Thyroid Hormone Stimulates the Proliferation of Sertoli Cells and Single Type A Spermatogonia in Adult Zebrafish (*Danio rerio*) Testis

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Thyroid hormones participate in regulating growth and homeostatic processes in vertebrates, including development and adult functioning of the reproductive system. Here we report a new stimulatory role of thyroid hormone on the proliferation of Sertoli cells (SCs) and single, type A undifferentiated spermatogonia (A_{und}) in adult zebrafish testes. A role for T_3 in zebrafish testis is suggested by in situ hybridization studies, which localized thyroid receptor α (*thr*α) in SCs and the β (*thr*β) mRNA in Sertoli and Leydig cells. Using a primary zebrafish testis tissue culture system, the effect of T_3 on steroid release, spermatogenesis, and the expression of selected genes was evaluated. Basal steroid release and Leydig cell gene expression did not change in response to T_3. However, in the presence of FSH, T_3 potentiated gonadotropin-stimulated androgen release as well as androgen receptor (*ar*) and 17α-hydroxylase/17,20 lyase (*cyp17a1*) gene expression. Moreover, T_3 alone stimulated the proliferation of both SCs and A_{und}, potentially resulting in newly formed spermatogonial cysts. Additional tissue culture studies demonstrated that Igf3, a new, gonad-specific member of the IGF family, mediated the stimulatory effect of T_3 on the proliferation of A_{und} and SCs. Finally, T_3 induced changes in *connexin 43* mRNA levels in the testis, a known T_3-responsive gene. Taken together, our studies suggest that T_3 expands the population of SCs and A_{und} involving Igf signaling and potentiates gonadotropin-stimulated testicular androgen production as well as androgen sensitivity. **(*Endocrinology* 154: 4365–4376, 2013)**

The relationship between thyroid hormones (THs) and reproduction has been investigated in different vertebrate species (1). The effects of THs are mediated by specific intracellular thyroid hormone receptors (THRs), members of the nuclear receptor superfamily, and similar to the situation in vertebrates in general, TH effects on reproduction in fish are complex (2).

Although potentially not valid for all mammalian species investigated (1), in rodents (rats, mice, and hamsters) T_3 (the biologically most active TH) regulates testicular growth and pubertal maturation by stimulating the terminal differentiation of Sertoli cells (SCs), thereby determining the period during which they can proliferate (3–5). However, the differentiation state of adult mammalian SCs seems less terminal than has been thought for many years (6). With regard to sex steroid signaling, SC differentiation also involves T_3-mediated down-regulation of aromatase gene transcription (7) and up-regulation of androgen receptor gene expression (8). In the early 1990s, Cooke and coworkers (9, 10) showed that testis size and sperm output were enhanced in hypothyroid rats. Further studies demonstrated that T_3 can directly suppress proliferation and induce differentiation of cultured neonatal rat SCs (11), involving up-regulation of cyclin-dependent ki-

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Abbreviations: A_{und}, type A undifferentiated spermatogonia; BrdU, bromodeoxyuridine; CX, connexins; DIG, digoxigenin; IGF1R, IGF 1 receptor; ISH, in situ hybridization; 11-KT, 11-ketotestosterone; qPCR, quantitative PCR; SC, Sertoli cell; TH, thyroid hormone; THR, thyroid hormone receptor.

nase inhibitors (12), thereby eliminating mitogenic effects of FSH (13). Moreover, TH reduced the expression of the neural cell adhesion molecule in SC-gonocyte cocultures isolated from neonatal rat testis (14). Another marker for SC differentiation/maturation in mammals is the expression of connexins (CXs) (15). Gilleron and coworkers (16) demonstrated that T_3 increased the levels of CX43, an important gap junctional protein that participates in the control of cell proliferation. Male mice with a SC-specific loss of *CX43* show an arrest of spermatogenesis at the level of spermatogonia or display a SC-only syndrome (17). In two fish species, rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*), four different Cxs/*cxs* were identified in the testis (18, 19), and Cx43 may be involved in maturation of trout testis because its expression levels increased during the onset of spermatogenesis.

TH plays a critical role in the onset of Leydig cell differentiation and stimulation of steroidogenesis in postnatal rat (20) by increasing the expression of steroidogenic acute regulatory protein. Stimulatory effects on gonadal steroidogenesis have been reported for different species, such as rat (21) and goldfish (22). TH effects on steroidogenesis can be direct or via modulation of gonadotropin-stimulated steroid release, at least in mammals (21).

In contrast to the situation in mammals, fish (and amphibians) show the cystic type of spermatogenesis (23), in which cytoplasmic extensions of SCs form cysts by enveloping a synchronously developing germ cell clone derived from a single spermatogonial cell, the type A undifferentiated spermatogonia (A_{und}). The cyst-forming SCs retain their capacity to proliferate also in the adult fish testis (24), and because growth and development of spermatogenic cysts in the adult testis involve an increase in the number of SCs per cyst (24, 25), SC proliferation can be expected in the adult, spermatogenetically active fish testis. The cytoskeleton of SCs and dynamic junctional complexes between SC-SC and SC-germ cell are important for SC functions (26, 27). Both gap junctions and tight junctions form between SC as part of the SC barrier (previously known as blood-testis barrier), which in fish forms later than in mammals, namely at the beginning of the spermiogenic phase of cyst development (25, 28). It is known that gap junctions between SCs and SCs and germ cells are essential for spermatogenesis and for male fertility (27). Because TH modulate the expression of CXs (19, 20), connexin gene expression may be a useful parameter in studies on T_3 effects in zebrafish testis tissue culture.

In all vertebrates, THRs are expressed by several different cell types, and hence, THs have pleiotropic effects, including effects on the gonads. In goldfish, recent in vivo and in vitro studies suggested that THs affect gonadal steroid synthesis and steroid receptor expression (2). In fact, most experimental studies to date have focused on TH effects on the developing testes and only limited data are available regarding to adult testis functions, whereas direct effects of TH on the fully mature testis have not been studied yet.

The present study addresses the following questions regarding potentially direct effects of TH on adult zebrafish testis: 1) Which testicular cell types express THRs? 2) Does TH modulate testicular expression of selected testicular target genes? 3) Does TH alone, or in association with recombinant zebrafish FSH (rzf FSH), modulate the steroidogenic process? 4) Does TH change the proliferation and differentiation status of SCs and germ cells?

