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A progestin (17α,20β-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish

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ABSTRACT

Recently, evidence has been provided for multiple regulatory functions of progestins during the late mitotic and meiotic phases of spermatogenesis in teleost fish. For example, our previous studies suggested that 17α,20β-dihydroxy-4-pregnen-3-one (DHP), potentially via Sertoli cells that express the progesterone receptor (*pgr*) gene, can contribute to the regulation of zebrafish spermatogenesis. To further our understanding of the function of DHP at early spermatogenetic stages, we investigated in the present study the expression of genes reflecting Sertoli cell function and spermatogenic development in adult zebrafish testis after DHP treatment in tissue culture. Moreover, using an *in vivo* model of estrogen-mediated down-regulation of androgen production to interrupt adult spermatogenesis, we studied the effects of DHP on estrogen-interrupted spermatogenesis. In this model, DHP treatment doubled the testis weight, and all differentiating germ cell types, such as type B spermatogonia and primary spermatocytes, were abundantly present and incorporated the DNA-synthesis marker (BrdU). Accordingly, transcript levels of germ cell marker genes were up-regulated. Moreover, transcripts of two Sertoli cell-derived genes *anti-müllerian hormone* (*amh*) and *gonadal soma-derived growth factor* (*gsdf*) were up-regulated, as were three genes of the insulin-like growth factor signaling system, *insulin-like growth factor 2b* (*igf2b*), *insulin-like growth factor 3* (*igf3*) and *insulin-like growth factor 1b receptor* (*igf1rb*). We further analyzed the relationship between these genes and DHP treatment using a primary zebrafish testis tissue culture system. In the presence of DHP, only *igf1rb* mRNA levels showed a significant increase among the somatic genes tested, and germ cell marker transcripts were again up-regulated. Taken together, our results show that DHP treatment induced the proliferation of early spermatogonia, their differentiation into late spermatogonia and spermatocytes as well as expression of marker genes for these germ cell stages. DHP-mediated stimulation of spermatogenesis and hence growth of spermatogenic cysts and the associated increase in Sertoli cell number may in part explain the elevated expression of Sertoli cell genes, but our data also suggest an up-regulation of the activity of the Igf signaling system.

1. Introduction

In teleost fish, progestins such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), are known to regulate spermiation, enhancement of sperm motility and to act as a pheromone in male cyprinids [54]. However, potential roles of DHP during early stages of spermatogenesis have been neglected for a long time, even though a small peak of DHP was found in male plasma in a number of teleost species [53,21,24,44,11] and germ cells at an early stage of spermatogenesis were able to synthesize DHP in rainbow trout [63]. Recently, DHP was shown to induce DNA synthesis and to initiate meiosis in an organ culture system for prepubertal Japanese eel (*Anguilla japonica*) testis explants [34]. Moreover, using the same experimental set-up, Miura and colleagues have reported that DHP induced 11β-hydroxysteroid dehydrogenase (11β-Hsd) activity, the enzyme catalyzing the final step in the production of the main androgen in fish, 11-ketotestosterone (11-KT) [40]. More recently, trypsinogen expression in the Sertoli cells surrounding spermatogonia was shown to be a critical element of the molecular mechanism of DHP-stimulated entry into meiosis in eel [36].

There are two types of progesterone receptors in fish. The classical nuclear progesterone receptor (Pgr) functions primarily as ligand-dependent transcription factor to regulate target gene expression [8], while membrane-associated progesterone receptors are potentially involved in non-genomic mechanisms [59]. While both types of progesterone receptors are highly expressed in fish testis [19,15], the membrane-associated progesterone receptor is restricted to germ cells [15,60] in contrast to a broader expression

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pattern of the nuclear Pgr forms. In Japanese eel, *pgr1* mRNA is expressed in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA is detected only in germ cells using RT-PCR [3]. In both Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) the *in situ* hybridization signal for *pgr* mRNA was restricted to Sertoli cells surrounding spermatogonia in testes at the onset of spermatogenesis [5,6]. In zebrafish (*Danio rerio*), there is only one type of Pgr [7,14], and Pgr protein expression in the testis was reported to be restricted to spermatogonia and spermatocytes. The latter finding is in contrast to our own observation, showing *pgr* mRNA expression in Leydig and Sertoli cells which was supported by the observation that germ cell-free mutant zebrafish testis did not show a significant decrease in *pgr* mRNA expression [7]. Functionally, our previous work indicated that in adult zebrafish, DHP induced 11β-Hsd activity in a Pgr-dependent manner [7]. Moreover, we have observed an increase in testicular *pgr* mRNA expression in association with the appearance of late type B spermatogonia in Atlantic salmon [6]. Since male germ cell development depends on their interaction with the somatic Sertoli cells, it is reasonable to assume that Sertoli cell-derived factors mediate DHP-regulated steps in spermatogenesis in fish as was shown in the case of trypsinogen expression [36].

To further understand the function of DHP during early stages of spermatogenesis in zebrafish, we have used an *in vivo* experimental model that is based on estrogen-induced interruption of spermatogenesis [9]. This approach and a primary zebrafish testis tissue *ex vivo* culture system [27] were used in the present study to investigate the effects of DHP on spermatogenesis, including determination of the expression of genes reflecting aspects of Sertoli cell function and spermatogenic development in adult zebrafish testis in order to identify potential factors mediating DHP action.

2. Material and methods

2.1. Fish stocks

Adult (>90 dpf) male outbred zebrafish were used for experimental purposes in the current study. Animal culture, performed using standard conditions for this species [65], handling and experimentation were consistent with the Dutch national regulations, and were approved by the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands).

