

Igf Binding Proteins Protect Undifferentiated Spermatogonia in the Zebrafish Testis Against Excessive Differentiation

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IGF binding proteins (IGFBPs) modulate the availability of IGFs for their cognate receptors. In zebrafish testes, IGF3 promotes the proliferation and differentiation of type A undifferentiated (A_{und}) spermatogonia, and *igf3* expression is strongly elevated by FSH but also responds to T_3 . Here we report the effects of FSH and T_3 on *igfbp* transcript levels in adult zebrafish testis. We then examined T_3 and FSH effects on zebrafish spermatogenesis and explored the relevance of IGFBPs in modulating these T_3 or FSH effects, using a primary tissue culture system for adult zebrafish testis. T_3 up-regulated *igfbp1a* and *igfbp3* expression, whereas FSH reduced *igfbp1a* transcript levels. To quantify effects on spermatogenesis, we determined the mitotic index and relative section areas occupied by A_{und} , type A differentiating, or type B spermatogonia. In general, T_3 and FSH stimulated spermatogonial proliferation and increased the areas occupied by spermatogonia, suggesting that both self-renewal and differentiating divisions were stimulated. Preventing IGF/IGFBP interaction by NBI-31772 further increased T_3 - or FSH-induced spermatogonial proliferation. However, under these conditions the more differentiated type A differentiating and B spermatogonia occupied larger surface areas at the expense of the area held by A_{und} spermatogonia. Clearly decreased *nanos2* transcript levels are in agreement with this finding, and reduced *amh* expression may have facilitated spermatogonial differentiation. We conclude that elevating IGF3 bioactivity by blocking IGFBPs shifted T_3 - or FSH-induced signaling from stimulating spermatogonial self-renewal as well as differentiation toward predominantly stimulating spermatogonial differentiation, which leads to a depletion of type A_{und} spermatogonia. (*Endocrinology* 157: 4423–4433, 2016)

Spermatogenesis is a complex cellular developmental process that requires complex regulatory mechanisms. In vertebrates, the endocrine system has evolved as master control system of spermatogenesis and signals mainly toward testicular somatic cells such as Leydig cells, Sertoli cells (SCs), and myoid cells, which then communicate with the germ cells via short-range signaling systems. The cellular basis of spermatogenesis is a population of spermatogonial stem cells, which form part of the population of the type A undifferentiated spermatogonia (A_{und}), usually present as single cells in close contact with Sertoli cells. Spermatogonial stem cells can be quiescent, self-renew to

produce more A_{und} , or differentiate into subsequent developmental stages to eventually produce spermatozoa (1). To sustain spermatogenesis, a balance between self-renewal and differentiation is required (1).

Spermatogenesis is primarily regulated by two pituitary hormones, FSH and LH. In tetrapod vertebrates, the FSH receptor (*Fshr*) is expressed by SCs, whereas the LH/choriogonadotropin receptor (*Lhcgr*) is expressed by Leydig cells. However, in fish, Leydig cells also express the *Fshr* (2, 3), and piscine FSH is a potent steroidogenic hormone (4). Recombinant eel FSH induces the proliferation and differentiation of spermatogonia by stimulating androgen

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Abbreviations: A_{diff} , type A differentiating spermatogonia; A_{und} , type A undifferentiated spermatogonia; BrdU, 5-bromo-2'-deoxyuridine; IGFBP, IGF binding protein; IGFR, IGF receptor; qPCR, quantitative PCR; SC, Sertoli cell.

production in explants of prepubertal eel testis tissue (2). In zebrafish, androgens also exert a stimulatory effect on spermatogenesis (5), and recombinant zebrafish FSH stimulated testicular androgen production (4). However, different from the eel model, FSH stimulated spermatogonial proliferation and differentiation also in an androgen-independent manner in the adult zebrafish testis (6), involving the SC-derived growth factor IGF3. Similarly, studies in rainbow trout showed that close to 100 genes responded to FSH in an androgen-independent manner (7), whereas more than 200 testicular genes fall into this category in the zebrafish (8). Genetic studies showed that inactivating a single gonadotropin or a single gonadotropin receptor did not result in spermatogenic defects, probably because FSH and LH regulate steroidogenesis (2–4), and both LH and FSH signaling had to be inactivated to achieve infertility in male zebrafish (9, 10). However, also other hormones and short-range signaling molecules are involved in regulating spermatogenesis.

Thyroid hormones are relevant for male reproduction in mammals and fish by modulating the number of functionally differentiated SCs (11–16), involving down-regulation of aromatase and up-regulation of androgen receptor gene expression (16, 17). In zebrafish, thyroid hormone receptors (*thrs*) are expressed in both SCs and Leydig cells and T₃ stimulated the formation of new cysts (self-renewal) by promoting the proliferation and accumulation of SCs and of A_{und} (18). The T₃ effects were mainly mediated by increasing SC *igf3* expression and were completely and partially blocked, respectively, for A_{und} and SC proliferation, by an IGF receptor (IGFR) inhibitor (18).

The IGF family of growth factors promotes proliferation and differentiation of diverse cell types. In most vertebrates, the IGF signaling system is composed of IGF1 and IGF2, two IGFRs, and the IGF binding proteins (IGFBPs) (19). Prior to IGF/IGFR interaction, IGFBPs bind IGF with equal or higher affinity than IGFRs (19, 20). Therefore, IGFBPs can modulate the bioavailability of IGFs, thereby inhibiting or potentiating IGF actions (20–22). IGF3 (previously referred to as IGF1b) is the most recently discovered ligand member of this system that has been identified in gonadal tissue of fish (23). As mentioned above, *igf3* is expressed in zebrafish SCs, and FSH and T₃ increased testicular *igf3* transcript levels (6, 18). Gonadotropin-stimulated *igf3* gene expression has also been reported for immature rainbow trout testis (7) and zebrafish ovarian tissue (24, 25). In zebrafish, we have shown recently that recombinant zebrafish IGF3 promoted in an androgen-independent manner the proliferation of type A_{und} and type A differentiating spermatogonia (A_{diff}) and up-regulated the expression of genes related to spermatogonial differentiation (6). Insulin/IGF signaling regulates early stages of male germ cell development in species representing diverse animal

groups, ranging from worms (26, 27) and insects (28) to mammals (29), underlining the relevance of further examining this system. Our previous work showed that testis tissue expressed *igf1*, *igf2a*, *igf2b*, and *igf3*, and both known zebrafish *igfr* variants (6, 30, 31). However, information about the IGFBPs and their potential implication in modulating spermatogenesis has not been addressed yet. Here we studied the effect of T₃ and FSH on spermatogonial proliferation and differentiation, on *igfbp* transcript levels, and we explored the potential contribution of IGFBPs to mediating T₃ or FSH effects by using an IGFBP inhibitor.