Materials and Methods

Animals

Sexually mature male zebrafish (*Danio rerio*) between 6 and 12 months of age were used in the present study. The animals were kept and handled according to the Dutch national regulation and experiments were approved by the Utrecht University animal use and care committee.

Testicular explants

To study effects of T_3 (Sigma-Aldrich) on androgen release, spermatogenesis, and gene expression, a previously described ex vivo organ culture system for zebrafish testis was used (29). Several experiments were conducted (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), including T_3 dose response (2, 10, and 50 ng/mL) experiments to study gene expression and androgen release. For morphological evaluation, a concentration of 50 ng T_3/mL was selected. The two testes from each fish were incubated in parallel, one testicle (randomly chosen) serving as control for the contralateral one; eight replicates were used for each of the different conditions.

Morphological analysis

After 4 days in culture, zebrafish testes (n = 8) were fixed in 4% buffered glutaraldehyde at 4°C overnight, dehydrated, embedded in Technovit 7100 (Heraeus Kulzer), sectioned at 4 μm thickness, and stained with toluidine blue, according to conventional histological procedures.

Germ and somatic cell proliferation analysis

To evaluate whether T_3 affects the proliferative activity of single spermatogonia A_{und} or SCs, 50 μg/mL bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the medium during the last 6 hours of culture (Supplemental Figure 1A). To study if T_3 action involves the IGF signaling pathway, zebrafish testes were exposed to T_3 in the absence or presence of 10 μM NVP-AEW541 (Selleckchem), an IGF 1 receptor (IGF1R) kinase inhibitor that prevents autophosphorylation of IGF1R and hence blocks IGF receptor function (30), also in zebrafish (31). For data presentation (Figures 5 and 6) and statistical evaluation, the proliferation results from the experiments shown in Supplemental

Figure 1, A and B, where tissue was exposed to 50 ng/mL of T_3, were compared. Because no significant difference was found, the data were combined to form a single group (n = 16). This group was compared to the basal condition (n = 8) and to T_3+NVP (n = 8). Zebrafish testes were fixed at 4°C overnight in freshly prepared methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% acetic acid), after which the tissue was dehydrated, embedded in Technovit 7100, sectioned at 3 μm thickness, and submitted to BrdU immunodetection, as described previously by Leal and coworkers (29).

In vitro 11-ketotestosterone (11-KT) release by zebrafish testes

The androgen release capacity of zebrafish testicular tissue was measured after 1 day of ex vivo culture in two experiments. First, zebrafish testes were incubated in control medium or medium containing 50 ng/mL T_3 (Supplemental Figure 1A). In the second experiment, testes were exposed to medium containing 25 ng/mL rzf FSH or to medium containing 25 ng/mL rzf FSH and 2, 10, or 50 ng/mL T_3 (Supplemental Figure 1C). The 11-KT release into culture medium was quantified using a steroid release bioassay previously adapted for zebrafish testis (32). The results were calculated as nanograms of 11-KT released per milligram of testis tissue.

Gene expression analysis by real-time, quantitative PCR (qPCR)

The capacity of T_3 (2, 10, or 50 ng/mL) to modulate testicular gene expression was investigated after incubation periods of 1 or 4 days. Total RNA was extracted from the samples using an RNAqueous-Micro Kit (Ambion), following the manufacturer's instructions. To estimate relative mRNA levels of selected genes (Table 1), qPCR was performed as described by de Waal and coworkers (32). The levels of elongation factor 1α (*ef1*α) mRNA served as endogenous control RNA, which remained stably expressed under the different experimental conditions (Supplemen-

Table 1. Primers Used for Gene Expression Studies and to Generate DNA Templates for DIG-labeled cRNA Probe Syntheses for In Situ Hybridization

Target Gene	Primers	Sequence (5′ → 3′)
Amh	AD (Fw)	CTCTGACCTTGATGAGCCTCATTT
	AE (Rv)	GGATGTCCCTTAAGAACTTTTGCA
	AF (probe)	FAM-ATTCCACAGGATGAGAGGCTCCCATCC-TAMRA
***Ef1*α**	AG (Fw)	GCCGTCCCACCGACAAG
	AH (Rv)	CCACACGACCCACAGGTACAG
	AI (probe)	FAM-CTCCAATTTTGTACACATCCTGAAGTGGCA-TAMRA
igf3	2680 (Fw)	TGTGCGGAGACAGAGGCTTT
	2681 (Rv)	CGCCGCACTTTCTTGGATT
***igf1r*α**	2362 (Fw)	TACATCGCTGGCAACAAGCA
	2363 (Rv)	TCATTGAAACTGGTCCTTATGCAAT
***igf1r*β**	2595 (Fw)	GTGCTGGTCCTCTCCACACTCT
	2596 (Rv)	TTACCGATGTCGTTGCCAATATC
cx43	3856 (Fw)	CTACAGGGCTCTCCACTCTTTACTTCT
	3858 (Rv)	CGCACTCCAGTCACCCATCT
cx43.4	3859 (Fw)	CGTAGCTGAGGAAAAGAGTGGAAA
	3860 (Rv)	CGTAAGAAAACTCCAGCTCATGGT
ar	2412 (Fw)	ACGTGCCTGGCGTGAAAA
	2413 (Rv)	CAAACCTGCCATCCGTGAAC
insl3	2466 (Fw)	TCGCATCGTGTGGGAGTTT
	2467 (Rv)	TGCACAACGAGGTCTCTATCCA
star	2546 (Fw)	CCTGGAATGCCTGAGCAGAA
	2547 (Rv)	ATCTGCACTTGGTCGCATGAC
cyp17a1	2773 (FW)	GGGAGGCCACGGACTGTTA
	2774 (Rv)	CCATGTGGAACTGTAGTCAGCAA
dazl	3104 (Fw)	AGTGCAGACTTTGCTAACCCTTATGTA
	3105 (Rv)	GTCCACTGCTCCAAGTTGCTCT
piwil1	2542 (Fw)	GATACCGCTGCTGGAAAAAGG
	2543 (Rv)	TGGTTCTCCAAGTGTGTCTTGC
piwil2	2994 (Fw)	TGATACCAGCAAGAAGAGCAGATCT
	2995 (Rv)	ATTTGGAAGGTCACCCTGGAGTA
***thr*α**	3691[a] (Fw)	T3Rpps-TCAAACAAATAACATACTAACACTTTCCTTCTAAGTGGA
	3692[b] (Rv)	T7Rpps-CCATTGCGTCTCATCTCCTTCTG
***thr*β**	3695[a] (Fw)	T3Rpps-TCATTTCAGGCCACGTATGTCCGATC
	3696[b] (Rv)	T7Rpps-TAACTTGGTATGTACCCATTCTGCATGGCCTC

[a] Primer 3691 and 3695 contain the T3 RNA polymerase promoter sequence (underlined) at its 5′-end (T3Rpps; 5′-GGGCGGGTGTTATTAACCCTCACTAAAGGG-3′).

