transcripts in adult zebrafish testes, we used a previously developed primary testis tissue culture system (Leal et al. 2009a). The testes from 12 adult zebrafish were kept in culture for four days such that from each male one testis was incubated with culture medium containing 10nM E2, while the other testis was cultured without E2. The medium was refreshed at the end of the second day of incubation. After four days, testes were collected for gene expression analysis, as described below.

Quantification of relative testicular mRNA levels

To examine the effects of steroid exposure on the levels of selected testicular mRNAs, we extracted total RNA from testes tissue samples of 12 fish per experimental group from the in vivo experiments and from tissue incubated during four days of primary culture (see above), using an RNAquous Micro kit (Ambio, Austin, TX, USA), following the manufacturer’s protocol. Two micrograms of RNA from each individual sample were used for cDNA syntheses according to a previously described protocol (de Waal et al. 2008). Transcripts encoding germ cell markers, growth factors and receptors (Table 1) were measured using real-time, quantitative PCR (RT-qPCR). All RT-qPCRs were performed in 20µL reactions and cycle threshold (Ct) values were obtained in a ViiA 7 Real-Time PCR system (Applied Biosystems) using default settings, and the relative gene expression data were analysed using the 2^−ΔΔCt method as previously described (Livak & Schmittgen 2001). Due to its stable expression among all groups investigated, ef1a mRNA levels were used as endogenous control. Samples from the control group (i.e. not receiving any treatment) were normalized to an arbitrary value of 1 and used as the negative control. The data were expressed as means of fold change over control (relative expression).

