Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3

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Growth factors modulate germ line stem cell self-renewal and differentiation behavior. We investigate the effects of Igf3, a fish-specific member of the *igf* family. Fsh increased in a steroid-independent manner the number and mitotic index of single type A undifferentiated spermatogonia and of clones of type A differentiating spermatogonia in adult zebrafish testis. All 4 *igf* gene family members in zebrafish are expressed in the testis but in tissue culture only *igf3* transcript levels increased in response to recombinant zebrafish Fsh. This occurred in a cAMP/protein kinase A-dependent manner, in line with the results of studies on the *igf3* gene promoter. Igf3 protein was detected in Sertoli cells. Recombinant zebrafish Igf3 increased the mitotic index of type A undifferentiated and type A differentiating spermatogonia and up-regulated the expression of genes related to spermatogonial differentiation and entry into meiosis, but Igf3 did not modulate testicular androgen release. An Igf receptor inhibitor blocked these effects of Igf3. Importantly, the Igf receptor inhibitor also blocked Fsh-induced spermatogonial proliferation. We conclude that Fsh stimulated Sertoli cell production of Igf3, which promoted via Igf receptor signaling spermatogonial proliferation and differentiation and their entry into meiosis. Because previous work showed that Fsh also released spermatogonia from an inhibitory signal by down-regulating anti-Müllerian hormone and by stimulating androgen production, we can now present a model, in which Fsh orchestrates the activity of stimulatory (Igf3, androgens) and inhibitory (anti-Müllerian hormone) signals to promote spermatogenesis. **(*Endocrinology* 156: 3804–3817, 2015)**

Spermatogenesis relies on the continuous proliferation of spermatogonial stem cells (SSCs), the male germ line stem cells in the testis. SSCs either self-renew, producing more stem cells or differentiate into committed progenitor cells ultimately developing into spermatozoa. The SSCs locate to specific areas of the seminiferous tubules, the so-called SSC niche (1–4). Growth factors released from somatic elements of the niche, such as Sertoli cells (glial cell line-derived neurotrophic factor [GDNF] [5–7], activin A, and bone morphogenetic protein 4 [8, 9]), Leydig or peritubular myoid cells (colony-stimulating factor 1 [10–11]), contribute to the niche homeostasis, and influence the balance between SSC self-renewal and differentiation (4). It is possible that yet unknown factors participate in regulating germ cell proliferation/differentiation or that known factors exert yet unknown functions in the testis.

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Abbreviations: A_{diff}, type A differentiating; Amh, anti-Müllerian hormone; ATF-1, activating transcription factor 1; A_{und}, type A undifferentiated; BrdU, 5-bromo-2-deoxyuridine; CLSM, confocal laser scanning microscopy; CRE, cAMP-response element; E_2, 17β-estradiol; GDNF, glial cell line-derived neurotrophic factor; Gfp, green fluorescent protein; HEK, human embryonic kidney; H89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; ISH, in situ hybridization; NVP-AEW541, 7-[3-(azetidin-1-ylmethyl)cyclobutyl]-5-(3-phenylmethoxyphenyl)pyrrolo[2,3-d]pyrimidin-4-amine; 11-KT, 11-ketotestosterone; PD98059, 2-(2-amino-3-methoxyphenyl)chromen-4-one; rzf, recombinant zebrafish; SSC, spermatogonial stem cell.

FSH and LH regulate Sertoli and Leydig cell functions, thereby also modulating SSC fate. In rodents, FSH stimulates Sertoli cell GDNF secretion which promotes SSC self-renewal (12). LH-regulated androgens seem to inhibit spermatogonial differentiation under certain conditions (13, 14), and the decrease of androgen levels improves the success of germ cell transplantation by increasing the number of SSC colonies (15). On the other hand, androgens are required for the completion of meiosis (16) and spermiogenesis in rodents (17). Analysis of mice models lacking FSH or its receptor (18–20) showed that puberty was delayed, Sertoli cell number and testis weight were reduced, and the percentage of misshaped sperm with compromised mobility was increased but mutant males were still able to sire offspring, though in lower numbers. Deleting the androgen receptor from Sertoli cells arrested spermatogenesis at the meiotic phase and caused infertility (17). Administering FSH to gonadotropin-deficient, androgen-insensitive mice resulted in increased numbers of spermatogonia and stimulated their entry into meiosis, but the germ cells failed to complete meiosis and spermiogenesis, for which androgen action is needed (21). Therefore, it is reasonable to assume that FSH modulates the balance among growth factors that mainly target spermatogonia and their entry into meiosis.