[b] Primer 3692 and 3696 contain the T7 RNA polymerase promoter sequence (underlined) at its 5′-end (T7Rpps; 5′-CCGGGGGGTGTAATACGACTCACTATAGGG-3′).

tal Figure 2). All qPCRs were performed in 20 μL reaction volume and quantification cycle (Cq) values were determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Relative mRNA levels were calculated as reported previously (32, 33).

In situ hybridization

Zebrafish *thrα* and *thrβ*-specific PCR products were generated with primers 3691–3692 and 3695–3696 (Table 1), respectively, containing either T3 (primers 3691 and 3695) or T7 (primers 3692 and 3696) RNA polymerase-promoter sequences attached at their 5′-ends. The ~450- and ~455-bp PCR products obtained were gel purified and served as templates for digoxigenin (DIG)-labeled cRNA probe syntheses, as described by Vischer and coworkers (34). Whole-mount in situ hybridization (ISH) was used to localize thyroid hormone receptor (*thrα* and *thrβ*) mRNAs in testicular tissue fixed in 4% paraformaldehyde in PBS (pH 7.4), according to previously described methods (35). The tissue was treated with proteinase K (20 μg/mL; Sigma-Aldrich) at 37°C for 20 minutes, and acetic anhydride (0.25% in 0.1 M triethanolamine [pH 8.0]; Merck) was included to reduce background after postfixation and before prehybridization. Hybridization with T3 and T7 DIG-riboprobes was performed overnight at 72°C, and DIG immunostaining was performed on the following day using anti-conjugated alkaline phosphatase (1:2000; Roche). Staining was revealed with nitro blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (both Sigma-Aldrich), followed by three consecutive PBS washings. Then, tissue was fixed in 4% paraformaldehyde in PBS, dehydrated, and embedded in Technovit 8100 (Heraeus Kulzer) for plastic sectioning. Sections of 7 μm thickness were counterstained with 0.1% Nuclear Fast Red (in 5% aluminum sulphate solution) for 2 minutes and washed in running tap water for 5 minutes. After a rinse in deionized water, air-dried sections were mounted with Aqueous Mounting Medium (Dako North America Inc).

Statistical analysis

For the ex vivo experiments, differences between control and treatment for the measured parameters (ie, BrdU-positive SCs [Figure 6C], relative mRNA levels, androgen release) were tested for statistical significance using the Student's *t* test for paired observations; significant differences are indicated by an asterisk. When comparisons were made over three or more conditions (ie, *connexin* mRNA levels, mitotic index of SCs, and type A spermatogonia), data were analyzed by one-way ANOVA, followed by a Tukey's multiple comparison test; significant differences are indicated by different lowercase letters. In some cases, data were log transformed to achieve an equal variance. A significance level (P) $< .05$ was applied in all statistical analyses, for which we used the Prism4 software package (GraphPad). Data are presented as the mean ± SEM.

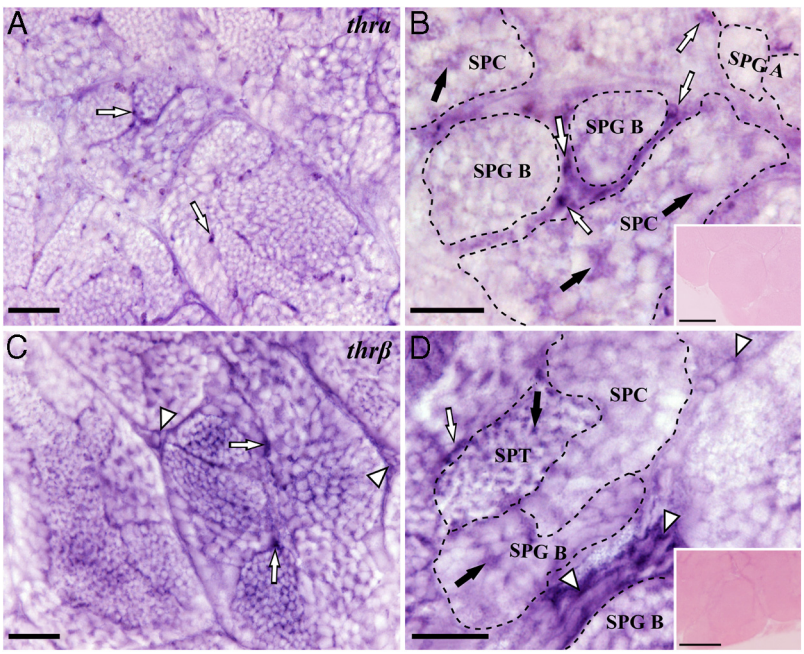

Figure 1. Localization of THR mRNAs in zebrafish testis using whole-mount ISH and post hybridization embedding and sectioning. Low (A and C) and high (B and D) magnifications of 7-μm-thick sections of zebrafish testis, hybridized with the *thrα* and *thrβ* antisense cRNA probes. Staining representing *thrα* mRNA (A and B) was found in SCs (*white arrows*) enveloping germ cells in different stages of differentiation, while *thrβ* mRNA staining (C and D) was found in both SCs (*white arrows*) and Leydig cells (*arrowheads*). Signals for both THRs were also observed in between germ cells (*black arrows*; see *Discussion*). No specific staining was obtained with the sense cRNA probes (*insets* in B and D). Dashed lines indicate cysts containing SPC, spermatocytes; SPG A, type A differentiated spermatogonia; SPG B, type B spermatogonia; SPT, spermatids. The different germ cell types were identified by a combination of their size and number of cells per cyst (see Ref. 25). *Bars*, 25 μm.