Morphometric analyses of spermatogenesis

Testicular morphometric analyses were carried out in testes (n=eight per experimental group) from the different hormonal treatments. During the last six hours of the treatments, the animals were exposed to 4mg/mL of the proliferation marker 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich). After determining body and testes weight to calculate the gonado-somatic index (GSI; gonad weight/total body weight×100%), one of the testis from each animal was fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid), and the other in 4% glutaraldehyde (Sigma-Aldrich). Samples were dehydrated, embedded in Technovit 7100, and then sectioned at 4µm thickness. Sections from testis fixed in glutaraldehyde were stained with toluidine blue (Leal et al. 2009b) and served to quantify the number of spermatogenic cysts per mm² as follows. Six sections were selected with a distance of at least 50 µm in between. The complete section area was photographed at 400-fold magnification, using a Zeiss Stemi SV11 equipped with a digital camera (Nikon DXM1200 and Nikon ACT-1 2.63 software). The pictures were analysed using ImageJ software (Image Processing and Analysis in Java). After first determining the surface area (mm²) of each section, the total number of spermatogenic cysts containing type A undifferentiated (Aund), type A differentiating (Adiff), type B spermatogonia, spermatocytes or spermatids was counted and subsequently normalized by the section area. Data from six sections of a single male were merged to calculate this individual’s mean number of cysts containing Aund, Adiff, B spermatogonia, spermatocytes or spermatids per mm². Afterwards, sections from testes fixed in methacarn were prepared for BrdU immunohistochemistry, as described previously (Leal et al. 2009a). To calculate the BrdU index of Aund and Adiff spermatogonia, 100 randomly selected Aund cells or 100 randomly selected Adiff cysts were counted, discriminating between BrdU-labelled and non-labelled germ cells/cyst.
Table 1  Primers used for mRNA levels measurement.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers</th>
<th>Sequence (5′–3′)</th>
<th>Ref seq and primers references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Müllerian hormone amh</td>
<td>4486 (Fw)</td>
<td>CTCTGACCTTGATGAGCCCTATT</td>
<td>GenBank:AY721604 (García-López et al. 2010)</td>
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<tr>
<td>Androgen receptor ar</td>
<td>4487 (Rv)</td>
<td>GAGTGGCTTAAAGAATTTCCTAAA</td>
<td>GenBank:EF153102</td>
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<tr>
<td>Deleted in azoospermatia-like dazl</td>
<td>2412 (Fw)</td>
<td>ACGTGCGCTGGGCTTAAA</td>
<td>(de Waal et al. 2008)</td>
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<tr>
<td>Elongation factor 1 alpha ef1a</td>
<td>2413 (Rv)</td>
<td>CAAACCTGCACTCGCTGAA</td>
<td>GenBank:AB018191 (Chen et al. 2013)</td>
</tr>
<tr>
<td>Follicle-stimulating hormone receptor fshr</td>
<td>2466 (Fw)</td>
<td>CCGTGCACCTGCAAGAACAG</td>
<td>GenBank:LA7469 (Morais et al. 2013)</td>
</tr>
<tr>
<td>Gonadal somatic cell derived factor gsdf</td>
<td>2476 (Rv)</td>
<td>TGCACACTGCAACAGAGTGACAG</td>
<td>GenBank:AY278107 (de Waal et al. 2009)</td>
</tr>
<tr>
<td>Insulin-like growth factor 1 igf1</td>
<td>2477 (Fw)</td>
<td>CATCTGGGAGACTGATTGAAA</td>
<td>GenBank:EU378916 (García-López et al. 2010)</td>
</tr>
<tr>
<td>Insulin-like growth factor 1a receptor igf1ra</td>
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<td>CGCCAGACACAAAGAACAGAAA</td>
<td>GenBank:NM_131825</td>
</tr>
<tr>
<td>Insulin-like growth factor 1b receptor igf1rb</td>
<td>2395 (Rv)</td>
<td>CATCTGGGAGACTGATTGAAA</td>
<td>GenBank:NM_152969 (Morais et al. 2013)</td>
</tr>
<tr>
<td>Insulin-like growth factor 2a igf2a</td>
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<td>CATCTGGGAGACTGATTGAAA</td>
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</tr>
<tr>
<td>Insulin-like growth factor 3 igf3</td>
<td>2367 (Rv)</td>
<td>CATCTGGGAGACTGATTGAAA</td>
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<tr>
<td>Luteinizing/choriogonadotropin receptor lhcg</td>
<td>2466 (Fw)</td>
<td>TTACGCGCTCGTCGCTGCAAT</td>
<td>GenBank:AY714133</td>
</tr>
<tr>
<td>Outer dense fibre of sperm tails 3b odf3b</td>
<td>2467 (Rv)</td>
<td>GCTGACCTGCTGCGCTGCAAT</td>
<td>(de Waal et al. 2009)</td>
</tr>
<tr>
<td>Relaxin/insulin-like family peptide receptor 2a rxfp2a</td>
<td>2548 (Fw)</td>
<td>TACGCGCTCGTCGCTGCAAT</td>
<td>GenBank:AF250289</td>
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<td>Relaxin/insulin-like family peptide receptor 2b rxfp2b</td>
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**Statistical analyses**

Statistical analyses were carried out using the GraphPad Prism 5.0 software package (GraphPad Software). Differences between control and experimental groups were tested for statistical significance by ANOVA (Post-test Tukey) for multiple group comparisons and by Student’s t-test for comparing two groups. P values of 0.05 or lower were considered as statistically significant.

**Results**

**Exposure to E2**

Five weeks of in vivo exposure to E2 induced clear effects on the morphology and physiology of mature testes in zebrafish. An 50% reduction of GSI (Fig. 1A) heralded a significant inhibitory effect of E2 on spermatogenesis, as further revealed by quantitative morphometric analysis: the number of spermatogenic cysts containing more advanced germ cells, such as B spermatogonia, spermatocytes and spermatids decreased (Figs 1B and 2); also, the number of spermatocytes in the lumen of the spermatogenic tubuli was strongly reduced (Fig. 2). No significant change occurred in response to E2 in the number of cysts containing A spermatozoa, spermatogonia, on the other hand, accumulated (Figs 1B and 2), and A spermatozoa, spermatogonia showed an increased proliferation activity in response to E2 (Fig. 3).