Materials and Methods

Animals

Adult male zebrafish between 3 and 12 months of age were used in this study. To study gene expression and for morphometric analyses, six to eight animals were used per experiment. All experiments followed the Dutch National regulations for animal care and use in experimentation, and the experimental protocols have been submitted to and were approved by the Utrecht University Experimental Animal Committee.

Tissue culture

Adult zebrafish testes were dissected for tissue culture experiments using a previously described system (5), in which one testis was incubated under control conditions, the contralateral one under experimental conditions (ie, with medium containing hormones and/or IGFBP inhibitor). In this experimental system, two conditions are compared at a time, which in the present study were basal vs T₃, T₃ vs T₃ in the presence of 10 μ M NBI-31772, basal vs FSH, or FSH vs FSH in the presence of 10 μ M NBI-31772. According to previous results (4, 18), T₃ (Sigma-Aldrich) was used at a concentration of 50 ng/mL, recombinant zebrafish FSH was used at a concentration of 100 ng/mL (see reference 4 for details on the production and characterization of recombinant zebrafish FSH), and testis tissue was incubated for 1 (gene expression) or 3 or 4 days (gene expression or morphology). NBI-31772 is a nonpeptide IGFBP ligand that exhibits high affinity for all six mammalian IGFBPs without interacting with the IGF receptors and thus increasing free biologically active IGF (32, 33). The affinity for NBI-31772 is quite similar among IGFBPs (K_i 1.18–5.64 nM), except for IGFBP6, which shows a lower affinity (K_i 16.06 nM) (32). In zebrafish, 10 μ M NBI-31772 increased cardiomyocyte proliferation during heart regeneration (34). As described above, we used NBI-31772 in combination with T₃ or FSH. Therefore, we also examined whether 10 μ M NBI-31772 alone (ie, comparing basal vs NBI-31772) affected the mitotic index, the section areas occupied by different spermatogonial generations, or testicular gene expression.

Finally, zebrafish testes were incubated for 3 days in the presence of recombinant zebrafish IGF3 (100 ng/mL; see reference 6 for details on the production and characterization of recombinant zebrafish IGF3) with or without 10 μ M NBI-31772 to examine *amb* transcript levels.

In all experiments with FSH, the production of biologically active steroids was blocked by including 25 μ g/mL trilostane

(Chemos), an inhibitor of 3β -hydroxysteroid dehydrogenase activity. At the end of the incubation period, testis tissue was snap frozen in liquid nitrogen and stored at -80°C until RNA extraction or fixed for morphological analyses.

Gene expression

The relative transcript levels of *igfbps*, germ cells markers and other genes of interest (Table 1) were quantified by real-time, quantitative PCR (qPCR) assays in testis tissue incubated with FSH or T_3 for 1 (*igfbps*) or 3 or 4 days (germ cell marker or growth factors). In the present study, specific qPCR primers to detect *igfbp1a*, *igfbp1b*, *igfbp2a*, *igfbp2b*, *igfbp5a*, *igfbp5b*, *igfbp6a*, and *igfbp6b* mRNAs were designed and validated; these primers were designed using Primer Express software (PE Biosystems) on different exons to avoid amplification of potential genomic DNA contaminations, as described previously (35). In addition, all zebrafish *igfbp* qPCR primer sets were also tested to confirm the lack of cross-reactivity on all cloned full-length zebrafish *igfbp* cDNAs as templates in the qPCR (data not shown). The expression of *igfbp3* was analyzed using a commercially available TaqMan gene expression assay (Applied Biosystems).

Total RNA was isolated from tissue using an RNAqueous microkit (Ambion), according to the manufacturer's protocol. cDNA synthesis from total RNA and quantification of transcript

levels were carried out as described previously (35). In brief, $2\text{ }\mu\text{g}$ of total RNA was reverse transcribed using 250 U of SuperScript II ribonuclease reverse transcriptase (Life Technologies). Each qPCR was performed in $20\text{ }\mu\text{L}$ $1\times$ SYBR Green assay mix (Applied Biosystems) and specific qPCR primers (each 900 nM), containing $5\text{ }\mu\text{L}$ cDNA. The quantification cycle values were determined in a Step One Plus real-time PCR system (Applied Biosystems) using default settings. The relative amounts of mRNA in the cDNA samples were calculated using the arithmetic comparative method ($\delta\delta\text{cycle threshold}$ method), according to Bogerd et al (35). Expression of the elongation factor 1 alpha (*ef1a*) was stable (Supplemental Figure 1); therefore, *ef1a* was used as endogenous control. Results on testicular gene expression were expressed as fold change with respect to the control group. In experiments in which we investigated the effects T_3 , FSH, or NBI-31772 alone, the control tissues were incubated with basal medium. In experiments examining the effects of NBI-31772 on the T_3 -, FSH-, or IGF3-induced changes in gene expression, control tissues were incubated in medium containing T_3 , FSH, or IGF3, respectively.

Morphological analysis

To quantify the proliferation activity of A_{und} , A_{diff} and type B spermatogonia, $100\text{ }\mu\text{g/mL}$ of the proliferation marker 5-bro-