Results

Thyroid hormone receptor mRNA localization in zebrafish testis

Identification of the cell types expressing the mRNAs coding for the two THRs described for zebrafish (*thrα* and *thrβ* mRNA) was accomplished by ISH. Based on the shape and localization of the stained cells in the tubular or intertubular compartments, the cells specifically labeled were identified as Sertoli and Leydig cells (Figure 1). The *thrα* mRNA signal was detected in SCs only (Figure 1A). Labeled SCs were in contact with germ cells at different stages of differentiation (Figure 1B). The *thrβ* mRNA signal was localized in both Sertoli and Leydig cells (Figure 1, C and D). For both receptors, labeling also appeared as thin stretches between germ cells in the lumen of spermatogenic cysts (eg, in cysts containing spermatocytes [Figure 1B] or spermatids [Figure 1D]). No specific

signal was obtained when sections were incubated with the sense *thrα* and *thrβ* probes (Figure 1, *insets*).

Triiodothyronine effects on gene expression and androgen release

To investigate if T_3 changes gene expression in the zebrafish testis, mRNA levels of selected Sertoli, Leydig, and germ cell genes (Table 1) were quantified. In a first experiment using 50 ng T_3/mL, only the mRNA levels for IGF 3 (*igf3*), a gonad-specific IGF subtype recently discovered in teleost fish (36), changed significantly on day 1 of the culture (Figure 2A). We repeated the analysis using different doses of T_3, which showed that *igf3* mRNA levels were up-regulated after 1 day in response to the intermediate and highest dose of T_3, 10 and 50 ng/mL, respectively (Figure 2C). No significant response to 50 ng/mL T_3 was observed after 4 days of incubation (Figure 2, A and B). Studying the effect of T_3 on the mRNA levels of two CXs, a transient down-regulation of *cx43* mRNA levels was observed after 1 day of culture (Figure 2D), which recovered after 4 days. No change was observed in the mRNA levels of *cx43.4* in response to T_3.

The release of 11-KT in culture medium was measured after 1 day of culture (Supplemental Figure 1, A and C). Exposure to T_3 alone did not change 11-KT release (Figure 3A). However, T_3-dose response studies in combination with rzf FSH demonstrated that at the highest dose (50 ng T_3/mL) the androgen release was significantly enhanced when compared to rzf FSH only, which in turn clearly stimulated androgen release above the levels found under basal conditions or in the presence of T_3 alone. Moreover, *ar* and *cyp17a1* gene expression was not different from the one under basal conditions when testis tissue was incubated with T_3 alone, while T_3 enhanced FSH-stimulated *ar* and *cyp17a1* gene expression significantly in the presence of 50 ng/mL T_3 (Figure 3B). Previous results showed that rzf FSH alone did not change *ar* mRNA levels but led to a fourfold increase in *cyp17a1* mRNA levels (37). These results suggest that T_3 plays a permissive role for stimu-

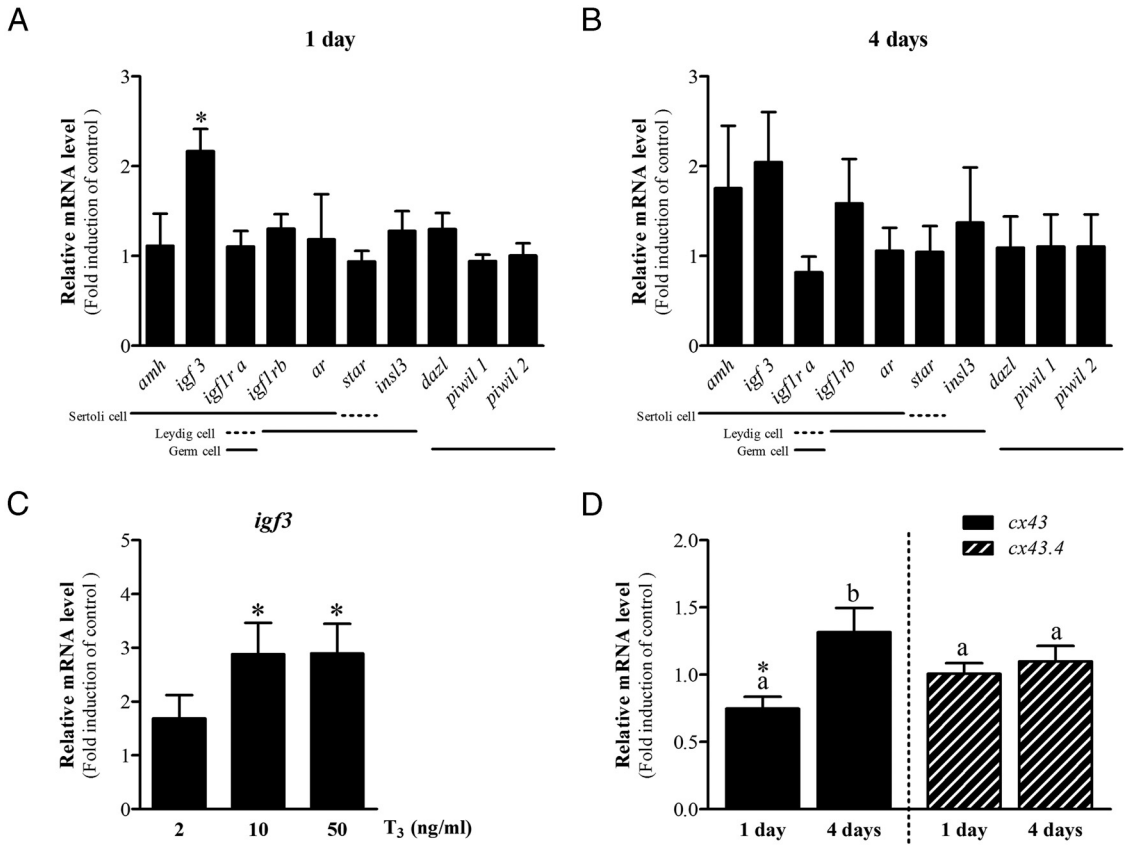

Figure 2. Gene expression analysis after 1 or 4 days of zebrafish testis tissue culture. (A, B) Relative mRNA levels of Sertoli, Leydig, and germ cell-marker genes in testicular explants incubated with T_3 (50 ng/mL) for 1 or 4 days. Bars represent the relative mRNA levels (fold of basal; mean ± SEM; n = 8). Solid lines (—) indicate genes repeatedly localized to the indicated cell types. Dotted lines (• • • •) indicate genes reported to be expressed in the indicated cell types in some but not all studies. (C) Relative mRNA levels of *igf3* after T3 dose response after 1 day of incubation (fold of control; mean ± SEM; n = 6). (D) Testicular mRNA levels of two CXs (*cx43* and *cx43.4*) in tissue after 1 or 4 days of exposure to T_3 (fold of basal; mean ± SEM; n = 8). For each gene, different letters indicate significant differences ($P < .05$) over time. *, significant difference compared to the respective basal condition ($P < .05$).

latory effects of FSH on androgen production and androgen sensitivity in adult zebrafish testis tissue.