As expected, based on the morphological data, testicular mRNA levels were clearly affected by the E2 exposure in vivo. The expression of genes preferentially transcribed by more advanced germ cells, e.g. sycp3, a maker for meiotic cells (Saito et al. 2011), and odf3b, a marker for spermatids (Leal et al. 2009a), were significantly downregulated (Fig. 4A), while that of dazl, expressed by A spermatozoa, and B spermatogonia (Chen et al. 2013), did not change. Considering signalling molecules, E2 exposure reduced the transcript levels of genes encoding...
Androgen rescues estrogen-blocked spermatogenesis

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Androgen-mediated stimulation of estrogen-inhibited spermatogenesis

Previous work showed that three weeks of estrogen exposure led to androgen insufficiency, probably resulting from a negative feedback on pituitary gonadotropin release (de Waal et al. 2009). Here, we tested if the administration of androgen can reverse these inhibitory effects in the continued presence of E2. Two weeks of androgen exposure in addition to E2 resulted in a partial recovery of the GSI values (Fig. 1A), in a complete recovery of the more advanced germ cell cysts containing B spermatogonia, spermatocytes and spermatids (Figs 1B and 2); also, the lumina of the spermatogenic tubules were again filled with spermatozoa (Fig. 2). However, the additional exposure to androgen did not revert the
Androgen rescues estrogen-blocked spermatogenesis

Figure 3
Quantification of the BrdU indices of type A and spermatogonia (A) and type A spermatogonia (B) after different hormone treatments in vitro. Different letters denote statistic differences (P<0.05; ANOVA, Post-test Tukey; n=8). E2: five weeks of exposure to 10 nM E2; E2 + OA: five weeks to 10 nM E2 and to 100 nM OA during the last two weeks; OA: three weeks in normal water followed by two weeks of exposure to 100 nM OA.

accumulation of A and spermatogonia nor their elevated BrdU index (Figs 1B, 2 and 3A). Interestingly, the number of cysts containing A spermatogonia was even elevated above control levels in the group receiving the combined estrogen and androgen treatment (Fig. 1B).

Figure 4
Expression levels of selected germ cell markers, growth factors and receptors genes in zebrafish testes explants cultured in the absence or presence 10 nM E2 from the control group following the additional exposure to OA (Fig. 4A) that also increased amh and gsdf and decreased igf1 transcript levels, which no longer differed significantly from control levels (Fig. 4A).

In line with the morphometric data, RT-qPCR analyses showed that the mRNA levels of late germ cells markers (synp3 and odf3b) returned to control levels after the additional exposure to OA (Fig. 4A) that also increased amh and gsdf and decreased igf1 transcript levels, which no longer differed significantly from control levels (Fig. 4A).

While the E2-triggered effects on the more advanced germ cell stages were fully reversed by androgen treatment, this was different for type A spermatogonia. One possibility to explain this observation is that E2 effects on type A spermatogonia are dominant over potential androgen effects on these cells. We have therefore also examined the effects of androgen treatment alone. While in vivo OA exposure did not significantly affect GSI values (Fig. 1A), we found significant decreases in the number of cysts containing A and B spermatogonia, while the number of cysts containing spermatids more than doubled (Figs 1C and 2).

RT-qPCR analyses of germ cell marker transcripts are in line with the prominent increase in spermatids (odf3b; Fig. 4B) while no statistically significant effects were observed for the other germ cell markers. Androgen exposure also affected growth factor transcript levels. The two more ubiquitously expressed Igf family members igf1 and igf2a were upregulated, but igf3 transcript levels did
not differ from the control group (Fig. 4B). Also, insl3 mRNA levels, a gene specifically expressed in Leydig cells, remained unchanged while gsdf mRNA levels (downregulated by E2; see earlier) increased in response to androgen treatment (Fig. 4B). Effects opposite to those observed after E2 treatment were also observed regarding the transcript levels of receptors for two reproductive hormones (ar and fshr) that were upregulated after androgen treatment (Fig. 4B). A clear upregulation was also observed for the igf1rb transcript (Fig. 4B), a receptor preferentially expressed by somatic cells in the zebrafish testis (Nòbrega et al. 2015).