To unravel the effects of a given growth factor, the mammalian testis with its multilayered germinal epithelium is a comparatively complex structure: a Sertoli cell is associated with several different stages of germ cell development at any given time (22). Here, we make use of the cystic mode of spermatogenesis in fish (23, 24) as an alternative model to study this question, mainly because a fish Sertoli cell usually contacts only 1 or 2 different germ cell clones at a time. In cystic spermatogenesis, a SSC is completely enveloped by Sertoli cells. Upon differentiation, the developing germ cell clone remains enveloped by and accompanied through spermatogenesis by its group of Sertoli cells that also proliferate in a predictable manner (24–26), thereby providing space and support for the proliferating/differentiating germ cell clone.

We sought to further simplify the situation in our search for testicular factors regulating spermatogonial development by investigating a species showing clear reproductive seasonality. Focusing on the transition from a quiescent, immature testis towards an activation of spermatogonial proliferation, we analyzed differentially expressed genes in Atlantic salmon testis tissue samples using microarrays (manuscript in preparation). Among the differentially expressed genes, potentially inhibitory and stimulatory growth factors were identified. The identity and biological activity of an inhibitory factor, anti-Müllerian hormone (Amh), has been described previously in juvenile eel (27) and adult zebrafish (3). Here, we report on a stimulatory factor, a member of the Igf family, Igf3 that appears to be found only in fish, and is predominantly expressed in adult gonads (28, 29).

Materials and Methods

Animals

The wild-type, transgenic, and mutant zebrafish lines used in the present study are described in Supplemental Materials and Methods. Handling and experimentation were consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands) approved the experimental protocols.

Testis tissue culture

A previously described ex vivo organ culture system for adult zebrafish testis (30) was used for short-term (20 h for steroid release and gene expression, Supplemental Figure 1), medium-term (2 days as in Figure 1, B and C), or long-term (7 d in all other cases) incubations. For experiments on steroid release or, in some cases, for gene expression studies, incubation periods of up to 2 days were used. However, to study effects on spermatogenesis (5-bromo-2-deoxyuridine [BrdU] incorporation and morphometric studies on spermatogonia, production of meiotic and/or postmeiotic cells), we used an incubation period of 7 days, because the meiotic and spermiogenic phase in zebrafish together spans 6 days (26), because the cell cycle time of spermatogonia is approximately 30 hours (31), so that only 1 cell cycle would occur during an incubation period of 2 days. In some cases (Figure 2, D–G, and Figures 5 and 6 below), the males were exposed for 3 weeks to 10nM 17β-estradiol (E_2), which results in an androgen insufficiency-related inhibition of spermatogenesis associated with an accumulation of type A spermatogonia (32), before their testis tissue was used for tissue culture.

Zebrafish testis tissue was incubated either with recombinant zebrafish (rzf)Fsh or rzfLh (33), both at 500 ng/mL (unless indicated otherwise), in the presence or absence of trilostane (Chemos; 25 μg/mL), androgen (11-ketotestosterone [11-KT], 400nM; Sigma-Aldrich), or rzfIgf3 (100 ng/mL, unless indicated otherwise; see Supplemental Materials and Methods for details on Igf3 production). In addition, an IGF-1 receptor kinase inhibitor, NVP-AEW541 (7-[3-(azetidin-1-ylmethyl)cyclobutyl]-5-(3-phenylmethoxyphenyl)pyrrolo[2,3-d]pyrimidin-4-amine; 10μM; Selleckchem), or cAMP pathway inhibitors, the protein kinase A (PKA) inhibitor H89 (N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; 100μM; Sigma-Aldrich), and the MAPK inhibitor PD98059 (2-(2-amino-3-methoxyphenyl)chromen-4-one; 50μM; InvivoGen) were added to the culture medium when proliferation (see Figure 6 below) or *igf3* gene expression (see Figure 4 below) were studied. BrdU (50 μg/mL; Sigma-Aldrich) was added for the last 6 hours of incubation (long term) to investigate the proliferation activity in the testis. Zebrafish testis was either used for testicular androgen release (measured as described by García-López et al [34]), morphological analyses or gene expression studies. A description of the morphological analyses (immunocytochemical detection of green fluorescent protein [Gfp] or BrdU after testis