Table 1. Primers Used for Gene Expression Studies

Target Genes	Primers Name	Sequence (5'–3')	Gene Information
<i>ef1a</i>	AG (Fw) AH (Rv)	GCCGTCCACCGACAAG CCACACGACCCACAGGTACAG	Reference gene (18)
<i>igfbp1a</i>	4194 (Fw) 4196 (Rv)	GAGCCCCGAGCCTAACCA TCTCATAACGGGCCGACG	This paper
<i>igfbp1b</i>	4199 (Fw) 4200 (Rv)	GTGGAGCACCACCTACTGAAG TGCATCACCTGCTGAGCC	This paper
<i>igfbp2a</i>	4206 (Fw) 4207 (Rv)	GACCCTAAAGCACCATGCTAA TTGACCAGGTGCTGAAAAGG	This paper
<i>igfbp2b</i>	4211 (Fw) 4213 (Rv)	GCCCACCATGACCAACCA GAAGTAAATGGCAGCGGTC	This paper
<i>igfbp5a</i>	4226 (Fw) 4227 (Rv)	CTCCCCCTCCCATCGACAA CAGAAGGAAGCTGGACGGAAT	This paper
<i>igfbp5b</i>	4333 (Fw) 4334 (Rv)	CGCAAACATGTAAGCCCTCTAG ATGGAGTTCAAATGCCGGG	This paper
<i>igfbp6a</i>	4955 (Fw) 4956 (Rv)	CCTCTGGTGGCGACAAATATG TGCATCAACTGCCAGAACTCTAA	This paper
<i>igfbp6b</i>	4928 (Fw) 4929 (Rv)	TGACATCTACATCCAACTGTGA GGAAAAAGCAGTGTCTCGGTCC	This paper
<i>nanos2</i>	4817 (Fw) 4818 (Rv)	AAACGGAGAGACTGCGCAGAT CGTCCGTCCCTTGCCCTT	Expressed in A_{und} (53,64)
<i>piwil1</i>	2542 (Fw) 2543 (Rv)	GATACCGCTGCTGGAAAAAGG TGGTTCTCCAAGTGTGCTTGC	Expressed in all generations of type A spermatogonia (54, 62)
<i>piwil2</i>	2994 (Fw) 2995 (Rv)	TGATACCAGCAAGAAGAGCAGATCT ATTGGAAAGGTCACCCTGGAGTA	Expressed in all germ cell types except A_{und} and spermatozoa (62)
<i>dazl</i>	3104 (Fw) 3105 (Rv)	AGTGCAGACTTTGCTAACCTTATGTA GTCCACTGCTCCAAGTTGCTCT	Expressed mainly in type B spermatogonia and primary spermatocytes (31)
<i>igf3</i>	2680(Fw) 2681 (Rv)	TGTGCGGAGACAGAGGCTTT CGCCGCACTTCTTGGAAT	Promotes differentiation (18)
<i>insl3</i>	2466 (Fw) 2467 (Rv)	TCGCATCGTGTGGGAGTTT TGCACAACGAGGTCTCTATCCA	Promotes differentiation (56)
<i>amh</i>	AD (Fw) AE (Rv)	CTCTGACCTTGATGAGCCTCATTT GGATGTCCCTTAAGAACTTTTGCA	Negative regulator of differentiation (48)
<i>gsdf</i>	2366 (Fw) 2367 (Rv)	CATCTGCGGGAGTCATTGAAA CAGAGTCCTCCGGCAAGCT	Expressed in SCs (63)

Abbreviations: Fw, forward; Rv, reverse.

mo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was added to the tissue culture medium during the last 6 hours of the 4-day incubation period. After fixation in methacarn (60% [vol/vol] absolute ethanol, 30% chloroform, and 10% acetic acid), the samples were dehydrated in graded ethanol (70%, 96%, and 100%), embedded in Technovit 7100 (Heraeus Kulzer), and sectioned at a thickness of 4 μ m. To determine the proliferation activity, one set of sections was used to localize BrdU as described previously (5). The mitotic index was determined by analyzing 100 A_{und} cells or 100 cysts (A_{diff} and B spermatogonia), discriminating between the BrdU-positive and BrdU-negative cells/cysts.

To quantify the proportions of section areas occupied by the different spermatogonial cell types, another set of sections was stained with toluidine blue, and 15 randomly chosen, nonoverlapping fields were photographed at 400-fold magnification. The images were analyzed quantitatively by counting the number of points over the three spermatogonial cell types investigated (A_{und}, A_{diff}, and B spermatogonia), using ImageJ software (National Institutes of Health, Bethesda, Maryland, <http://rsbweb.nih.gov/ij>) with a 540-point grid.

The samples were coded such that the person evaluating the BrdU labeling or section surface areas was not aware of the treatment group to which the samples belonged. All results were expressed as fold change of control. In case of incubations with T₃ and FSH, the control tissue was incubated in basal medium without hormone to evaluate the effects of the two hormones. To evaluate the potential modulation of T₃ or FSH effects by NBI-31772, the control tissue was incubated in medium containing T₃ or FSH, respectively. In addition to expressing the results as fold change, the individual data of the pair-wise comparisons (basal vs T₃, T₃ vs T₃ and NBI-31772, basal vs FSH, or FSH vs FSH and NBI-31772) were presented in Supplemental Figure 2, thereby showing the variation between individuals.

Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 5 software package (GraphPad Inc). Differences between control and experimental groups were tested for statistical significance using a Student's *t* test for paired observation, comparing the two testes of one male that were incubated under two different conditions. Morphological (mitotic index and the proportions of section areas occupied by the different spermatogonial cell types) and gene expression data are represented as fold change of control (mean \pm SEM). The morphological data are also shown as individual data in Supplemental Figure 2, in which the testes of a single male that were incubated under control (open symbols) or experimental (filled symbols) conditions are connected by a stippled line to illustrate the individual response patterns.

Results

T₃ and FSH modulate the expression of specific IGFBPs

The expression of the *igfbps* was analyzed by qPCR in zebrafish testis treated for 1 day with T₃ (50 ng/mL) or FSH (100 ng/mL) in the presence of 25 μ g/mL trilostane to block the production of biologically active

steroids. All nine *igfbps* known in the zebrafish genome were expressed in testis tissue (Figure 1). Transcript levels of *igfbp1a* and *igfbp3* were up-regulated in response to T₃ (Figure 1A), whereas *igfbp1a* transcript levels were down-regulated in response to FSH (Figure 1B). Neither T₃ nor FSH modulated the transcript levels of any of the other *igfbp* genes at the concentration and time evaluated here (Figure 1, A and B).