Discussion

As expected, exposure to E2 for five weeks induced an interruption of spermatogenesis, including reduced GSI values and drastic changes of testis histology. This expectation was based on a previous study, in which three weeks of E2 exposure induced a negative feedback on the brain–pituitary system, androgen insufficiency and inhibition of spermatogenesis (de Waal et al. 2009). Based on this model (Van der Ven et al. 2003, 2007, de Waal et al. 2009), we evaluated the morphological changes in the testes by morphometric analyses, and related histological changes to the expression of selected genes. Moreover, we introduced two new scenarios: one to investigate if androgen treatment in addition to estrogen exposure (i.e. keeping up the negative feedback on the brain-pituitary system) can rescue spermatogenesis, and one to examine the effects of androgen exposure without E2-induced negative feedback.

Our morphometric data corroborate previous results, in which a more time-consuming approach was used to determine the effects of E2 exposure on the mass of the different germ cell types (de Waal et al. 2009). However, in the latter study, we did not discriminate between type A
\( A_{\text{ind}} \) and A
\( A_{\text{diff}} \) spermatogonia. The present study shows that E2 exposure induced a 6.5-fold increase specifically of type A
\( A_{\text{ind}} \) spermatogonia. The present study shows that E2 exposure induced a 6.5-fold increase specifically of type A
\( A_{\text{ind}} \) spermatogonia. The SSCs are a subpopulation of A
\( A_{\text{ind}} \) spermatogonia also in zebrafish (Nòbrega et al. 2010). As stem cells, they can produce either more SSCs by self-renewal or produce progenitors committed to differentiate into spermatozoa. In juvenile eel (Anguilla anguilla), estrogen promoted SSC self-renewal both in vivo and in tissue culture (Miura et al. 1999, 2003). In amphibians, which share the same cystic type of spermatogenesis as teleost fish, estrogen treatment also stimulated spermatogonial proliferation (Minucci et al. 1997, Cobellis et al. 1999, Chieffi et al. 2000). Previously, we found no effects of E2 on spermatogenesis in testes tissue culture (de Waal et al. 2009) and direct E2 effects on testicular gene expression were limited in this study (Fig. 5). However, in vivo exposure to E2 increased the mitotic index of A
\( A_{\text{ind}} \) but not of A
\( A_{\text{diff}} \) spermatogonia. In combination with the increased number of spermatogenic cysts containing A
\( A_{\text{ind}} \) but not of A
\( A_{\text{diff}} \), we concluded that E2 favoured, probably indirectly via the brain–pituitary axis rather than via direct action on the testis, type A
\( A_{\text{ind}} \) self-renewal divisions while blocking their further differentiation, leading to an accumulation of A
\( A_{\text{ind}} \) spermatogonia. The decrease in the number of cysts with type B spermatogonia and all further differentiated germ cell stages also suggests that the further differentiation of the A
\( A_{\text{diff}} \) spermatogonia was compromised in E2-treated fish.

E2-induced reductions in the transcript levels of spermatocyte and spermatid markers sycp3 and odf3b, respectively, were in line with the morphometric data. Previous work showed that dazl expression is visible in A
\( A_{\text{diff}} \). B spermatogonia and spermatocytes (Chen et al. 2013), but we did not find an E2-induced decrease in its transcript levels. It is possible that dazl mRNA levels not simply reflect the quantity of later spermatogonial generations and spermatocytes in zebrafish but also responds to so far unidentified conditions related to the E2 treatment.