Triiodothyronine stimulates germ and SC proliferation

Morphological analysis showed that in testes incubated with basal medium or with T_3, the testicular cyto-architecture remained intact. The spermatogenic process appeared normal and we observed cysts with germ cells at all stages of differentiation, including free spermatozoa in the lumen of seminiferous tubules (Figure 4, A and B). Analyzing the effects of T_3 on spermatogenesis after 4 days of tissue culture (Figure 4C) revealed a higher volume fraction ($P < .05$) of single type A_{und} spermatogonia cysts and fewer cysts with spermatids ($P < .05$) in comparison with the control testes. To determine if T_3 also changed the proliferation activity of these spermatogonia, their mitotic index was obtained. Quantitative evaluation of the sections after immunocytochemical detection of BrdU showed a significantly higher proportion of BrdU-positive type A_{und} spermatogonia in T_3-treated tissue (Figure 5), suggesting that a stimulation of proliferation of this germ cell type is the basis for the increased volume fraction of these cells in morphometric analysis (Figure 4).

Proliferation of SCs was observed as well (Figure 6A). Quantitative evaluation showed that ~30% of the SCs were BrdU-positive after T_3 treatment compared with only ~9% in the control group (Figure 6B). BrdU-labeled SCs can occur in association with germ cells forming spermatogenic cysts, or as "free" SCs (ie, not associated with germ cells). Intriguingly, closer analysis of the BrdU-labeled SCs showed that the majority was either free or associated with type A spermatogonia, whereas the minority was associated with type B spermatogonia, spermatocytes, and spermatids (Figure 6C).

Members of the IGF family are known for their mitogenic activity and we observed that T_3 induced an increase of testicular *igf3* mRNA levels (Figure 2, A and C). Therefore, we studied whether T_3-stimulated proliferation of A_{und} and SCs depended on the functioning of IGF receptors (Supplemental Figure 1B). Incubating testis tissue with T_3 in the absence or presence of the IGF receptor inhibitor NVP-AEW541 showed that T_3-induced BrdU incorporation into A_{und} (Figure 5) or SCs (Figure 6) was abolished or reduced, respectively, leading to a significant decrease ($P < .05$) of the BrdU-labeling index of these two cell types.

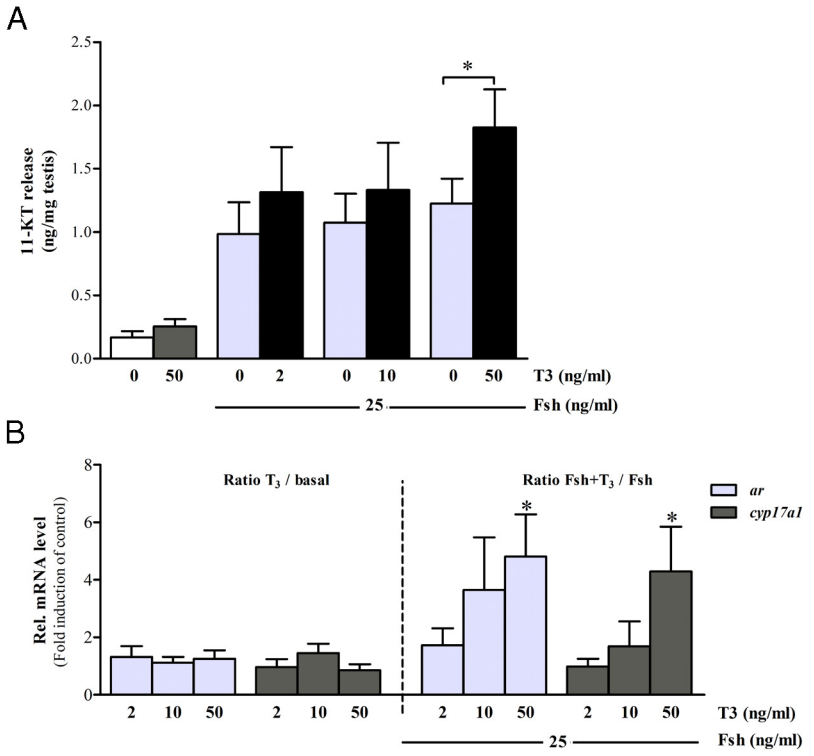

Figure 3. Ex vivo androgen release and gene expression analysis in adult zebrafish testis. (A) Androgen release after 1 day of incubation under basal conditions and with 50 ng/mL T_3 or after exposure to different doses of T3 in the presence or absence of 25 ng/mL rzf FSH. Results are given as the amount of 11-KT produced in nanogram per milligram weight of testis tissue incubated. (B) Testicular *ar* and *cyp17α1* mRNAs levels (fold of basal; mean ± SEM; n = 8) after 1 day of exposure to different doses of T_3, alone or in combination with rzf FSH. *, significant difference from the respective control ($P < .05$).

Discussion

In vertebrates, THs have several functions and THR expression in the testis suggests THs participate in regulating male reproduction. In the present study, we investigated potential roles of T_3, alone or in combination with rzf FSH, on adult zebrafish steroidogenesis and spermatogenesis, using morphological, physiological, and molecular approaches.