T₃ and FSH effects on spermatogonial development

A previous study indicated that T₃ increased the mitotic index of A_{und} spermatogonia and led to their accumulation after 4 days of testis tissue culture (18). However, the proliferation activity and potential accumulation of A_{diff} and B spermatogonia was not addressed. As expected from previous studies, the mitotic index of A_{und} increased in the presence of 50 ng/mL of T₃ after 4 days of incubation. The mitotic index of A_{diff} spermatogonia also increased, whereas it remained con-

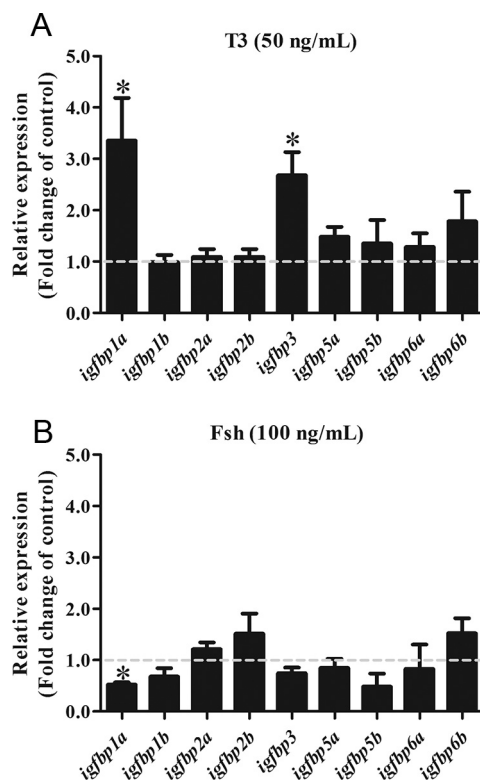


Figure 1. Levels of nine *igfbp* transcripts after 1 day of adult zebrafish testis tissue culture. Effect of T₃ (50 ng/mL) (A) and FSH (100 ng/mL) in the presence of 25 μ g/mL trilostane (B) on *igfbp* expression. Dotted line and bars represent control group (basal conditions) and the relative mRNA levels (fold of basal; mean \pm SEM), respectively. The production of biologically active steroids was blocked by trilostane (25 μ g/mL) in panel B. Asterisks indicate significant differences ($P < .05$) between control and experimental group ($n = 7$).

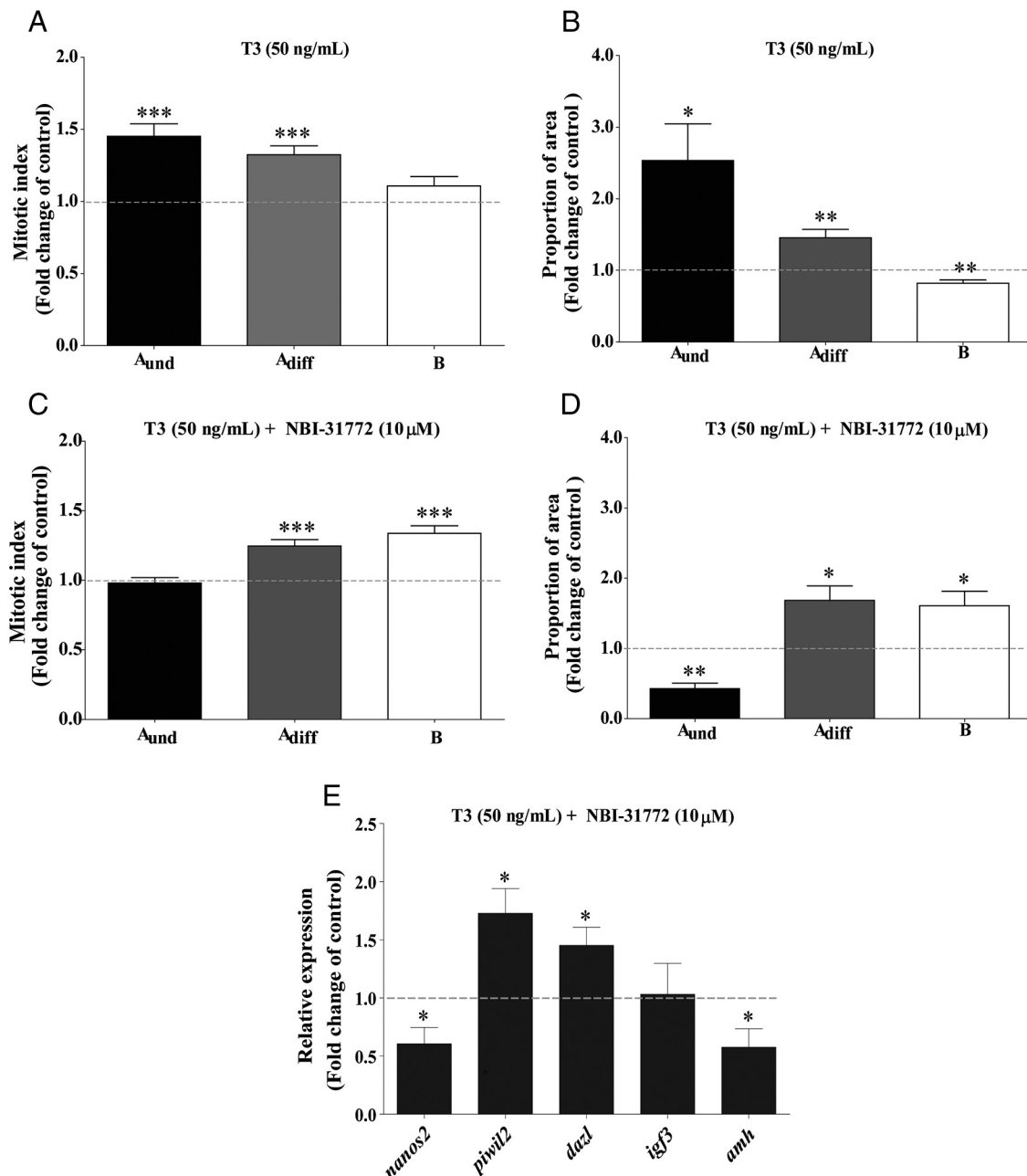


Figure 2. Effects of T_3 (A and B) and T_3 in the presence of the IGF1P inhibitor (C–E) after 4 days of adult zebrafish testis tissue culture on spermatogonial proliferation, accumulation, and gene expression. A, Mitotic indices of type A_{und} , type A_{diff} , and type B spermatogonia after incubation under basal conditions (dotted line) or in the presence of T_3 (50 ng/mL) (bars) ($n = 12$). B, Proportion of section surface area with spermatogenic cysts containing type A_{und} , type A_{diff} , and type B spermatogonia after incubation under basal conditions (dotted line) or in medium containing T_3 (50 ng/mL) (bars) ($n = 6$). C, Mitotic indices of type A_{und} , type A_{diff} , and type B spermatogonia in the presence of T_3 (50 ng/mL) (dotted line) or T_3 (50 ng/mL) and 10 μ M NBI-31772 (bars) ($n = 13$). D, Proportion of section surface area with cysts of type A_{und} , type A_{diff} , and type B spermatogonia in the presence of T_3 (50 ng/mL) (dotted line) or T_3 (50 ng/mL) and 10 μ M NBI-31772 (bars) ($n = 6$). E, Gene expression analysis in adult zebrafish testis after 4 days of tissue culture in the presence of T_3 (50 ng/mL) (dotted line) or T_3 (50 ng/mL) and 10 μ M NBI-31772 (bars) ($n = 7$). Results are presented as fold changes with respect to the control group (basal or T_3). Asterisks indicate significant differences. *, $P < .05$, **, $P < .01$, ***, $P < .001$ compared with the respective control group.