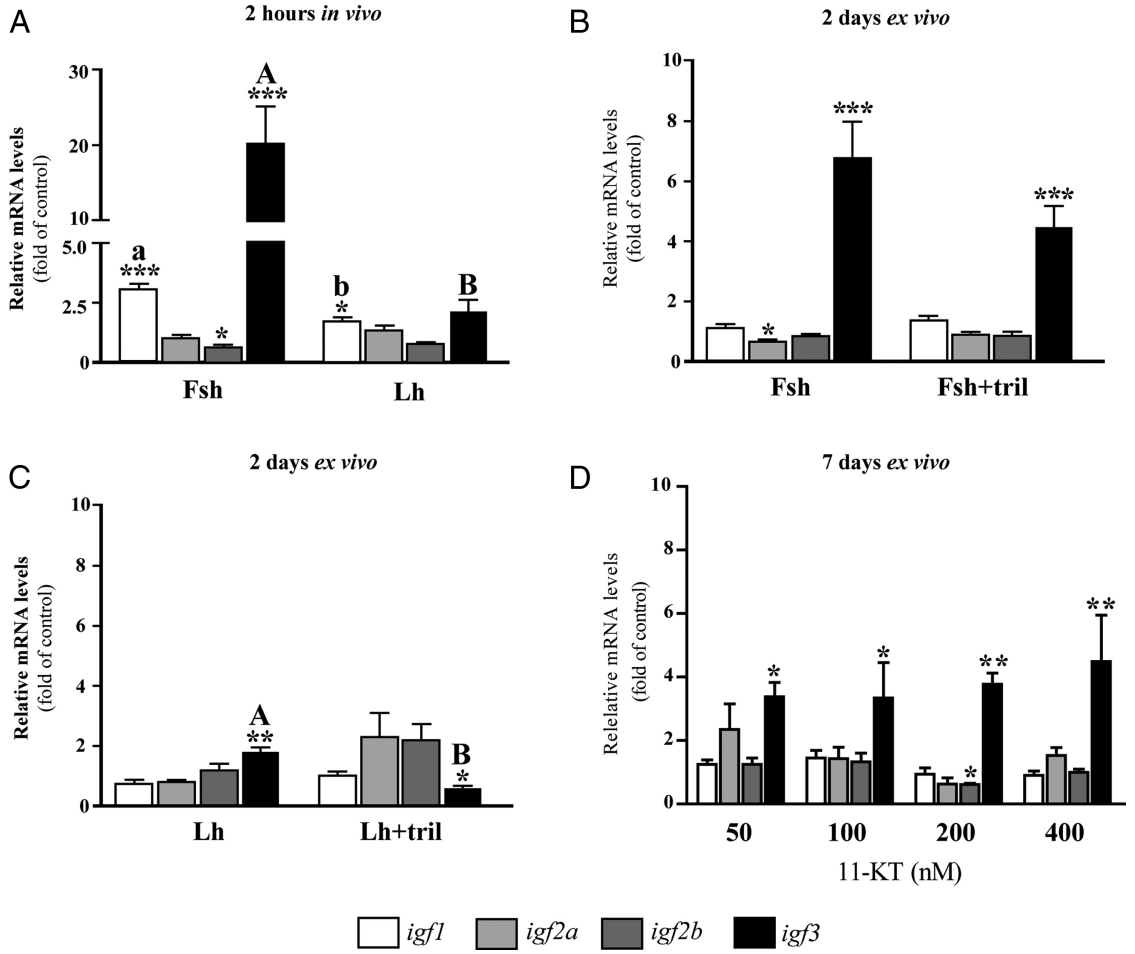

Figure 1. Testicular transcript levels of *igf* gene family members. A, Transcript levels of *igf1*, *igf2a*, *igf2b*, and *igf3*, 2 hours after injecting rzfFsh or rzfLh (100 ng/g body weight; n = 6–8 fish per treatment) into the body cavity. Transcript levels of *igf* genes after 2 days of testis tissue culture in the presence of 100 ng/mL of rzfFsh (B) or 500 ng/mL of rzfLh (C) in the absence or presence of 25-μg/mL trilostane (tril) (n = 6–8 per treatment). D, Transcript levels of *igf1*, *igf2a*, *igf2b*, and *igf3* in testicular explants incubated with increasing concentrations of 11-KT ex vivo (n = 4–7 explants per dose) for 7 days. The columns represent the fold change of the relative mRNA levels in the treated group over its respective control (mean ± SEM). Bars marked with *, $P < .05$; **, $P < .01$; or ***, $P < .001$ are significantly different from control condition, and bars marked with different letters denote statistically significant differences between Fsh and Lh responses.