2.2. In vivo exposure to sex steroids

In order to investigate DHP-induced spermatogenesis *in vivo*, zebrafish were subjected to a two-step treatment. First, the fish were kept for a period of 3 weeks in water containing 10 nM estradiol (E_2) (Sigma–Aldrich, Zwijndrecht, The Netherlands). This regime down-regulated testicular androgen production and interrupted spermatogenesis [9]. For the second step that lasted another 2 weeks, fish were either exposed to 10 nM E_2 (control group) or exposed to 10 nM E_2 in combination with 100 nM DHP (Sigma-Aldrich) (experimental group).

As described by de Waal et al. [9], exposure was performed in a semi-static system in 13 L glass tanks at a temperature of 27 °C. The water was refreshed daily and both 10 mM E_2 and 100 mM DHP stock solution were prepared in deionized water separately by sonication, and then further diluted in aquarium water. During the last 6 h of exposure, fish were transferred to a glass beaker containing water with 5-bromo-2-deoxyuridine (BrdU, a DNA synthesis/proliferation marker) (Sigma–Aldrich) at a concentration of 3 mg/ml.

Fish were euthanized in ice water and total body weight was measured. Both testes of each animal were excised, weighed and the gonado-somatic index (GSI; *i.e.* the ratio between testis weight and body weight) was calculated. One testis was snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction for gene expression analysis. The other testis was first used for *ex vivo* steroid release bioassays, and then processed for morphological analysis or immunocytochemical BrdU detection, as described previously [27].

2.3. Ex vivo exposure

To determine if DHP directly regulates the expression of Sertoli cell-derived factors that showed a change in expression after DHP treatment *in vivo*, an *ex vivo* organ culture system for adult zebrafish testis explants was used [27]. In this system, the basal medium is free of growth factors, and the testicular steroidogenic capacity is down-regulated spontaneously during the first 48 h, so that there is little interference from endogenously produced steroids. Moreover, for the *ex vivo* experiment, adult fish were first kept in water containing 10 nM E_2 for a period of 3 weeks, leading to a down-regulation of Leydig cell activity and an interruption of spermatogenesis [9]. For each individual, one testis served as control for the contralateral one, such that one testis was incubated under control conditions (basal medium only), while the other one was incubated in the presence of 200 nM DHP. Incubations took place in a humidified air atmosphere at 25 °C for 7 days, and medium was refreshed once after three or four days. After incubation, testicular tissue explants were snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction for gene expression analysis.

2.4. Basal ex vivo steroid secretion by zebrafish testicular explants

The acute *ex vivo* steroid release bioassay, described initially for African catfish [51] was adapted recently for zebrafish testis [9]. Incubations lasted 6 h in a humidified air atmosphere at 25 °C in 96-well flat-bottom plates (Corning Inc., New York, USA) using a final volume of 200 µl. Basal culture medium consisted of 15 g/L Leibovitz L-15 (Invitrogen) supplemented with 10 mM Hepes (Merck), 0.5% w/v bovine serum albumin fraction V (Roche, Mannheim, Germany), 0.4 mg/L amphotericine B (Fungizone®; Invitrogen), 200,000 U/L penicillin/streptomycin (Invitrogen) and the pH adjusted to 7.4. Testis tissue was incubated in basal medium to study basal androgen release. Testis tissue was sampled from untreated adults as well as from fish either exposed to 10 nM E_2 or from fish exposed to 10 nM E_2 and 100 nM DHP *in vivo*, as described above. After 6 h of incubation, the medium was harvested and used for quantification of 11-KT ($n = 8 \sim 10$ per condition). The experiment served to study if the previously reported, DHP-induced stimulation of testicular 11β-Hsd activity would be reflected in significant differences of basal androgen release.

2.5. Immunocytochemistry

After *ex vivo* incubation, testicular explants were fixed, embedded, sectioned, and then processed for BrdU-immunodetection, as described previously [27]. The detection of Piwi-like 1 (Piwil1) protein by immunocytochemistry has been carried out as described previously by Houwing et al. [18] using 5 µm paraffin sections from adult zebrafish testis after fixation in 4% w/v phosphate-buffered paraformaldehyde.

2.6. In situ hybridization

Localization of *gonadal soma-derived growth factor* (*gsdf*) mRNA by *in situ* hybridization was performed as described previously [7] using 10 µm cryo-sections prepared from 4% w/v paraformaldehyde-fixed testis tissue from sexually mature zebrafish testis. The

localization of *daz-like gene* (*dazl*) mRNA was performed in a similar way as for *gsdf* mRNA, except that testis tissue was dehydrated and embedded in paraffin, according to conventional techniques, before 5 μm thick sections were used for hybridization with the antisense *dazl* cRNA probe. Neighboring sections were stained with hematoxylin-eosin to distinguish the type of germ cells showing *in situ* hybridization signal. Specific primers for *gsdf* (2643, 5′-GGGCGGGTGTTA*TTAACCCTCACTAA*AGGGCTGGAGCATCTGCGGGAG TCATTGAA-3′, with the T3 RNA polymerase promoter in italics; and 2685, 5′-CCGGG GGGTG*TAATACGACTCACTATA*GGGCCAGTGATGCT-GAACTACGGCTAGTTTG TGTT-3′, with the T7 RNA polymerase promoter in italics) were designed to PCR-amplify a 560 bp cDNA fragment that was used as a template for sense and antisense digoxigenin-labeled cRNA probe synthesis, according to Vischer et al. [62]. Sense and antisense *dazl* cRNA probes were generated as described in Bontems et al. [4].