The transcript levels of genes encoding growth factors promoting spermatogonial differentiation, such as igf3 (Nòbrega et al. 2015), insl3 (Assis et al. 2016, Crespo et al. 2016) and gsdf (Sawatari et al. 2007, Yan et al. 2017) were downregulated following E2 exposure in vivo, very prominently (~150 fold) in the case of insl3. Considering that Fsh is a potent stimulator of Sertoli cell igf3 (Nòbrega et al. 2015) and of Leydig cell insl3 mRNA levels (Crespo et al. 2016), the decrease of these two transcripts may reflect a negative feedback exerted by E2 on gonadotropin release. A reduced input by gonadotropins and stimulatory growth factors, in conjunction with an androgen insufficiency (de Waal et al. 2009), may explain the inhibition of spermatogenesis. Moreover, our gene expression data suggest that E2 treatment reduced the testicular sensitivity for gonadotropin and androgen stimulation, viz. the reduced fshr, lhcgr and ar transcript levels, also limiting endocrine stimulation of spermatogenesis.

The E2-induced downregulation of amh transcript levels, on the other hand, is more difficult to understand. In adult zebrafish, Fsh downregulated amh transcript levels, and experiments with recombinant Amh showed that it blocked the differentiation of A
\( A_{\text{ind}} \) spermatogonia.
Androgen rescues estrogen-blocked spermatogenesis (Skaar et al. 2011). A low gonadotropin tone following E2 treatment therefore would have been compatible with a de-inhibition of amh mRNA levels, but we found reduced amh expression instead. On the other hand, estrogen treatment reduced testicular amh mRNA levels in fish (e.g. Schulz et al. 2007, Vizziano et al. 2008), and gonadal expression of amh and of the estrogen-producing enzyme cyp19a1a were mutually exclusive in zebrafish (Rodríguez-Mari et al. 2005). However, these observations were made during or shortly after gonadal sex differentiation. Thereafter, co-expression of amh and cyp19a1a occurs in zebrafish ovarian granulosa cells (Rodríguez-Mari et al. 2005). Similarly, the lack of an E2-effect on testicular amh transcript levels in our testis tissue culture experiment supports the idea that E2 does not have a direct inhibitory effect on amh gene expression in adult testes. Considering possible indirect E2-effects, we propose that the E2-related downregulation of amh mRNA levels as well as their upregulation following the additional androgen treatment, reflect changes in the number of amh-expressing Sertoli cells. The number of Sertoli cells per spermatogenic cyst increased in a predictable manner with the progress of the cyst through spermatogenesis (Leal et al. 2009b). Therefore, we infer that reduced spermatogenic activity following E2 treatment and its re-increase following additional androgen treatment are associated with a decrease and a re-increase, respectively, in the number of amh-expressing Sertoli cells. Seen in this light, it is remarkable that three hormone receptors (ar, fshr, lhcgr) and one other growth factor transcript (gsdf) share the same pattern of E2-induced reduction and subsequent re-increase after additional androgen exposure. Three of these genes (ar, fshr, gsdf) are co-expressed with amh in Sertoli cells, suggesting that their expression dynamics may reflect changes in Sertoli cell number as well. As regards lhcgr, it is interesting to note that in fish, this gene is not only expressed by Leydig cells, but also by Sertoli cells (García-López et al. 2010, Xie et al. 2017) and by spermatids (Chauvigné et al. 2014). Spermatid numbers also decreased and re-increased with the treatments applied, so that among the growth factor and receptor genes examined, a true E2-induced downregulation may be restricted to igf3 and insl3 transcript levels that largely depend on indirect effects, reflecting reduced gonadotropin stimulation.