It is known that THs work via their specific intracellular THRs, and studies on the *thrα* and *thrβ* mRNA levels in testis tissue of the seasonally reproducing brook trout demonstrated seasonal variations with a constant expression throughout spermatogenesis while the higher expression pattern was observed after spawning season (20). However, the testicular cell types expressing the THRs have not been clearly identified in fish. In mammals, THRs have been localized to SCs by different laboratories, while the staining in

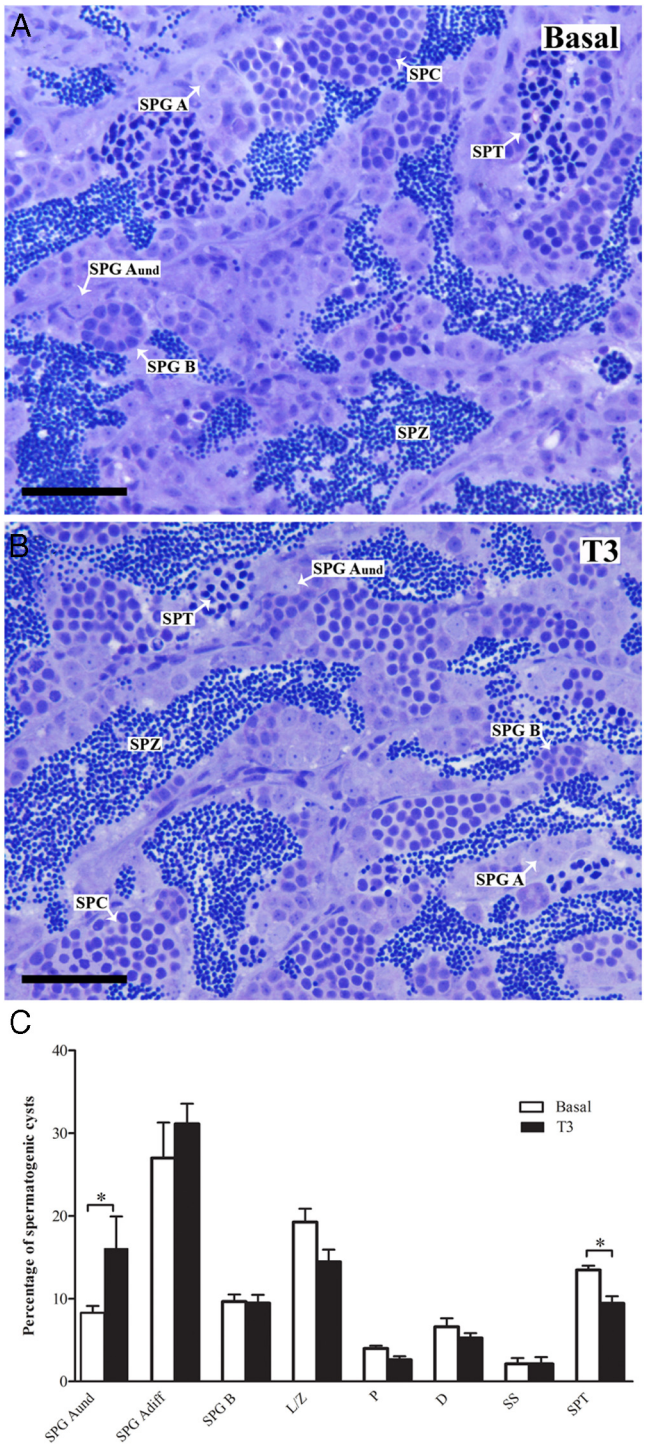

Figure 4. Morphological analysis of zebrafish testis after tissue culture. (A, B) Histological sections stained with toluidine blue showing normal cyto-architecture of zebrafish testis after 4 days of tissue culture with or without 50 ng/mL of T_3. All germ cell types were present in the testis. (C) Morphometric quantification (% of the total number of cysts at different stages of germ cell development; mean ± SEM; n = 8) of the different germ cell cysts in zebrafish. D, diplotene primary spermatocytes; L/Z, leptotene/zygotene primary spermatocytes; P, pachytene primary spermatocytes; SPG A_{diff}, type A differentiated spermatogonia; SPG A_{und}, type A undifferentiated spermatogonia; SPG B; type B spermatogonia; SPT, spermatids; SPZ, spermatozoa; SS, secondary spermatocytes. Bars marked with asterisk are significantly different from respective controls ($P < .05$). *Bars*, 50 μm.

germ cells and in interstitial cells is debated (38, 39). Also in zebrafish, information on the testicular cell types expressing THRs is missing but is needed to direct further studies as regards physiological functions of THs in the testis. We found that SCs, contacting germ cells in different stages of spermatogenesis, expressed both THR forms while Leydig cells expressed *thrβ* only. We also found stained areas between germ cells in a number of (often larger) cysts. In cod testis intermediate cytoskeletal protein of SCs, vimentin, also was detected in between germ cells (40). Similarly, FSH receptor protein localization by immunohistochemistry in eel testis revealed staining in between germ cells (41). These data suggest that SCs forming a spermatogenic cyst do not form a simple, saclike space but may show a more complex structure toward the germ cell-contacting luminal side of the cyst, perhaps with cytoplasmic extensions inserted between germ cells. Future morphological/ultrastructural work may provide evidence for this assumption. Collectively, our data indicate that THs modulate testicular functions via two important somatic cell types, the Sertoli and Leydig cells, whereas direct effects on germ cells seem unlikely in zebrafish. As in other vertebrates, the localization of *thrα* also in zebrafish SCs indicates that this cell type is an evolutionary conserved target for THs. As in some (but not all) other models (42, 43), we detected *thrβ* also in Leydig cells. In zebrafish germ cells were THR negative, different from reports in rat where germ cells from intermediate spermatogonia to pachytene spermatocytes expressed *Thrα1* (38, 43). Our results suggest that TH effects on SC and germ cell proliferation are mediated by THRs in SC, whereas T_3-modulation of steroidogenesis is mediated by Thrβ in Leydig cells. Modulatory effects of TH on androgen levels were described in rat in vivo (44), but our study provides original information on the fact that TH directly modulates zebrafish testicular androgen production (see next page).

After 1 day of tissue culture, T_3 significantly increased the *igf3* mRNA levels. Interestingly, this gonad-specific *igf* type, recently discovered in fish but not present in tetrapod vertebrates (36), has been localized to SCs in adult zebrafish testis and may play a role in the recovery following a cytotoxic insult in adult zebrafish testis (45, 46). In hypothyroid rats T_3 greatly stimulated SC IGF1 production in vivo, also incubation of cultured SCs with T_3 increased the production of IGF1 (47). It appears that a stimulatory role of TH on the release of IGF family members by SCs is an evolutionary conserved mechanism in vertebrates.