stant for type B spermatogonia (Figure 2A and Supplemental Figure 2A). Furthermore, the relative surface areas occupied by A_{und} and A_{diff} spermatogonia increased, whereas the one for type B spermatogonia decreased slightly in response to T_3 (Figure 2B and Supplemental Figure 2B).

Exposure to FSH increased the mitotic indices of A_{und} , A_{diff} and type B spermatogonia after 4 days of incubation (Figure 3A and Supplemental Figure 2E). The proportion of area occupied by A_{und} did not change but the areas occupied by A_{diff} and B spermatogonia increased (Figure 3B and Supplemental Figure 2F).

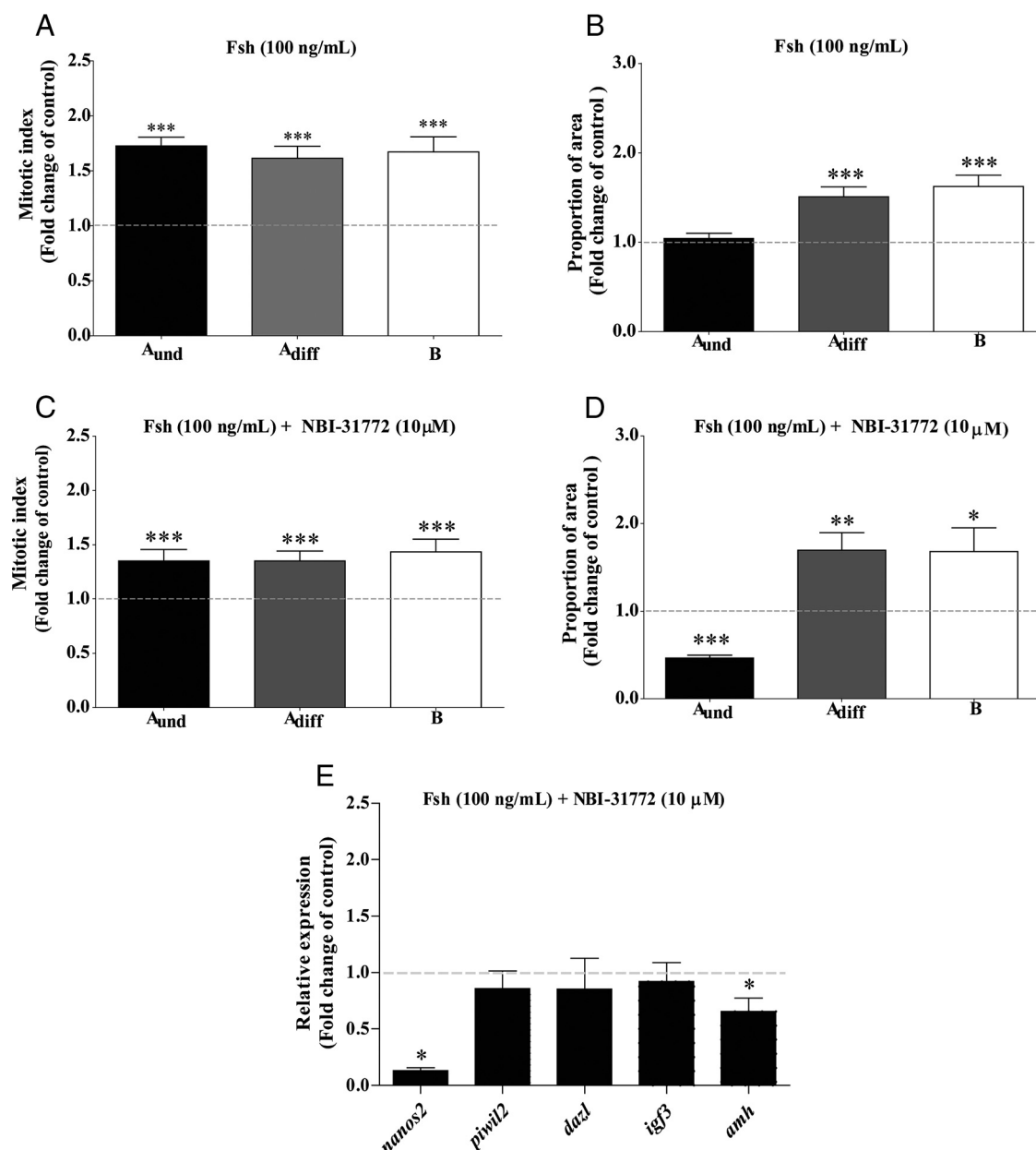


Figure 3. Effects of FSH (A and B) and FSH in the presence of the IGFBP inhibitor NBI-31772 (C–E) on FSH-stimulated spermatogenesis after 4 days of adult zebrafish testis tissue culture on spermatogonial proliferation, accumulation, and gene expression. A, Mitotic indices of type A_{und}, type A_{diff}, and type B spermatogonia in the presence of trilostane (25 μg/mL) or in the absence (dotted line) or presence (bars) of FSH (100 ng/mL) (n = 15). B, Proportion of section surface area with spermatogenic cysts containing type A_{und}, type A_{diff}, and type B spermatogonia in the presence of trilostane (25 μg/mL), in the absence (dotted line) or presence (bars) of FSH (100 ng/mL) (black bars) (n = 8). C, Mitotic indices of type A_{und}, type A_{diff}, and type B spermatogonia in presence of FSH (100 ng/mL) (dotted line) or FSH (100 ng/mL) in combination with 10 μM NBI-31772 (bars) (n = 14). D, Proportion of section surface area with cysts of type A_{und}, type A_{diff}, and type B spermatogonia in the presence of FSH (100 ng/mL) (dotted line) or FSH (100 ng/mL) in combination with 10 μM NBI-31772 (bars) (n = 7). E, Gene expression analysis in adult zebrafish testis after 4 days of tissue culture in the presence of FSH (100 ng/mL) (dotted line) or FSH (100 ng/mL) and 10 μM NBI-31772 (bars) (n = 8). The production of biologically active steroids by FSH was blocked by including trilostane (25 μg/mL). Results are presented as fold changes with respect to the control group (basal or FSH). Asterisks indicate significant differences. *, $P < .05$, **, $P < .01$, ***, $P < .001$ compared with the respective control group.