2.7. Gene expression analysis

For gene expression analysis, total RNA was extracted from testicular samples using RNAqueous®-Micro Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Synthesis of cDNA from total RNA was performed as described previously [7]. Specific primers for zebrafish *steroidogenic acute regulatory protein* (*star*), *piwil1*, *dazl*, *synaptonemal complex protein 3 like* (*sycp3l*), *gsdf*, *insulin-like growth factor 1* (*igf1*), *insulin-like growth factor 2b* (*igf2b*), *insulin-like growth factor 3* (*igf3*), *insulin-like growth factor 1 receptors* (*igf1ra* and *igf1rb*) and the internal control gene *bactin1* were designed for SYBR green expression analysis (Applied Biosystems). Specific primers and FAM-labelled probes for *amh* and the two endogenous controls (*i.e. 18S ribosomal RNA* [18srRNA] and *elongation factor 1α* [*ef1 α*]) were designed for TaqMan expression analysis. The specificity and amplification efficiency of each primer combination was confirmed on serial dilutions of testis cDNA (results not shown). Respective primer and probe sequences are shown in Supplementary Table 1. All real-time, quantitative PCRs (qPCR) were performed in 20 μl reactions and C_t values determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Before normalizing expression data, we analyzed the stability of the internal control genes from *in vivo* and *ex vivo* experiments. *In vivo*, only *18s rRNA* showed stable expression between experimental groups. *Ex vivo*, all three internal control genes showed stable expression between experimental groups. Therefore, *18s rRNA* was used to normalize data from the *in vivo* experiment, while the geometric mean [61] of the three internal control genes was used to normalize the *ex vivo* data (Supplementary Fig. 1). The comparative C_t method was used to calculate the relative mRNA levels [50].

2.8. Statistical analysis

For the *in vivo* exposure experiment, differences between treatment groups for the measured parameters (*i.e.* relative mRNA level, GSI) were compared by Student's *t*-test. For the *ex vivo* gene expression experiments, differences between treatment groups were tested for statistical significance using the paired *t*-test. The analyses were performed using GraphPad Prism4 software package (GraphPad Software, San Diego, CA). Data are presented as the mean ± SEM.

3. Results

3.1. Localization of Piwil1 protein and of gsdf and dazl mRNA in zebrafish testis

In order to evaluate the suitability as germ cell markers, we localized Piwil1 protein and *dazl* mRNA in adult testis tissue. The strongest signal for Piwil1 protein was found in the cytoplasm of type A spermatogonia (Fig. 1A and B), notably in single cells, pairs and 4 type A spermatogonia-containing cysts. In later spermatogonial generations, the labeling was very weak. No labeling was found in meiotic or post-meiotic germ cells.

An *in situ* hybridization signal for *dazl* mRNA was detected in all spermatogonial generations. However, the labeling was less intense in small cysts with type A spermatogonia, while large cysts with type B spermatogonia were intensely stained (Fig. 1C). The labeling intensity was reduced when germ cells entered meiosis, so that we observed only a weak labeling of early (leptotene/zygotene) spermatocytes (Fig. 1D). Starting with pachytene spermatocytes, all subsequent meiotic and postmeiotic stages remained unlabeled.

In contrast to Piwil1 protein and *dazl* mRNA, *gsdf* mRNA was not expressed in germ cells, but was found throughout the testis in thin cytoplasmic extensions that surrounded groups of germ cells (Fig. 1E). Higher magnifications showed that triangular areas often close to the tubular wall were also stained intensely (Fig. 1F). This staining pattern corresponds to the thin cytoplasmic extensions of Sertoli cells and their perinuclear cytoplasmic areas, respectively. There were no differences in the staining intensity between Sertoli cells contacting germ cells at different stages of the spermatogenesis.

The above-described staining patterns of Piwil1 protein and *dazl* mRNA are summarized schematically in relation to the stage of spermatogenesis in Supplementary Fig. 2.

3.2. In vivo effects of E_2 on zebrafish testicular genes expression

Previous studies indicated that after 21 days of exposure of adult male zebrafish to E_2, testis weight decreased significantly and spermatogenesis was inhibited [9]. In the present study, we measured the mRNA levels of the Sertoli cell-specific genes *gsdf* and *amh,* and the insulin-like growth factor signaling-related genes *igf1*, *igf3*, *igf1ra* and *igf1rb*. After 21 days of *in vivo* exposure to E_2, there is a significant decrease of the mRNA expression levels of *gsdf* and *amh* (Fig. 2). Among the insulin-like growth factor signaling-related genes analyzed, *igf1ra* and *igf1rb*, but not the mRNAs coding for the insulin-like growth factors, showed a significant decrease.

3.3. In vivo effects of DHP on zebrafish testis

The GSI of zebrafish exposed to both E_2 and DHP was twice as high as in the group exposed to E_2 only (Fig. 3A, $P < 0.01$). Qualitative morphological analysis of samples collected from zebrafish exposed for 5 weeks to 10 nM E_2 showed that differentiating germ cell types (*i.e.* type B spermatogonia, spermatocytes and spermatids) usually present in the testis of untreated adults (Supplementary Fig. 3) were drastically reduced or absent (Fig. 3C and D). Testis samples collected from fish exposed also to DHP during the last 2 weeks of the 5 weeks long E_2 exposures, on the other hand, showed all these types of differentiating germ cells in abundant numbers (Fig. 3E and F).