tissue culture) is provided in the Supplemental Materials and Methods. To evaluate the percentage of haploid, diploid and S-phase cells in response to Fsh or 11-KT, propidium iodide DNA staining was applied to testicular cell suspensions. The DNA content of the cells was analyzed by flow cytometry (see details in Supplemental Materials and Methods).

Testicular gene expression

Total RNA extraction from testes was performed using the RNAqueous-Micro kit (Ambion), following the manufacturer's instructions. To estimate the relative mRNA expression levels of a number of selected genes (Supplemental Table 1) by validated real-time, quantitative PCR assays, samples were processed as previously described (35, 36). To visualize the cellular expression sites of *igf3* mRNA in zebrafish testis, in situ hybridization (ISH) was carried out as described previously (37), using digoxigenin-labeled sense and antisense cRNA synthesized from a *igf3*-specific PCR product generated with primers 2878–2879 (Supplemental Table 2).

For ISH (*igf3* mRNA localization) and immunohistochemistry (Igf3 or Igf3 and vimentin protein colocalization) analyses, zebrafish received an injection of rzfFsh (100 ng/g body weight) into the coelomic cavity. After 6 hours, zebrafish were euthanized, and testis tissue was collected and processed for ISH or immunohistochemistry purposes (see Supplemental Materials and Methods).

Regulatory elements in the zebrafish *igf3* gene

Tissue culture experiments suggested that cAMP is involved in the Fsh-mediated increase of *igf3* transcript levels, so that we decided to launch in silico promoter analyses followed by functional assays. To analyze whether cAMP is involved in regulating the *igf3* gene, promoter constructs of approximately 2.0 kb and approximately 1.0 kb were PCR amplified with zebrafish genomic DNA as template using forward primers 3482 and 3484 in combination with reverse primer 3486 (Supplemental Table 2) and cloned into pGL3 basic vector (Promega). Putative cAMP-response element (CRE) and activating transcription factor 1 (ATF-1) transcription factor-binding sites were mutated using primers 3770–3771 and 3775–3776 (Supplemental Table 2). Twenty-four hours after transfection of wild-type and mutated