T₃ and FSH stimulate spermatogonial differentiation in the presence of an IGFBP inhibitor

To better understand the function of T₃/FSH and IGF3 signaling systems in modulating spermatogenesis, the

main modulators of IGF bioavailability, the IGFBPs, were studied using a specific inhibitor (NBI-31772) blocking IGF interaction with IGFBPs. The effect of NBI-31772 on spermatogenesis was first tested by analyzing BrdU incorporation in spermatogonia in zebrafish testis incubated

under basal condition or in the presence of 10 μ M NBI-31772. NBI-31772 alone did not modulate spermatogonial proliferation, relative section surface areas occupied by spermatogonia, or gene expression (Supplemental Figure 3). Then we analyzed whether T₃-stimulated BrdU incorporation into spermatogonia was modulated in the presence of NBI-31772. The mitotic index of A_{und} spermatogonia remained unchanged but increased for A_{diff} and B spermatogonia (Figure 2C and Supplemental Figure 2C). The relative area occupied by A_{und} spermatogonia decreased, whereas those of the more differentiated spermatogonial generations increased in the additional presence of NBI-31772 (Figure 2D and Supplemental Figure 2D).

In parallel experiments, we quantified the transcript levels of selected genes. In line with the morphological analysis, *nanos2* (a marker for A_{und} spermatogonia) was down-regulated, whereas *piwil2* (expressed by all germ cells except A_{und} spermatogonia and spermatozoa) and *dazl* (expressed by type B spermatogonia and primary spermatocytes) were up-regulated in the presence of T₃ and NBI-31772 (Figure 2E). The transcript levels of *igf3* did not change, but antimüllerian hormone (*amh*) transcript levels decreased significantly (Figure 2E); the transcript levels of two other growth factors reported to promote germ cell differentiation (ie, *gsdf* and *insl3*) also did not change (data not shown).

When testis tissue was incubated with FSH (and trilostane), the additional presence of NBI-31772 further elevated the mitotic indices of all spermatogonia (Figure 3C and Supplemental Figure 2G). The relative area occupied by A_{und} spermatogonia decreased, whereas those occupied by A_{diff} and type B spermatogonia increased in the additional presence of NBI-31772 (Figure 3D and Supplemental Figure 2H). Finally, the transcript levels of *nanos2* were strongly down-regulated, and also those of *amh* were significantly lower than in the presence of FSH alone (Figure 3E); the transcript levels of *gsdf* and *insl3* did not change (data not shown).

IGF3 down-regulates *amh* expression in the presence of the IGFBP inhibitor

Our previous studies suggested that FSH (6) but not T₃ (18) down-regulated *amh* expression. Because in the experiments with the IGFBP inhibitor T₃ did reduce, and FSH further reduced, *amh* transcript levels, we hypothesized that the increased availability of IGF3 may have down-regulated *amh* expression. To test this, zebrafish testis was incubated for 3 days in presence of IGF3 (100 ng/mL; this concentration did not affect *amh* transcript levels; see reference 6) with or without the NBI-31772 (10 μ M). The testicular expression of *amh* was down-regu-

lated in response to IGF3 when the IGFBP inhibitor was also present (Supplemental Figure 4).

Discussion

In mammals, six IGFBPs with high affinity for IGF family members and ubiquitous expression have been described (19–22). Several studies on IGFBP function have been published, but information about potential functions in testis tissue is limited. *Igfbp2-4* transcripts have been detected in purified Leydig cells and seminiferous tubules in rats (36); *Igfbp2-6* transcripts were detected in sheep testis after in vivo treatment with IGF1 (37). Nine *igfbp* genes have been reported in the zebrafish genome (38); the additional paralogs probably have arisen in context with the additional, fish-specific genome duplication. As in mammals, zebrafish *igfbps* are expressed in several tissues (39–43), including the testis, suggesting that IGFBPs have short-range roles in addition to systemic functions. Here we report testicular expression of all nine *igfbps* and their regulation by T₃ and FSH in zebrafish testis. T₃ up-regulated the transcript levels of *igfbp1a* and *igfbp3*, whereas FSH down-regulated the expression of *igfbp1a*. Studies in pigs (44) and rats (45) have reported that thyroid hormone and FSH, respectively, modulated the expression of testicular *Igfbps*. Thyroid hormone induced *Igfbp4* expression in prepubertal porcine SCs. Hypophysectomy elevated rat testicular *Igfbp3* transcripts levels that returned to control levels after FSH treatment. In fish, information on the regulation of testicular *igfbp* expression is scarce. In rainbow trout, the expression of *igfbp6* was up-regulated by FSH-induced stimulation of sex steroid production (7); other *igfbps* were not analyzed in this study. In rainbow trout ovaries, a partially purified salmon gonadotropin that induced oocyte maturation also down-regulated the expression of *igfbp2b*, *igfbp4*, and *igfbp5* (46). GH treatment of female sea bream, on the other hand, elevated ovarian *igfbp2* transcript levels (47). Taken together, these studies may indicate that thyroid hormone (and GH/IGF) signaling supports gonadal IGFBP levels, whereas FSH effects via Sertoli or granulosa cells (ie, excluding steroidogenic effects of FSH in fish) reduce IGFBP levels. This is similar to the response pattern evoked by T₃ and FSH that we have found for the zebrafish testis and might suggest that it represents an evolutionarily conserved response of physiological relevance. The use of trilostane may have allowed detecting the FSH-mediated reduction of *igfbp1a* expression in our studies.