To further confirm that DHP treatment stimulated germ cell development *in vivo*, we monitored BrdU incorporation. While BrdU-labeling was found in only a limited number of spermatogonia in testis under E_2-treated conditions (Fig. 3C and D), BrdU-labeled germ cells were abundantly present in testis of fish exposed to both E_2 and DHP (Fig. 3E and F). BrdU-labeling was found in type A and type B spermatogonia as well as in primary spermatocytes, *i.e.* in all germ cell types theoretically able to incorporate BrdU during the short-term exposure of 6 h. Collectively, the morphological analysis shows that DHP induced the proliferation and differentiation of germ cells, resulting in the abundant appearance of type B spermatogonia and primary spermatocytes.

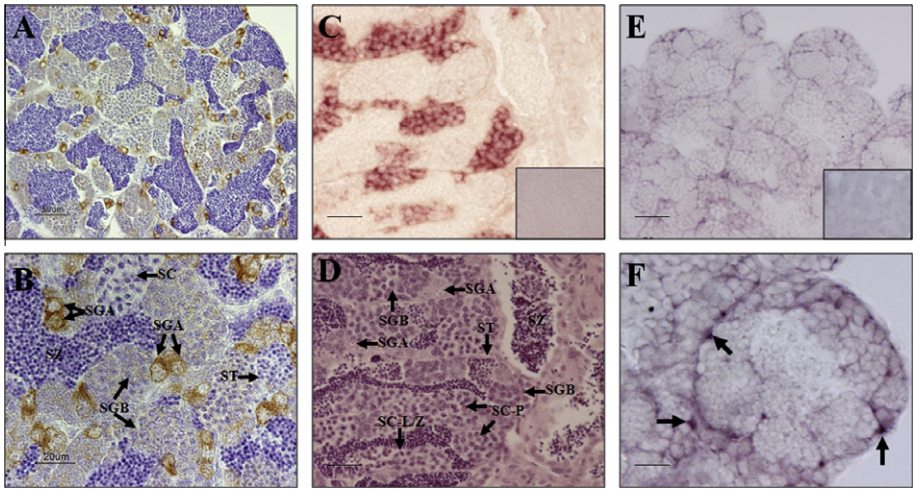

Fig. 1. Localization of Piwil1 protein by immunohistochemistry, and of *gsdf* and *dazl* mRNA by *in situ* hybridization in adult zebrafish testis. Low (A) and high (B) magnification of paraffin sections stained for Piwil1 showed strong cytoplasmic labeling of type A spermatogonia, and very weak labeling of type B spermatogonia. Spermatocytes, spermatids, and spermatozoa remained unstained. Consecutive paraffin-sections, hybridized with the *dazl* antisense cRNA probe (C), or stained with hematoxylin-eosin (D), showed strong staining in the cytoplasm of type B spermatogonia, but a less intense staining in type A spermatogonia. Note that the staining became rather weak in early (leptotene/zygotene) spermatocytes, and had faded completely in pachytene spermatocytes. All subsequent germ cell stages remained unstained. Inset in (C) shows that the sense cRNA probe did not result in specific staining. Low (E) and high (F) magnifications of cryosections hybridized with the *gsdf* antisense cRNA probe showed signal in the cytoplasmic extensions of Sertoli cells forming spermatogonial cysts containing germ cells at different stages of development. Inset in (E) shows that the sense cRNA probe did not result in specific staining. Black block arrows in (F) indicate perinuclear cytoplasm of Sertoli cells. SGA, type A spermatogonia; SGB, type B spermatogonia; SC, spermatocytes; SC-L/Z, primary spermatocytes in the transition from leptonema to zygonema (L/Z); SC-P, primary spermatocytes in pachynema; ST, spermatids; SZ, spermatozoa. Bars indicate 50 μm in (A) and (E), 25 μm in (C) and (D), and 20 μm in (B) and (F).

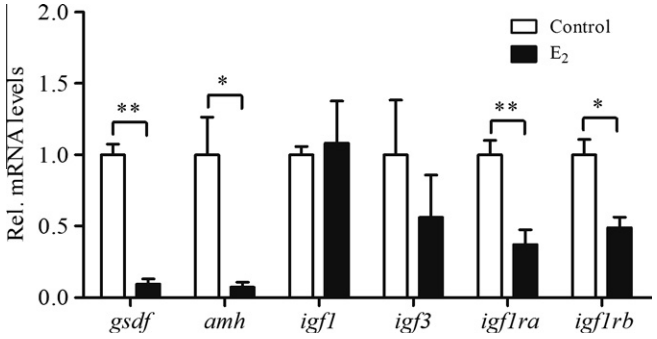

Fig. 2. E_2 effect on mRNA levels of selected testicular genes. Relative (Rel.) mRNA levels of Sertoli cell-specific genes (*gsdf*, *amh*), and of genes of the insulin-like growth factor signalling-related system (*igf1*, *igf3*, *igf1ra*, *igf1rb*) in zebrafish testis after 3 weeks of *in vivo* exposure to E_2 (10 nM). The levels of respective mRNAs were determined by qPCR and normalized to an internal reference (*18s rRNA*). Data are expressed as the mean (±SE; $n = 6–12$) relative to respective transcript levels measured in control fish. Bars marked with $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ are significantly different (Student's *t*-test) from their respective controls.

In order to examine the possibility that DHP exposure may have, at least in part, re-activated the E_2-inhibited steroidogenic system, we quantified the mRNA level of *star*, which controls the rate-limiting step in gonadotropin-stimulated steroidogenesis (*i.e.* the transfer of cholesterol from the outer to the inner mitochondrial membrane); we also quantified the amount of 11-KT released from testis tissue. There was no difference in *star* mRNA levels between control and DHP-treated fish (Fig. 3B). While the basal release of 11-KT from testes of untreated controls usually is ~100 pg 11-KT/mg tissue and hence clearly (~10-fold) above the limit of detection [12], no 11-KT was detected in medium harvested from testes of males exposed to E_2 alone or in combination with DHP. We conclude that DHP treatment did not modulate the previously observed [9] E_2-induced inhibition of testicular androgen production.