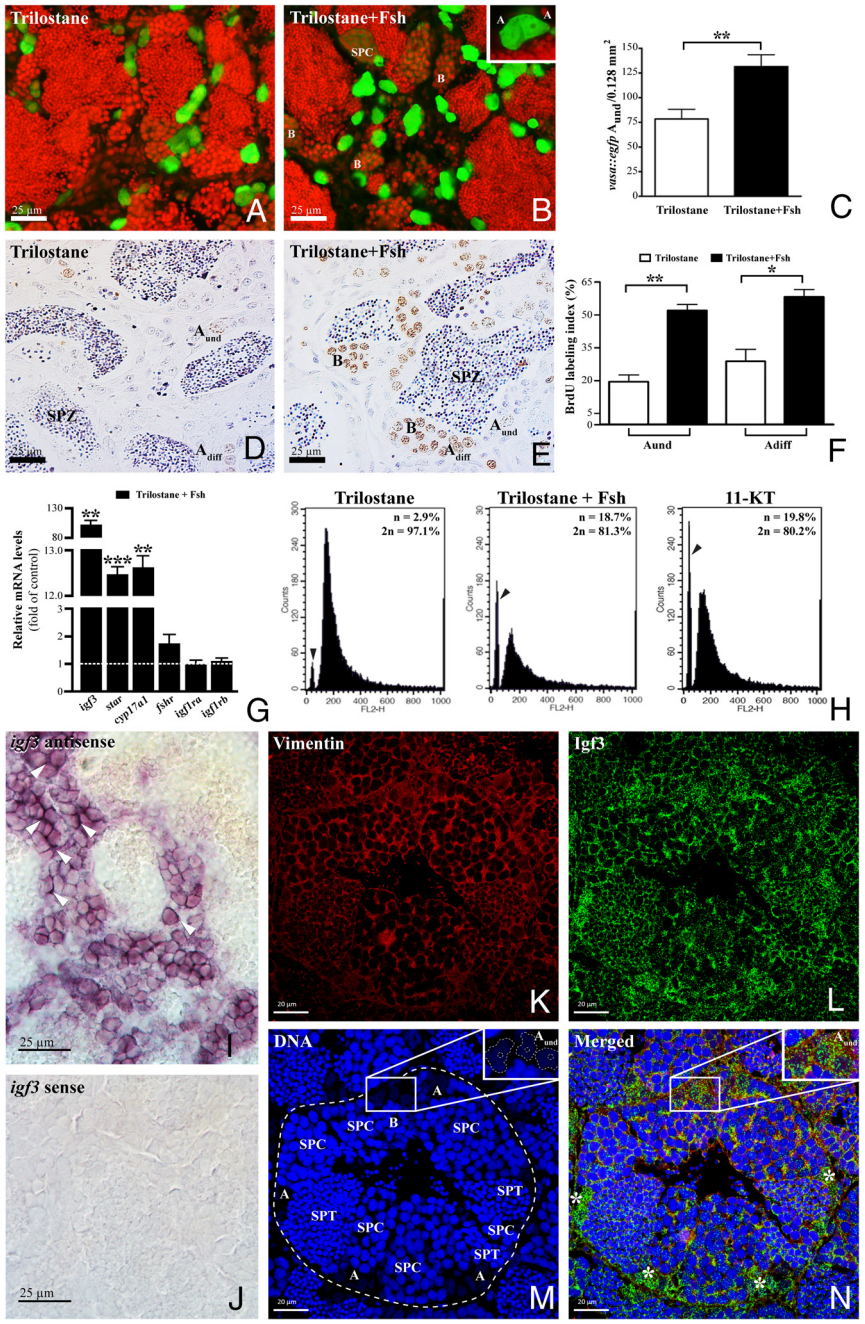

Figure 2. Effects of rzfFsh on germ cell development and localization of *igf3* mRNA and Igf3 protein in testis tissue of Fsh-treated zebrafish. Qualitative (A and B) and quantitative (C) evaluation of the number of A_{und} spermatogonia in *vasa::egfp* zebrafish testes incubated for 7 days in the absence or presence of 500-ng/mL rzfFsh; trilostane (25 μg/mL) was added to prevent the production of biologically active steroids (n = 8). D–F, Morphological analysis and BrdU labeling index of spermatogonia type A_{und} and A_{diff} in zebrafish testis. Males were preexposed to 10nM E_2 in vivo for 21 days, and testis tissue was cultured for 7 days with 25-μg/mL trilostane in the absence or presence of 500-ng/mL rzfFsh (n = 8). G, Fold changes in transcript levels of selected genes after ex vivo incubation of zebrafish testes for 7 days in the absence or presence of 500-ng/mL rzfFsh; the medium also contained 25-μg/mL trilostane (n = 8). The males were preexposed to E_2 in vivo for 21 days. H, Proportion of haploid cells (spermatids and spermatozoa), indicated by arrowheads, after incubating testicular cell suspensions with 25-μg/mL trilostane (negative control), 400nM 11-KT (positive control), or with 100-ng/mL rzfFsh+trilostane (n = 4) for 7 days. I and J, ISH localized *igf3* mRNA expression in the zebrafish testis. White arrowheads indicate intensely labeled Sertoli cell cytoplasm. No specific staining was obtained with a sense cRNA probe. K–N, Immunofluorescence staining showing vimentin (red) and Igf3 (green) expression pattern in cytoplasmic extensions of Sertoli cells contacting germ cells

constructs, human embryonic kidney (HEK) 293T cells were incubated in DMEM in the absence (basal) and presence (stimulation) of 10μM forskolin (Sigma-Aldrich) in order to determine the respective firefly luciferase activities. The complete procedure is described in more detail in Supplemental Materials and Methods.

Statistical analysis

All data are represented as mean ± SEM. Significant differences between 2 groups were identified using Student's *t* test (paired or unpaired, as appropriate) ($P < .05$). Comparisons of more than 2 groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($P < .05$). GraphPad Prism 4.0 (GraphPad Software, Inc) was used for all statistical analyses.