Thyroid hormone receptors (*thra* and *thrβ*) are expressed in adult zebrafish testis in Sertoli and/or Leydig cells, and T₃ promoted the formation of new spermatogonia.

gonial cysts by stimulating the proliferation of SCs and A_{und} via SC-derived IGF3 (18). However, this study did not examine the effect of T₃ on other spermatogonial generations. Here we found that T₃ promoted the proliferation of both A_{und} and A_{diff} but not type B spermatogonia. In agreement with our previous study (18), the proportion of A_{und} increased in response to T₃, but different from the previous study, we find here that T₃ also increased the relative section area occupied by A_{diff} but decreased the one occupied by type B spermatogonia. This discrepancy could reflect the different methodological approaches: previously we determined the percentage of spermatogenic cysts with spermatogonia in different stages of development, whereas here we quantified the proportion of the total section area occupied by the different spermatogonial generations. Our data suggest that T₃ promoted the formation of new cysts with type A_{und} spermatogonia but also stimulated the accumulation of A_{diff} by increasing their proliferation while slightly reducing their further de-

velopment into type B spermatogonia (Figure 4A). Overall, T₃ exposure resulted in expanding preferentially the type A spermatogonial population. This was associated with elevated transcript levels of selected IGFBPs (discussed further below).

FSH promoted spermatogonial proliferation and differentiation in zebrafish in androgen-dependent and -independent manners (4, 6, 48), and stimulatory effects of FSH on spermatogenesis, either via androgens (2) or also independent of androgens (7) were reported in eel or trout, respectively. Whereas the steroidogenic potency of FSH is a fish-specific feature, (nonsteroidogenic) FSH bioactivity is required for normal premeiotic germ cell numbers also in mammals (49). In the present study, zebrafish testes were incubated with FSH while blocking the production of biologically active steroids. Under these conditions, FSH increased the mitotic indices of type A and B spermatogonia and also elevated the surface areas occupied by these cells, except for type A_{und} spermatogonia. An increased prolifer-

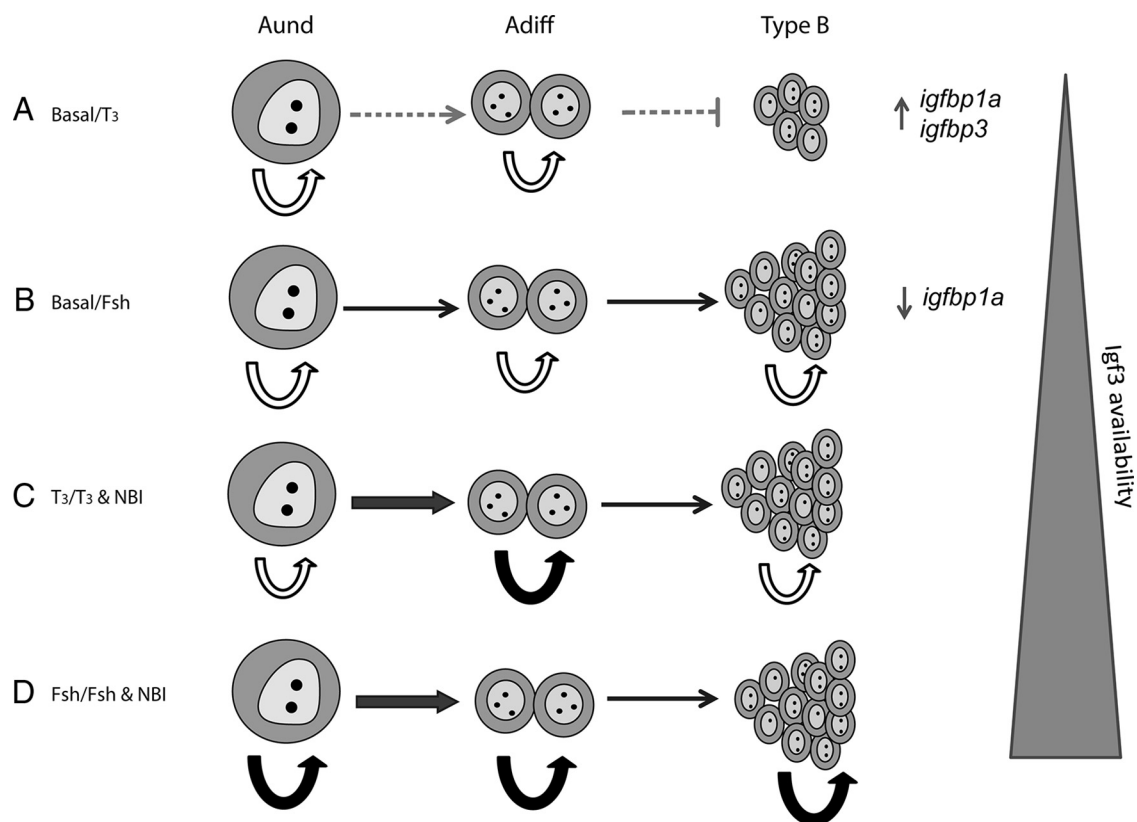


Figure 4. Schematic representation of the T₃/FSH and IGFBP inhibitor (NBI-31772) effects on zebrafish spermatogenesis. A, T₃ expands type A spermatogonia by increasing their proliferation and accumulation and by slightly reducing their further development into type B spermatogonia. These effects were accompanied by the increased expression of two *igfbp* transcripts. B, FSH stimulates part of the type A_{und} spermatogonia to self-renew, whereas other type A_{und} spermatogonia differentiated toward A_{diff} and type B spermatogonia. This concomitant stimulation of self-renewal and differentiation is associated with the selective reduction of *igfbp1a* transcript levels that may allow differentiation of type A spermatogonia. C, T₃ in the presence of the IGFBP inhibitor further promoted differentiation of type A_{und} spermatogonia into the more differentiated spermatogonial stages. D, In the presence of the IGFBP inhibitor, the prodifferentiation effects of FSH dominate and type A_{und} spermatogonia become partially depleted. Curved white arrow, self-renewal proliferation; curved black arrow, strong self-renewal proliferation; dotted arrow, weak differentiation effect; dotted flat-tipped arrow, reduced differentiation; black arrows, differentiation; thick black arrows, strong differentiation.

ation activity but a stable surface area proportion suggest that FSH stimulated some type A_{und} spermatogonia to self-renew, whereas other type A_{und} spermatogonia differentiated toward A_{diff} and type B spermatogonia. It therefore appears that FSH triggered a balanced activation of both self-renewal and differentiation of type A spermatogonia while also promoting further differentiation to type B spermatogonia. This was associated with reduced transcript levels for *igfbp1a* (discussed further below) (Figure 4B).