Analyzing the results of germ cell-marker gene expression showed that the morphological observations described above were in accordance with significantly elevated expression levels of all three germ cell markers used: *piwil1* (~2-fold), *dazl* (~2-fold), and *sycp3l* (~4-fold) in testis of fish exposed to E_2 and DHP compared to the E_2-treated control group (Fig. 4A); these three genes represent early generations of type A spermatogonia, mainly late spermatogonial generations and spermatocytes, respectively. Moreover, the mRNA expression levels of the Sertoli cell-specific genes *gsdf* and *amh*, and insulin-like growth factor signaling related genes *igf2b*, *igf3* and *igf1rb*, were all significantly increased in testis of fish exposed to E_2 and DHP (Fig. 4A).

3.4. Effect of DHP on zebrafish testicular physiology ex vivo

The limited tissue mass after *ex vivo* culture does not allow carrying out gene expression as well as morphological analysis on the same samples. We decided to analyze testicular explants for gene expression, since the results from our *in vivo* experiments indicated that data on the expression of germ cell markers for spermatogonia and spermatocytes were in accordance with morphological observations (*i.e.* both reflected increased mass and proliferation activity of spermatogonia and spermatocytes). After 7 days of DHP treatment *ex vivo*, testicular explants collected from E_2-pretreated animals showed significantly increased expression levels of germ cell marker genes (*i.e. piwil1*, *dazl* and *sycp3l*) compared with the contralateral (control) testis incubated in the absence of DHP (Fig. 4B, $P < 0.05$). Next to the germ cell markers, only *igf1rb* expression levels showed a significant increase compared with controls (Fig. 4B, $P < 0.05$).

4. Discussion

The effects of substances regulating spermatogenesis are often determined by morphological analysis of testis tissue. Although this is a powerful approach, it is also time-consuming and requires well-trained observers. Quantifying gene expression of mRNAs

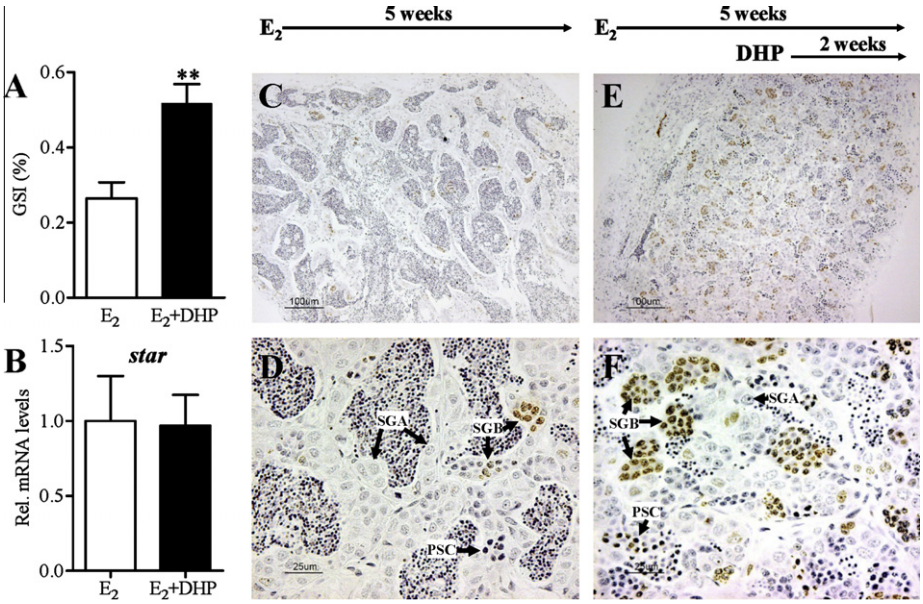

Fig. 3. DHP treatment *in vivo* reversed the inhibitory effect of E_2 treatment on adult zebrafish spermatogenesis. (A) Gonado-somatic index (GSI) of zebrafish maintained in the absence or presence of 100 nM DHP during the last 2 weeks of an in total 5 week-long exposure to E_2 (10 nM) *in vivo*. Bars represent mean values (±SE; $n = 5$). (B) Relative (Rel.) mRNA levels of *star* in testicular tissue of zebrafish maintained in the presence of E_2 alone or in the additional presence of 100 nM DHP (E_2 + DHP) during the last 2 weeks of an in total 5 week-long exposure to E_2 (10 nM) *in vivo*. The level of *star* transcript was determined by qPCR and normalized to an internal control (*18s rRNA*). Data are expressed as mean (±SE; $n = 5$) relative to *star* transcript levels measured in E_2-treated control fish. Immunocytochemical detection of BrdU, an S-phase proliferation marker, in testis tissue from zebrafish exposed to 10 nM E_2 for 5 weeks *in vivo* (C, D), or also exposed to 100 nM DHP (E, F) during the last 2 weeks of a 5 week-long period. For both groups, BrdU was present during the last 6 h of exposure. Note that BrdU-labeled germ cells are much more abundant in DHP-treated testis. SGA, type A spermatogonia; SGB, type B spermatogonia; PSC, primary spermatocytes; Bars marked with ** are significantly different ($P < 0.01$, Student's *t*-test) from their respective controls.