Results

Fsh triggers zebrafish spermatogenesis in an androgen-independent manner

Previous work in different fish species suggested that Igf1 directly stimulated germ cell proliferation (38) or supported permissively (39) gonadotropin-stimulated spermatogenesis. Therefore, we examined whether exposure to Fsh resulted in changes in the testicular transcript levels of the 4 Igf family members in zebrafish (*igf1*, *igf2a*, *igf2b*, and *igf3*). We found that Fsh strongly increased testicular *igf3* transcript levels in vivo (2 h) (Figure 1A) and after 2 days of tissue culture (Figure 1B). Other Igf family members showed no or much less prominent changes. The *igf2a/b* transcript levels did not change or were reduced in response to Fsh or androgens, so that we dismissed these Igf family members from further analyses in this study. Although a short-term elevation of *igf1* transcript levels was recorded (Figure 1A), comparative analysis of the transcript abundances for *igf1* and *igf3* indicated that already under

basal conditions and certainly after Fsh or androgen stimulation, *igf3* transcripts were more abundant than *igf1* transcripts (see Discussion section). The response to rzfLh (34) was clearly distinct (Figure 1, A and C): in vivo, only a slight stimulatory effect on *igf1* mRNA levels was observed, whereas ex vivo the *igf3* mRNA levels were increased slightly but significantly. Because both gonadotropins stimulated Leydig cell steroid release in zebrafish (34), we examined whether part of the stimulatory response was attributable to steroid production. To this end, we blocked the production of biologically active steroids with trilostane, a 3β-hydroxysteroid dehydrogenase inhibitor. The Fsh-induced increase in *igf3* mRNA levels was slightly but not significantly reduced (Figure 1B), whereas the weaker, Lh-mediated increase in *igf3* mRNA levels was blocked completely (Figure 1C). Quantifying *igf3* mRNA levels under the influence of androgens confirmed that *igf3* transcript levels were up-regulated by 11-KT (Figure 1D).

We then examined whether Fsh stimulated spermatogenesis ex vivo, using different approaches and wild-type or *vasa::egfp* transgenic zebrafish. To address the androgen-independent effects of rzfFsh on zebrafish spermatogenesis, 2 experimental settings were used: 1) trilostane was included in the incubation medium to block the production of biologically active steroids, or 2) testis tissue from an E_2-induced androgen-insufficiency model was used (32). In *vasa::gfp* transgenic animals, the single type A undifferentiated (A_{und}) spermatogonia and the first generations of type A differentiating (A_{diff}) spermatogonia show a high level of Gfp expression compared with germ cells in more advanced stages of differentiation (spermatogonia B and spermatocytes) (see Figure 2B). Examining whole testes from this transgenic line (not E_2 pretreated) by confocal laser scanning microscopy (CLSM) after 7 days of incubation with Fsh (500 ng/mL) (Figure 2, A and B) and trilostane revealed an increase in the number of strongly Gfp-positive spermatogonia. After the initial CLSM examination, the tissue was fixed and prepared for immunocytochemical detection of Gfp protein (Supplemental Figure 2) to quantify the number of strongly Gfp-positive, single type A_{und} spermatogonia. This approach confirmed the increase in the number of spermatogonia type A_{und} in response to Fsh (Figure 2C). Using the androgen-insufficiency model, we examined Fsh effects on BrdU-incorporation and found increased mitotic indices of both type A_{und} and type A_{diff} spermatogonia (Figure 2, D–F). Also type B spermatogonia, either rarely seen or even absent in control incubations in this androgen-insufficiency model, were present and proliferated in the presence of Fsh (Figure 2E). We conclude that Fsh stimulated spermatogonial development in an androgen-independent manner. Analyzing gene expression in testis tissue of the androgen-insufficiency model, we found approximately 100-fold increases in testicular *igf3* mRNA and approximately 13-fold increases in *star* and *cyp17a1* transcript levels (Figure 2G), whereas no changes were observed in *fshr*, *igf1ra*, and *igf1rb* transcript levels. The E_2-induced androgen-insufficiency model is based on changes in Leydig and Sertoli cell activity, resulting in an inhibition of spermatogenesis, associated with an accumulation of type A spermatogonia (32). We also examined the proliferation response of spermatogonia type A_{und} to the same dose of Fsh (500 ng/mL) using testis tissue from animals not preexposed to estrogens. Not unexpectedly, the basal activity was higher, but we again found a clear Fsh-mediated increase of the mitotic index of spermatogonia type A_{und}.