The level of two transcripts, those of igf1 and one of its receptors, igf1rb, was upregulated following E2 treatment. While the cellular site of expression is not known for igf1 in the zebrafish testis, igf1rb is expressed preferentially by somatic cells (Nóbrega et al. 2015). Currently, there is no information on the question whether the different igf ligands activate the two igf receptors differently in zebrafish. All four igf family members and both receptors are expressed in zebrafish testis, but considering that only igf3 expression increased in response to Fsh and none of them responded to Lh (Nóbrega et al. 2015), it is unlikely that the increased igf1 and igf1rb transcript levels reflect a response to the low gonadotropin levels induced by E2. The levels of igf1 and igf1rb transcripts stayed elevated somewhat after additional androgen treatment but were not significantly different from control levels. In response to androgen treatment alone, however, igf1 and igf1rb transcript levels were significantly upregulated, while the number of type A spermatogonia increased after E2 but decreased after androgen treatment. Clearly, understanding the functional context of steroid treatment effects on igf1 and igf1rb transcript levels requires more work.

In teleost fish, estrogen receptors are expressed in testicular somatic cells and haploid germ cells (Chang et al. 1999, Miura et al. 1999, Bouma & Nagler 2001, Wu et al. 2001, Menuet et al. 2002, Ito et al. 2007), opening the possibility for direct effects. As mentioned earlier, neither a previous study (de Waal et al. 2009) nor the present experiments on testicular gene expression provided evidence for direct effects of E2 on spermatogenesis in tissue culture. When instead of applying estrogens, estrogen production was blocked genetically (Dranow et al. 2016, Lau et al. 2016, Tang et al. 2017) or nuclear estrogen receptor function was lost (Lu et al. 2017), spermatogenesis proceeded normally in male zebrafish. This suggests that testicular genes directly regulated by E2 are not required for spermatogenesis. In our experiments, E2 elevated lhcgr transcript levels. Since there was no change in odf3b transcript levels, large changes in spermatid numbers (also expressing lhcgr; see above) are unlikely to have occurred, as supported by an earlier study (de Waal et al. 2009), suggesting that lhcgr changes ensued in somatic cells, but had no effect on spermatogenesis in tissue culture.

We have proposed previously that the inhibitory effects of E2 on spermatogenesis in vivo reflect first a gonadotropin, and then the ensuing androgen insufficiency (de Waal et al. 2009). As experimental proof of this hypothesis is lacking, we attempted to restore spermatogenesis by exposing E2-treated fish also to androgen during the last two weeks of a five weeks treatment period. This additional androgen exposure regime partially restored GSI and fully restored the number of cysts per area for B spermatogonia, spermatocytes and spermatids, as well...
as the transcript levels of late germ cells marker genes (sycp3 and odf3b). However, we also observed interesting differences between androgen effects in the absence vs the presence of E2. For example, type A und spermatogonia were partially depleted by androgens in the absence of E2, suggesting that androgens also promote the differentiation of this germ cell type. With the high number of this cell type in E2-treated males, it is possible that the increase in the number of type A diff spermatogonial cysts in the group treated with both estrogen and androgen reflects androgen-induced differentiation of A und, present in a much higher number than usual, into A diff while a depletion of A und was prevented by the continued presence of E2. A strong bias towards differentiation following treatment with androgen alone is also suggested by the reduction in the number of cysts containing type B spermatogonia and the increase in the number of cysts containing spermatids.

As mentioned earlier, we assume that one of the main effects of the E2-treatment is a reduction of circulating gonadotropin levels. In salmon, E2 treatment in vivo reduced Fsh plasma levels in both males and females (Dickey & Swanson 1998). E2 also reduced pituitary fshb transcript levels in the zebrafish (Wang et al. 2016), although information on steroid effects on Fsh plasma levels in this species is lacking. While it has been reported that androgen treatment increases pituitary lhβ transcript levels in different fish species, including zebrafish (Lin & Ge 2009, Shao et al. 2013, Melo et al. 2015), this increase is, in salmonids, not followed by increases in Lh plasma levels, which remained undetectable with or without steroid treatment in the 10–5 months preceding spawning (Dickey & Swanson 1998). Since genetic ablation of lhβ or of its cognate receptor did not compromise spermatogenesis in zebrafish (Zhang et al. 2015a,b, Xie et al. 2017), a hypothetical change in Lh release in response to the steroid treatments applied here would have been of limited relevance for spermatogenesis. Hence, the stimulation of spermatogenesis observed after additional androgen treatment may reflect direct androgen effects on the testis. It seems that without E2-pretreatment when normal levels of pro-differentiation growth factors are found, possibly reflecting normal Fsh stimulation of the testis, androgen treatment represents a strong, additional pro-differentiation signal.