representing specific germ cell types opens the possibility to study the effects of such substances faster. Piwil1 is a highly conserved (from insects to mammals) protein as a marker for early spermatogonia [18]. Our immunocytochemical studies showed that Piwil1 protein is particularly highly expressed in the first 3 generations of type A spermatogonia in zebrafish. *In situ* hybridization studies indicated that *piwil1* mRNA expression is restricted to spermatogonia, with the strongest signal in type A spermatogonia, and a much weaker signal in type B spermatogonia [18]; hence, *piwil1* mRNA and protein expression patterns are very similar. Since loss of Piwil1 protein leads to a loss of germ cells as soon as they attempt to enter the differentiation pathway, the main Piwil1 function (i.e. silencing transposons) seems particularly relevant in early spermatogonia, reinforcing the suitability of this gene (product) as marker for early spermatogonia in zebrafish. The axial/lateral element of the synaptonemal complex is encoded by *sycp3l*, and was chosen as a marker for spermatocytes [41]. Moreover, *dazl* was expressed particularly in the later spermatogonial generations but also, though less strongly, in early spermatocytes and can serve as a marker for rapidly proliferating germ cell generations. This expression pattern in zebrafish conforms with those reported for *dazl* homologues in other fish species, including medaka [67], gibel carp [42] and rainbow trout [30], and in different tetrapod vertebrates, including amphibia [17], birds [22] and mammals [68]. Similar to other members of the *daz* (deleted in azoospermia) family of genes, Dazl is an RNA-binding protein involved in controlling spermatogenesis by regulating the translation of specific mRNAs in germ cells [33]. The DHP-induced changes in the expression levels of the germ cells markers were in accordance with our morphological and BrdU-incorporation studies in the *in vivo* trial, suggesting that analyzing marker gene expression can serve as a reliable tool to quantify clear changes in testicular germ cell composition, such as induced by the steroid hormone treatment used in the present experiments.

Another innovative aspect of the present study is the use of an (E_2-induced) androgen insufficiency model to first interrupt spermatogenesis [9] before starting the treatment with the compound of interest, DHP in this case, to stimulate spermatogenesis. Since DHP induced an increase in testicular 11β-Hsd activity in eel [40] and zebrafish [7] *in vitro*, we tested if DHP treatment increased androgen output. However, we found no evidence for a DHP-mediated increase of 11-KT production above the detection level of our assay system and we can conclude that the stimulatory effect of DHP on zebrafish spermatogenesis is not mediated by an activation of androgen production. Apparently, the increase of 11β-Hsd activity alone [7] is insufficient to generate an elevated androgen output.

Survival and development of germ cells strictly depend on the constant support by testicular somatic cells, in particular Sertoli cells. Previously, we showed *pgr* mRNA expression in Sertoli cells contacting spermatogonia in zebrafish, and we hypothesized that DHP might regulate steps in early spermatogenesis indirectly via Sertoli cells. Indeed, three Sertoli cell-expressed genes, *i.e. amh, gsdf* and *igf3*, were up regulated in testes of animals exposed to DHP *in vivo*. Amh (also known as Müllerian-inhibiting substance) belongs to the Tgf-beta superfamily of signaling molecules. In tetrapods, AMH induces regression of the Müllerian ducts in male embryos. In addition to this early role during sex differentiation, AMH inhibited the differentiation of mesenchymal cells into Leydig cells and decreased the expression of steroidogenic enzymes [20]. In teleost fish, where Müllerian ducts are not formed [26], *amh* genes have been identified in several species (e.g. Japanese eel [35]; Japanese flounder [69]; zebrafish [45]; medaka [23]; rainbow trout [46]), and showed a male-biased pattern of expression in most species (except medaka). Hence, Amh may function during testis differentiation [16]. Moreover, androgen-induced [35] or natural [46] initiation of spermatogonial proliferation and differentiation was associated with a down-regulation of *amh* expression, while