To examine whether Fsh can also induce the development of haploid cells, aliquots of a testicular cell suspension ($\sim 2 \times 10^5$ cells) prepared from adult males in a way that they contain only about 3% of haploid cells (31), were incubated for 7 days in the absence or presence of 100-ng Fsh/mL in medium containing trilostane. Incubations with 11-KT (400nM) served as positive control; this androgen was previously shown to result in the production of BrdU-positive spermatids and spermatozoa in tissue culture (26). After 7 days, the cells were DNA-stained with propidium iodide and analyzed using a flow cytometer (Figure 2H). Both Fsh and androgen clearly increased the proportion of haploid cells from 3% to approximately 19%.

Figure 2. (Continued). in different stages of development. Propidium iodide (in blue) was used as a nuclear counterstaining. Colocalization with vimentin shows that Igf3 staining can cover the germ cell nucleus in particular in the case of large type A spermatogonia (inset in M and N) but is usually found in between germ cells in the cytoplasm of Sertoli cells (N). The cell types were identified mainly based on the nuclear diameter, according to Ref. 26. Dotted lines delimit testicular tubular compartment. Dotted lines in the inset (K) delimit type A spermatogonia nuclei and their nucleoli. A, type A spermatogonia; SPG A_{und}, undifferentiated type A spermatogonia; SPG A_{diff}, differentiated type A spermatogonia; SPG B or B, type B spermatogonia; SPC, spermatocytes; SPT, spermatids; SPZ, spermatozoa. Asterisks indicates intense Igf3 staining in the cytoplasm of SCs contacting type A SPG. Bars marked with *, $P < .05$; **, $P < .01$; or ***, $P < .001$ indicate significant differences between treated and control groups.

Igf3 and Igf receptor expression

One approach to study whether Igf family members and their receptors are expressed in germ cells and/or testicular somatic cells is to examine gene expression in the germ cell-free homozygous *piwil1* mutant (*piwil1*$^{(-/-)}$) (40) in comparison with wild-type fish. Initial PCR studies showed that the 2 Igf receptor genes present in the zebrafish genome, *igf1ra* and *igf1rb*, are both expressed in the testis (data not shown). We assumed that Igf or Igf

Table 1. Antibody Table

Peptide/Protein Target	Antigen Sequence (if known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
E domain of Igf3	C-ADLQRDEESASQRIRER	Anti-Igf3 (E domain)	Pacific Immunology	Rabbit	2 μg/mL
Gfp		Anti-Gfp	Torrey Pines Biolabs, Inc (catalog no. TP401)	Rabbit	1 μg/mL
Mouse/rabbit/rat IgG		PowerVision Poly-HRP	Immunologic, Klinipath (catalog no. DPVO110HRP)	Goat	1:200
Rabbit IgG		Antirabbit Alexa-Fluor 488	Life Technologies (catalog no. A-11034)	Goat	8 μg/mL
Vimentin		Antivimentin	DSHB (catalog no. H5)	Chicken	3 μg/mL
Mouse IgM		Antimouse Alexa Fluor 405	Life Technologies (catalog no. A-31553)	Goat	8 μg/mL
BrdU		Anti-BrdU	BD Bioscience (catalog no. 347580)	Mouse	1:80

receptor gene expression clearly below the levels found in wild-type fish would indicate predominant germ cell expression of that gene(s). The *piwil2* mRNA levels in the *piwil1*$^{(-/-)}$ mutant were very low, compatible with the germ cell depletion; the *piwil2* gene is expressed in most germ cells except spermatozoa (41). The data revealed that neither ligands nor receptor genes followed a pattern, suggesting predominant germ cell expression (Supplemental Figure 3). With respect to the ligands, only the reductions for *igf2b* mRNA levels reached statistical significance in the germ cell-free mutant males but were still clearly measurable. Considering the receptors, *igf1ra* transcript levels were significantly reduced, but *igf1rb* mRNA levels remained unchanged in the mutant (Supplemental Figure 3). One possible explanation for this pattern is that *igf1ra* mRNA is expressed by germ and somatic cells, whereas *igf1rb* is mainly expressed by somatic cells. However, the background for a reduction in *igf1ra* transcript levels could also be that Sertoli cell expression of this receptor depends in part on the presence of germ cells. This suggests that none of these genes is predominantly expressed in germ cells but does not exclude germ cell expression.