IGF3 mediated stimulatory effects of T₃ and FSH on spermatogonial proliferation in the adult zebrafish testis (6, 18), making both hormones interesting test cases for examining the potential role of IGFBPs. NBI-31772 displaces IGFs from the IGF-IGFBP complex, increasing the levels of free bioactive IGF (33). In mice, NBI-31772 produced neuropharmacological effects similar to those observed after experimentally increasing circulating IGF1 levels (50), and in vivo administration of NBI-31772 enhanced muscle regeneration (51). In the rat, IGF1 increased the survival of motor neurons, and cell survival improved further in the presence of NBI-31772 (52). Effects of NBI-31772 are not restricted to mammals. In zebrafish embryos treated with NBI-31772, cardiomyocyte proliferation increased by 47% (34). In the present study, NBI-31772 did not change basal spermatogonial proliferation, suggesting that IGFBP occupancy with IGF family members is low in the zebrafish testis under basal conditions. However, NBI-31772 clearly modulated spermatogonial development in response to T₃ or FSH that both were shown previously to increase *igf3* expression and IGF3 release (6, 18).

As discussed above, T₃ promoted self-renewal of type A_{und} spermatogonia that accumulated (Figure 2, A and B). But when combined with the IGFBP inhibitor, T₃ decreased the area occupied by type A_{und} spermatogonia. The morphological evidence is supported by the decrease of *nanos2* transcript levels, a molecular marker for single type A_{und} spermatogonia (53). The *amh* transcript levels decreased as well, which is usually observed in teleost testis when hormone treatment recruits germ cells into differentiation (eg, zebrafish [6]; trout [7]; eel [54]; sea bass [55]). Also, the elevated mitotic indices and areas occupied by type A_{diff} and type B spermatogonia and the expression of *dazl*, a germ cell marker reflecting differentiation beyond type A spermatogonia, indicate that blocking IGFBPs resulted in an additional (ie, on top of T₃) prodifferentiation signal (Figure 4C). Overall, the preferential expansion of the type A spermatogonial population seen after exposure to T₃ alone shifted to more differentiated germ cells at the expense of type A_{und} spermatogonia in the presence of the IGFBP inhibitor.

Compared with T₃, FSH exerted a stronger but still balanced prodifferentiation effect. Blocking IGFBPs in the presence of FSH further increased the proliferation activity of all spermatogonial cells, including A_{und} cells. Also, the areas occupied by A_{diff} and B-type cells increased, whereas the one of type A_{und} spermatogonia was reduced, despite their increased proliferation activity. These data suggest that the balanced effect of FSH alone, ie, promoting both self-renewal and differentiation, is lost when blocking the IGFBP inhibitor, such that unimpeded IGF3 bioactivity together with other prodifferentiation effects of FSH (eg, increased *insl3* transcript levels [56]), jointly reduce type A_{und} spermatogonia and tip the balance toward favoring the production of type A_{diff} and B spermatogonia (Figure 4D).

Information on the functional role(s) of IGFBPs in gonads is limited, but several studies in other tissues in mammals and fish have suggested that IGFBPs restrict the biological activity of IGFs. Transgenic mice overexpressing *Igfbp1* showed growth retardation (57). Also, when driven by a liver-specific promoter, rats overexpressing *Igfbp1* displayed growth retardation but also small gonads and a reduced fertility (58, 59). Moreover, IGFBP3 and IGFBP4 blocked the steroidogenic effects of IGF1 in rat Leydig cells (44), and IGFBP3 inhibited IGF1-stimulated lactate production in rat SC cultures (45). In addition, IGFBP3 has been implicated in promoting spermatogonial stem cell apoptosis in rats and mice (60, 61). In zebrafish, overexpression of *igfbp1a*, *igfbp1b*, *igfbp2a*, *igfbp2b*, *igfbp4*, *igf 6a*, and *igfbp6b* delayed embryonic development (40–43). Also, IGFBP1a and IGFBP1b inhibited IGF1-mediated proliferation in cultured zebrafish embryonic cells (41), whereas zebrafish IGFBP5a and IGFBP5b decreased the IGF1-mediated viability of U2OS and human embryonic kidney-293 cells (40). Seen in context with the present data, we propose that the effects of blocking IGFBPs can be understood in the following way: T₃- (18) or FSH-induced (6) stimulation of IGF3 release, in combination with the increased availability of IGF3 for its cognate receptors when blocking IGFBP activity and the resulting drop of *amh* transcript levels, jointly facilitate the additional stimulatory effects on spermatogonial proliferation and differentiation. This resulted in a strong prodifferentiation signal associated with a depletion of type A_{und} spermatogonia. On the other hand, we propose that an IGFBP function is to fine-tune the biological activity of the prodifferentiation factor, IGF3, to prevent exhaustion of the type A_{und} spermatogonial pool.

In this regard, it is very interesting to note that in the presence of the IGFBP inhibitor, depletion of type A_{und} spermatogonia and the production of A_{diff} and B spermatogonia is taking place irrespective of using T₃ or FSH, whereas the

two hormones have clearly different effects when the inhibitor is absent. We propose that this difference is related to the differential effects the two hormones exert on IGFBP expression. T₃ elevated IGFBP activity that would increase the protection of type A_{und} spermatogonia against IGF3-mediated differentiation and explain the accumulation of type A_{und} spermatogonia. On the other hand, FSH may achieve the activation of self-renewal and differentiation by selectively decreasing IGFBP activity.

In conclusion, we have shown that all IGFBPs known in the zebrafish genome are expressed in the testis and that T₃ and FSH modulate, each in a specific manner, the expression of selected *igfbp* genes. We also report that T₃ stimulates the self-renewal of type A_{und} spermatogonia and the accumulation of type A_{diff} spermatogonia, whereas FSH promotes both self-renewal and differentiation in zebrafish testis. However, both hormones promote the depletion of type A_{und} spermatogonia and the accumulation of type A_{diff} and type B spermatogonia in the presence of an IGFBP inhibitor, suggesting that IGFBPs protect type A_{und} spermatogonia from excessive differentiation in response to IGF3.

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