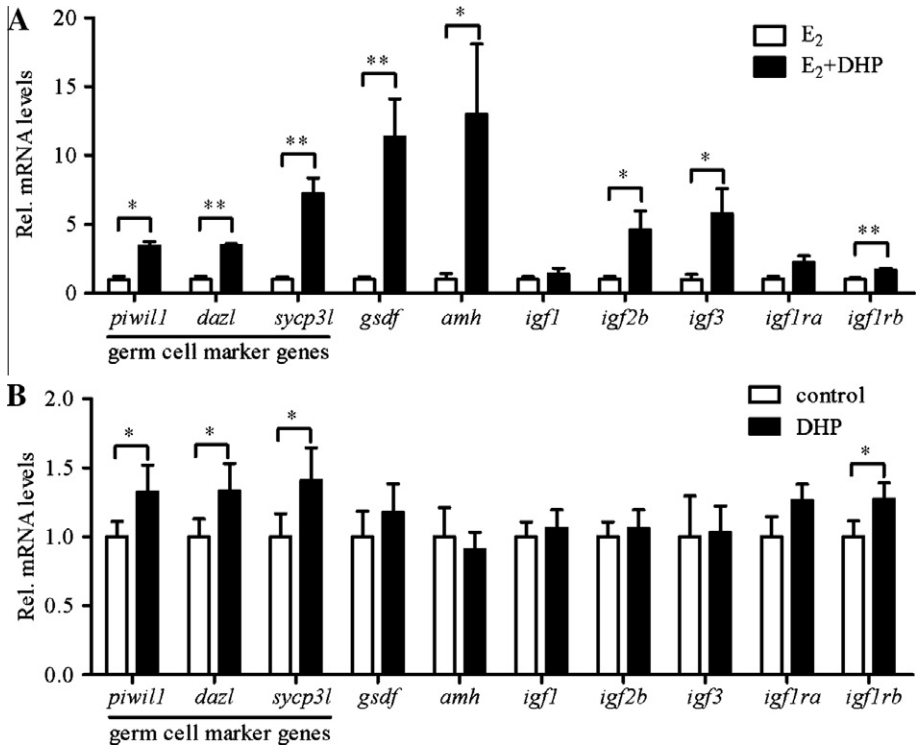

Fig. 4. DHP effect on mRNA levels of selected testicular genes. (A) Testicular relative (Rel.) mRNA levels of germ cell marker genes (*piwil1*, *dazl* and *sycp3l*), of Sertoli cell-specific genes (*gsdf*, *amh*), and of genes related to the insulin-like growth factor signalling system (*igf1*, *igf2b*, *igf3*, *igf1ra*, *igf1rb*) of zebrafish maintained in the presence of E_2 alone or in the additional presence of 100 nM DHP (E_2 + DHP) during last 2 weeks of an in total 5 week-long E_2 (10 nM) *in vivo* exposure period. The levels of respective mRNAs were determined by qPCR and normalized to an internal control (*18s rRNA*). Data are expressed as mean (±SE; $n = 5$) relative to respective transcript levels measured in E_2-treated control fish. Bars marked with $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ are significantly different (Student's *t*-test) from their respective controls. (B) Relative (Rel.) mRNA levels of germ cell marker genes (*piwil1*, *dazl*, and *sycp3l*), of Sertoli cell-specific genes (*gsdf*, *amh*), and of genes related to the insulin-like growth factor signalling system (*igf1*, *igf2b*, *igf3*, *igf1ra*, *igf1rb*) of testicular explants incubated in the absence (control) or the presence of 200 nM DHP for 7 days *ex vivo*. Tissue donors were adult zebrafish kept in water containing 10 nM E_2 for a period of 3 weeks prior to tissue culture. The levels of respective gene was determined by qPCR and normalized to the geometric mean of three internal controls (*18s rRNA*, *ef1* α and *bactin1*). Data are expressed as mean (± SE; $n = 9$) relative to respective transcript levels measured in control fish. Bars marked with $^{*}(P < 0.05)$ are significantly different (paired *t*-test) from their respective controls.

recombinant Amh suppressed the androgen-induced start of the clonal proliferation of spermatogonia in prepubertal Japanese eel [35]. On the other hand, recombinant eel Amh stimulated the single cell proliferation of germ cells in juvenile medaka [56], suggesting that Amh may not only prevent spermatogonial differentiation but may also stimulate expansion of the population of single germ cells, at least in juveniles, while in adults, an inhibitory effect on germ cell proliferation was recorded [38]. Similarly, in adult zebrafish, recombinant Amh inhibits Leydig cell androgen production, decreases the mitotic index of single type A spermatogonia and blocks their differentiation, so that these early spermatogonia accumulate in the testis [57]. This data set suggests that Amh effects on germ cells are converging on early spermatogonia, which is consistent with the location of the protein in Sertoli cells contacting type A spermatogonia [57]. Germ and Sertoli cell numbers increase in a spermatogenic cyst during its development, with a predictable numerical ratio between germ and Sertoli cell number [27], the latter increasing from 1–2 to 14 from the start of cyst development to the completion of meiosis. Our morphological and molecular analyzes showed that DHP treatment clearly increased the number of type A and B spermatogonia, but also of spermatocytes and spermatids, associated with a duplication of the GSI values. Hence, DHP treatment stimulated germ cell proliferation and differentiation through the consecutive steps of mitotic cell cycles and entry into meiosis. Consequently, the number of Sertoli cells must have increased considerably, making it difficult to differentiate between two possibilities of explaining the increased *amh* mRNA levels: they can reflect an increased number of *amh*-expressing Sertoli cells present in the testes of DHP-treated males, or a DHP-induced increase in *amh* expression per Sertoli cell. The expression level of *amh* was decreased 14-fold following *in vivo* exposure to E_2, and then increased 14-fold in response to DHP. This setting may indicated that *amh* expression levels are not directly affected by DHP, but reflect – at least for a considerable part – the elevated number of spermatogenic cysts with type A spermatogonia in testes from DHP-treated males.

Gsdf is also a Tgf-beta superfamily member. In contrast to *amh*, *gsdf* is a teleost-specific gene. Two forms of *gsdf* have been identified recently in rainbow trout and some other salmonid species, while a single form is found in the genome of other fish species [25]. Both *gsdf1* transcript and Gsdf1 protein were expressed in somatic cells of the genital ridge surrounding primordial germ cells during embryogenesis, and in both granulosa and Sertoli cells at later stages. In contrast, *gsdf2* transcript is restricted to testis and to the Sertoli cells. Potentially diverging data exist as regards the possible roles of the Gsdf forms. Sawatari et al. [49] described a stimulatory effect of Gsdf (possibly Gsdf2), on spermatogonial proliferation, while Lareyre et al. [25] reported that *gsdf1* mRNA is highly expressed in Sertoli cells surrounding quiescent type A spermatogonia but becomes down-regulated by androgens in association with the start of spermatogenesis. Gautier et al. [13] recently described the localization of *gsdf* mRNA in the zebrafish testis and found a pattern similar to the one described here, *i.e.* a Sertoli cell-specific expression not dependent on the stage of spermatogenesis. However, there is only a single *gsdf* in zebrafish, and no information is available yet on Gsdf function in zebrafish testis.