Incubation of testicular fragments collected from maturing brook trout during the rapid growth phase of the testis with T_3 increased *cx43* mRNA levels (19). In our study, exposure of the fully grown adult testis to T_3 did not

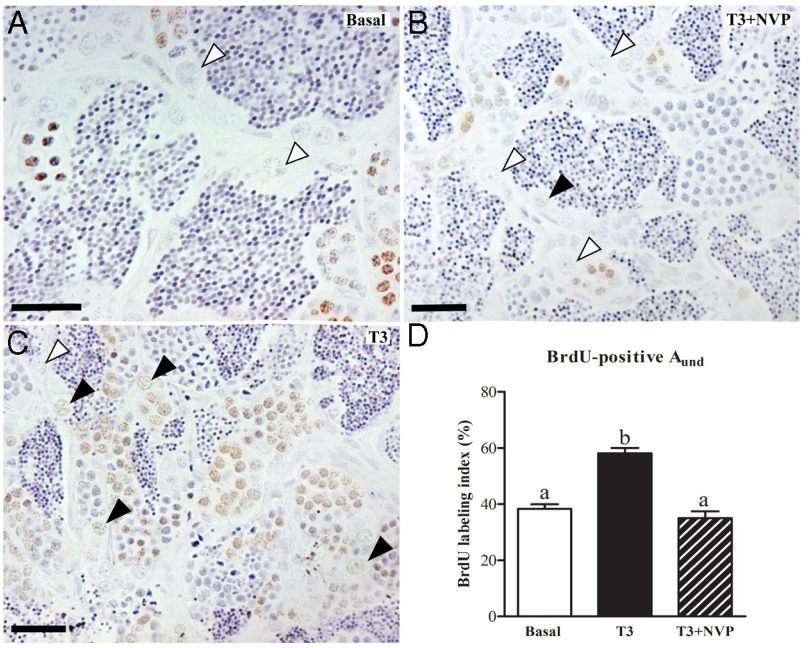

Figure 5. Localization and quantification of BrdU incorporated during the last 6 hours of incubation into zebrafish testis tissue explants after 4 days in culture. Transversal section showing BrdU immunodetection in tissue incubated in basal medium (A), in the presence of 50 ng/mL T_3 (B), or in medium containing 50 ng/mL T_3 + 10 μM NVP-AEW541 (C). (*black arrowheads*) BrdU-positive single type A undifferentiated spermatogonia (A_{und}). (*white arrowheads*) unlabeled A_{und}. (D) BrdU labeling index of type A_{und} (% of total number of BrdU-positive A_{und}). Bars, mean ± SEM (n = 8 or 16; see *Materials and Methods*). Different letters indicate significant differences ($P < .05$) between incubation conditions. *Bars*, 25 μm.

increase *cx43* mRNA levels significantly above control levels. However, comparing *cx43* mRNA levels over time, the levels first decreased after 1 day, before increasing in T_3-exposed tissue fragments toward day 4. In a murine SC line, THs increased *Cx43* mRNA levels and (16, 48) inhibited SC proliferation, which was associated with the expression of cell-cycle regulatory proteins such as S-phase kinase-associated protein 2 and cyclin-dependent kinase inhibitor p27Kip1 (also called CDKN1B) (16, 48–50). Conversely, in the SC-specific connexin 43 knockout mice (SC-*Cx43* KO), SC number was increased 73% compared with wild-type mice, indicating that CX43 is involved in the pubertal cessation of SC proliferation (13). In adult zebrafish testis, on the other hand, THs stimulated SC proliferation. Although THs may play distinct roles in piscine and mammalian testes, the downstream mechanisms underlying SC proliferation in both models may involve the down-regulation of Cx43. However, no changes were recorded for another extracellular matrix gene transcript (*cx43.4* mRNA).

THRs are ligand-regulated transcription factors that bind TH while being bound to DNA sequences of target genes where they can then interact with corepressor and coactivator complexes (51). Analyzing the T_3 effects on the expression of selected target genes, we observed that the two Leydig cell genes *star* and *insl3* did not change significantly, and neither did basal androgen release. Similarly, in goldfish, in vitro experiments demonstrated that T_3 did not change androgen levels significantly (2). However, studying gonadotropin-stimulated estrogen release from maturing (early vitellogenic) ovarian follicles isolated from rainbow trout THs were found to amplify the steroidogenic gonadotropin effect (52). The present study showed that also for the adult zebrafish testis, T_3 potentiated FSH effects on steroid release and gene expression. In the adult rat testis recovering from a cytotoxin-induced loss of Leydig cells, TH treatment stimulated Leydig cell recovery and increased androgen release (53), whereas in adult rat rendered transiently hypothyroid, the circulating testosterone levels were significantly decreased (44). In zebrafish, T_3 alone did not modulate the acute androgen release, but clearly enhanced FSH-stimulated *cyp17a1* and *ar* gene expression and androgen release. Piscine Leydig cells express FSHr/*fshr* (41, 54) and FSH is a potent steroidogenic hormone in fish (37, 55), while previous studies (42) have shown that androgens do not stimulate *cyp17a1* gene expression, so that we can attribute the increased expression of this gene to FSH. Hence, our data suggest that in the presence of rzf FSH, T_3 potentiates the trophic effects of FSH on the steroidogenic system in the adult zebrafish testis. At the same time, T_3 had a permissive effect because it was only in concert with T_3 that FSH increased *ar* gene expression. Hence, T_3 increased both androgen production and sensitivity of adult zebrafish testis tissue. In general, our findings support the concept of a direct, stimulatory crosstalk between THs and the androgen-producing system that was recently proposed to operate in vertebrates (56).

In testes treated with T_3 for 4 days, the volume fraction of type A_{und} spermatogonia increased. Analyzing the BrdU-labeling index showed that proliferation of this particular germ cell type (but also of SCs) was stimulated. Interestingly, treatment of African catfish (*Clarias gariepinus*) with thiourea, a TH inhibitor, compromised spermatogenesis, leading to a decrease in spermatid/spermatozoa counts (57). This observation is compatible with the assumption that TH-stimulated production of type A spermatogonia and SCs (in conjunction with the enhanced