We show here that many of the inhibitory effects of E2 exposure on spermatogenesis in vivo can be reversed by additional androgen treatment, suggesting that the E2-induced androgen insufficiency, rather than direct E2 actions on testes, is responsible for the inhibitory effects of E2 on zebrafish spermatogenesis. However, the accumulation of type A und spermatogonia is not reversed and may reflect an effect of the continued presence of E2. E2-induced downregulation of mRNA levels for important pro-differentiation growth factors (Igf3, Ins3, Gsdf) was observed in vivo, but not in tissue culture, probably reflects reduced Fsh release, and may – together with the androgen insufficiency – be part of the background for the accumulation of A und spermatogonia. Since the loss of nuclear estrogen receptors or of gonadal aromatase activity had no repercussions on spermatogenesis, the E2 effects we observed may indicate a negative feedback on the brain–pituitary system. This view is strengthened by recent studies: only following brain aromatase gene knock out (in addition to the gonadal form of the gene) did mutants show increased pituitary Fsh beta subunit, plasma androgen, as well as testicular igf3 and insl3 transcript levels (Tang et al. 2017). This suggests that physiologically, locally produced estrogens in the brain–pituitary system mediate the negative feedback effects that we mimicked by E2 treatment in this study. Testicular androgen release and igf3 and insl3 transcript levels are exquisitely sensitive to Fsh stimulation in zebrafish (García-López et al. 2010, Nóbrega et al. 2015, Crespo et al. 2016) while igf3 (Nóbrega et al. 2015) and insl3 (García-López et al. 2010) do not respond to Lh. It therefore appears that the inhibitory effects of estrogen treatment on spermatogenesis can be explained by negative feedback effects on pituitary Fsh release. These effects became apparent during in vivo exposure of adult animals but not in testis tissue culture approaches. This difference seems relevant when designing experiments to evaluate the effects of compounds present in the environment that potentially interfere with processes regulated by the endocrine system and involve different tissues/ organs. Our results also show that androgen treatment promoted germ cell differentiation independent of the transcript levels of growth factors promoting germ cell differentiation (e.g. igf3, insl3 and gsdf), showing normal or elevated levels in the absence of, or reduced levels in the presence of E2. This suggests on the one hand that androgens and growth factors can stimulate germ cell development through independent pathways, and on the other hand, that E2 treatment in vivo is an effective method to inhibit spermatogenesis, since the inhibition of gonadotropin release combines androgen insufficiency with reduced levels of stimulatory growth factors. Still, androgen treatment promoted spermatogenesis, but at the expense of spermatogonia, while previous work suggested that the gonadotropins (Crespo et al. 2016) and downstream growth factors (Nóbrega et al. 2015, Assis...
et al. 2016) mainly target spermatogonial generations and their entry into meiosis. There is a (slight) redundancy in Fsh and androgen effects with respect to igf3 expression. In tests tissue culture studies, androgens slightly (compared to Fsh) increased igf3 transcript level (Nóbrega et al. 2015); however, we did not observe this effect in the present study. It is possible that this discrepancy is related to the different experimental approaches (androgen exposure in tissue culture for a few days vs in vivo for a few weeks). The independent though not fully redundant (viz. differential effects on spermatogonia) stimulation of spermatogenesis by androgens and growth factors seems to be in line with recent conclusions based on genetic models in zebrafish. Xie et al. (2017) showed that either Lh or Fsh signalling alone is sufficient to support male fertility, and Crowder et al. (2018) and Tang et al. (2018) showed that the loss of the androgen receptor gene clearly reduced testis weight, although the spermatogenic process proceeded and mutant testes contained spermatooza.

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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