Similar to the setting in the case of *amh* expression, E_2–mediated inhibition of spermatogenesis first decreased *gsdf* mRNA level 11-fold, while a 12-fold increase occurred in response to DHP-mediated stimulation of spermatogenesis. Therefore, the increased *gsdf* mRNA in a DHP-stimulated testis may reflect, as discussed for *amh* above, the increased number of Sertoli cells in the testes of DHP-treated animals.

Insulin-like growth factors (IGFs), including IGF1 and IGF2, are evolutionarily conserved peptides across vertebrates and act through a conserved signaling pathway [66]. As a consequence of an additional round of genome duplication, two *igf2* genes (*igf2a* and *igf2b*), and two igf1r genes (*igf1ra* and *igf1rb*) have been reported in zebrafish [48]. Moreover, *igf3* (also known as *igf1b* [70]), which is expressed specifically in gonadal tissue, was discovered in zebrafish, medaka and Nile tilapia, teleost species from different taxonomic orders [64]. Information on the roles of testicular Igfs in adult spermatogenesis is limited in teleosts. Igf1 is required as permissive factor for androgen-stimulated spermatogenesis in a tissue culture system using immature eel testis [37]. Recombinant human IGF1 stimulated the proliferation of rainbow trout spermatogonia in primary cell culture [32]. Our preliminary data indicated that recombinant sea bream Igf1 stimulated zebrafish spermatogenesis in a primary testis tissue culture system [28]. Information on the regulation of expression and on the bioactivity of other Igf peptides (Igf2a, Igf2b and Igf3) in the testis is scarce. It was suggested that Igf3 may be involved in regulating testicular functions in tilapia, as inferred from down-regulation of its mRNA levels by estrogen treatment [2]. Moreover, we have found an up-regulation of *igf3* mRNA in the testis of adult zebrafish following depletion of germ cell numbers by a cytotoxic insult [39]. In the present study, the expression levels of *igf2b*, *igf3* and *igf1rb* were significantly higher in testis showing clearly elevated spermatogenetic activity in response to exposure to DHP *in vivo*. Unfortunately, we do not have information on the testicular cell types expressing Igf peptides in the zebrafish. However, in other fish species (e.g. tilapia [3]; gilthead seabream [43]; trout [29]), Igf peptides are expressed in germ and somatic cells. The concerted response of two ligands and one receptor in testes from DHP-exposed fish makes the Igf signaling pathway an interesting candidate for further studies on its role in zebrafish spermatogenesis.

As a first step in this direction, we examined the effect of DHP on the expression of these genes using a primary testis tissue culture system for zebrafish [27]. After 7 days of incubation with DHP *ex vivo*, testicular explants showed significantly increased expression of the germ cell marker genes. This is in line with a recent study, in which *ex vivo* incubation of primary zebrafish testis tissue fragments with DHP resulted in significantly increased incorporation of BrdU [14]. Next to the germ cell markers, only *igf1rb* mRNA showed a significant increase, which suggests that DHP acts directly on the testis level to up-regulate *igf1rb* expression. The observations that (i) up-regulation of germ cell markers was more prominent in the *in vivo* than in the *ex vivo* experiment, and (ii) that except for *igf1rb* mRNA, growth factor and receptor expression did not change in the *ex vivo* experiment may indicate that the DHP-mediated stimulation of spermatogenesis has a relatively weak direct component, and a relatively strong indirect component that becomes obvious in the *in vivo* experiment. In addition, unpublished work from our laboratory shows that there are no short-term effects (exposure 1 day) of DHP on the testicular *amh* and *gsdf* expression in *ex vivo* studies.

What might be the background for the difference between the *in vivo* and *ex vivo* situation? In a mammalian pituitary cell line, progesterone enhanced FSHβ but repressed LHβ gene expression [58]. A recent study demonstrated direct effects of androgens and estrogens on pituitary gonadotrophs in zebrafish [31], while there is no information on the possible direct effects of DHP on the brain and/or pituitary level. However, membrane progesterone receptors are expressed in zebrafish brain and pituitary [15] and the nuclear Pgr is expressed in the brain [7] in areas known to be involved in the regulation of reproduction, *e.g.* in the preoptic region of the hypothalamus [14] and is up-regulated by estrogen exposure [10]. If also in zebrafish DHP should stimulate *fshb* mRNA expression, the stronger effect of DHP *in vivo* might reflect a combined effect of DHP and Fsh. FSH is a major stimulator of Sertoli cell proliferation in mammals [55] and probably also in fish [52]. Moreover, in fish, Fsh appears to be the pituitary gonadotropin regulating both spermato- and steroidogenesis [12]. A recent study in zebrafish indicated a role for Fsh in stimulating the expression of the *igf3* gene [1], and a study in rainbow trout concluded that Fsh coordinates the expression of stimulatory paracrine factors such as those belonging to the Igf signaling system (including *igf3* mRNA) known to modulate early germ cell proliferation and differentiation [29,32,47]. It therefore seems possible that the stronger effects of DHP when administered *in vivo* may reflect the combined direct action of DHP on the testis level and the effects of triggering the release of an endogenous hormone, possibly Fsh. In view of the E_2-mediated inhibitory effect on steroidogenesis, such Fsh effects would have to be mediated via Sertoli cells.

In summary, we show that DHP strongly stimulates spermatogenesis in zebrafish *in vivo*, involving the up-regulation of expression of somatic and germ cell genes. To our knowledge, this is the first report showing that DHP stimulates proliferation and differentiation of early spermatogonial generations. Moreover, *igf1rb* transcripts levels are up regulated after DHP treatment *in vivo* and *ex vivo*, which suggest that part of the stimulatory effect on spermatogenesis may be mediated via the Igf signaling system.

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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.2013.01.005.

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