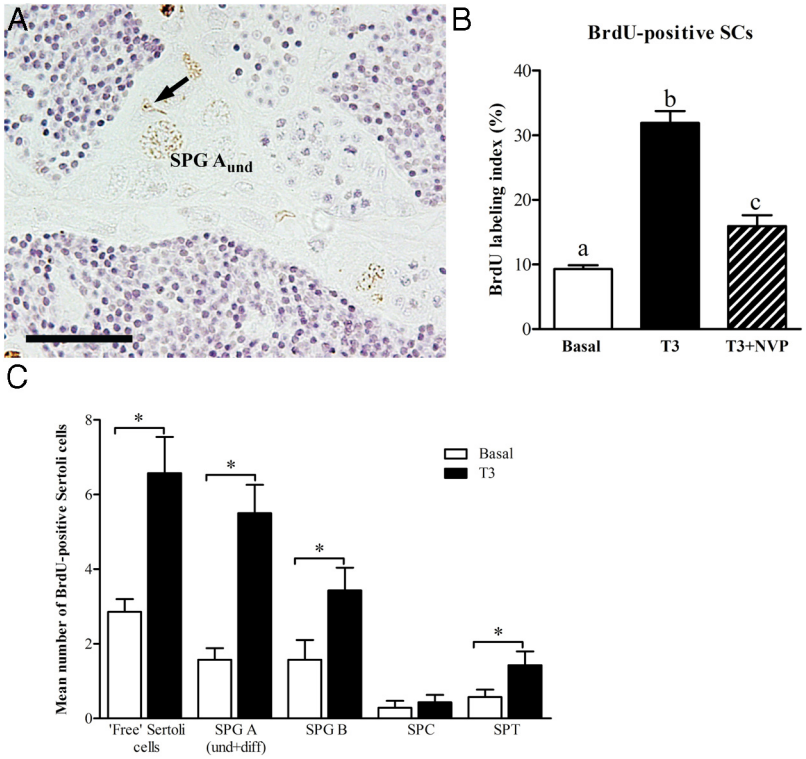

Figure 6. Localization and quantification of BrdU incorporated during the last 6 hours of incubation into zebrafish testis explants incubated with 50 ng/mL T_3 in the absence or presence of 10 μM NVP-AEW541 ex vivo for 4 days. (A) Labeled SC nucleus (*arrow*) associated with a BrdU-positive single type A_{und} spermatogonium. (B) BrdU labeling index (% of total number of cells) of SCs. Different letters indicate significant differences ($P < .05$) between incubation conditions. (C) Treatment with T_3 increased the labeling index of BrdU-labeled SCs associated, or not ("free"), with different germ cell cysts. SPG A (und+diff), type A undifferentiated and differentiated spermatogonia; SPG B, type B spermatogonia; SPC, spermatocytes; SPT, spermatids. "Free" SCs are apparently not associated with germ cells. *Bars*, mean ± SEM (n = 8 or 16; see *Materials and Methods*). Bars marked with asterisk are significantly different from its respective basal condition control ($P < .05$). Bar = 30 μm.

androgen signaling) are relevant in vivo. Remarkably, in zebrafish, an IGF receptor inhibitor abolished the T_3-stimulated increase of the mitotic index of type A_{und} spermatogonia, demonstrating that T_3-stimulated proliferation of A_{und} spermatogonia is mediated via a factor activating IGF receptors in the zebrafish testis.

In mammals, the effect of T_3 on SC proliferation has been comprehensively discussed. Several studies demonstrated that neonatal hypothyroidism and hyperthyroidism affect the number of SC by extending or shortening, respectively, the period of proliferation (3, 58, 59). Our observation of T_3-stimulated proliferation of SCs contrasts with the mammalian situation. On the other hand, SC proliferation is an expected event in the adult fish testis that accompanies growth and development of spermatogenic cysts (24) and probably involves activation of FSH signaling (60). Interestingly, in the present study, an important fraction of proliferating SCs was either associated with type A spermatogonia or was not associated with germ cells at all (referred to as "free" SCs). This suggests that this type of SC proliferation created additional germ cell support capacity, perhaps mediated by the THR forms expressed by SCs. In addition, analyzing SC proliferation in the presence of the IGF receptor inhibitor, the BrdU-labeling index decreased, suggesting that T_3-stimulated SC proliferation is in part mediated by IGF/Igfr signaling. Our observations in zebrafish seem congruent with conclusions based on in vitro studies on mouse SC, where inactivation of the IGF1 receptor gene decreased SC proliferation and also increased SC apoptosis (30). Moreover, a recent study demonstrated that SC-specific loss of insulin/IGF signaling in mice strongly reduced SC proliferation and testis size (61). We propose that an evolutionary conserved mechanism to stimulate SC proliferation is mediated in zebrafish by the gonad-specific Igf3.

Considering that germ cells are THR negative, the T_3-induced increase in number and mitotic index of type A_{und} spermatogonia could be mediated by somatic cells, possibly SC. Elevated SC numbers may have allowed this spermatogonial population to expand, potentially supported by an increased availability of SC-derived growth factors stimulating spermatogonial development. Because our results have shown that the IGF signaling pathway mediated T_3-stimulated proliferation of SC and type A_{und} spermatogonia proliferation, it is tempting to speculate that T_3 triggered an autocrine Igf3 loop to stimulate SC proliferation, and a paracrine loop to stimulate spermatogonial proliferation. Interestingly, in rainbow trout testis IGF receptor expression has been found in both somatic and germ cells (62). Assuming that newly formed SCs associate with single type A_{und} spermatogonia and hence form new spermatogenic cysts, the spermatogenic capacity of the testis would increase. On the other hand, hypothyroidism induced in vivo in pubertal Nile tilapia (*Oreochromis niloticus*) increased SC and germ cell numbers per cyst (63). This effect is not exactly opposite to the one that we have observed in adult zebrafish, because it was the size, not the number, of cysts that changed in pubertal tilapia testis. Hence, as described previously for mammals

(14), T_3 may play different roles depending on the ontogenetic stage of development and the species investigated (14).

Conclusion

The direct effects of T_3 on the zebrafish testis are coordinated by receptors expressed by Sertoli and Leydig cells. When present alone, T_3 stimulates SC and type A_{und} spermatogonia proliferation. When T_3 and FSH are present at the same time, we can expect an increase in the number of SC and single type A_{und} spermatogonia, but also a potentiation of the steroidogenic activity of FSH and of the androgen sensitivity of the testis. Because androgens are known to stimulate in particular the somewhat more advanced stages of germ cell differentiation in fish (23, 64) and mammals (65), we speculate that the joint action of T_3 and FSH would result in more newly formed spermatogenic cysts that would then be stimulated to differentiate in an androgen-driven manner.

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Important aspect of the paper: The direct effects of T_3 on adult zebrafish testis include stimulation of proliferation of SCs and type A undifferentiated spermatogonia as well as potentiation of gonadotropin-stimulated androgen signaling.

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