ISH experiments with testis tissue collected 6 hours after injecting 100-ng Fsh/g body weight showed that *igf3* mRNA was localized in the cytoplasm of Sertoli cells contacting single germ cells, small and larger groups of germ cells, representing type A and type B spermatogonia and spermatocytes (Figure 2I). The *igf3* mRNA expression was very weak or absent in Sertoli cells contacting spermatids, and no staining was found associated with spermatozoa (Figure 2I).

Igf3 protein was detected with an antibody raised against the E domain of Igf3 (Supplemental Materials and Methods). Using an immunofluorescence approach and analysis by CLSM allowed detecting Igf3 protein in Sertoli cells in association with all germ cell types except spermatozoa (Supplemental Figure 4, A–C). The fluorescence showed a small granular appearance in Sertoli cells contacting type A and B spermatogonia, but changed into a staining of larger vesicles when Sertoli cells contacted spermatocytes and in particular spermatids (Supplemental Figure 4C). To confirm the Sertoli cell localization of the Igf3 staining, we carried out double labeling experiments, using an antiserum against vimentin, a type III intermediate filament that is not expressed by germ cells. These studies showed that the large type A spermatogonia were enveloped by Igf3-positive Sertoli cell cytoplasm (see insets on Figure 2, M and N), and that all Igf3 staining was located in Sertoli cell cytoplasm between more advanced germ cell stages up until spermatids (Figure 2, K–N). The specificity of the staining obtained with the Igf3 antibody was confirmed by preincubating it with the peptide used for immunization (for antibodies, please see Table 1), which blocked the vast majority of the staining (Supplemental Figure 4).

CRE-binding protein- and ATF-1-response elements in the *igf3* promoter are involved in regulating *igf3* gene expression

The PKA inhibitor H89 (100μM) and the MAPK inhibitor PD98059 (50μM) clearly reduced the Fsh-induced (500 ng/mL) increases in testicular *igf3* mRNA levels (Figure 3). The strong down-regulation of *igf3* expression by H89 led us to investigate cAMP/PKA-related elements in the *igf3* promoter sequence.

To study the regulation of *igf3* gene expression in more detail, 2 distinct (ie, polymorphic) zebrafish *igf3* promoter sequences (see Supplemental Materials and Methods) as well as truncated and mutated versions (Figure 4, A and B) were cloned in front of a luciferase reporter gene and investigated after transfection in HEK 293T cells. After stimulation with 10μM forskolin, luciferase activity was

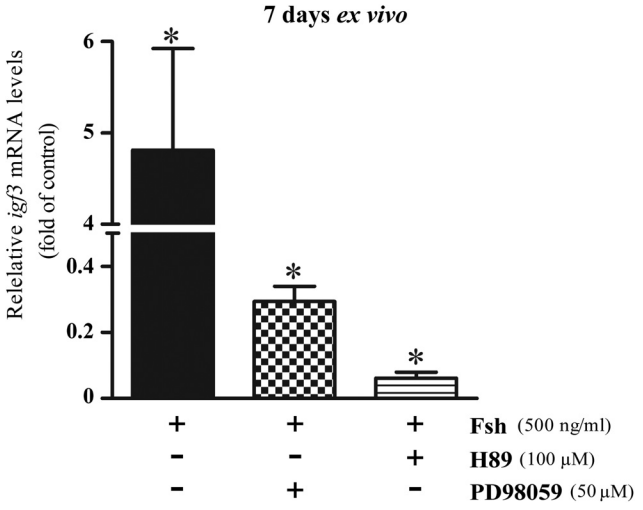

Figure 3. Relative mRNA levels of *igf3*. Testicular *igf3* transcript levels after incubation of tissue explants for 7 days with rzfFsh in the presence or absence of a PKA inhibitor (H89, 100μM) or a MAPK inhibitor (PD98059, 50μM; n = 4–7 explants per treatment). The columns represent the fold change of the relative mRNA levels in the treated group over its respective control (mean ± SEM). Asterisks indicate significant differences ($P < .05$) between treated and control groups.

reduced in cells transfected with the promoters harboring mutations in the response element for ATF-1 or the CRE. Moreover, the transcription factors binding to these elements seem to interact with each other depending on the length (2.0 or 1.0 kb) of the *igf3* promoter sequences. In the longer promoter sequences (Figure 4, C and E), the loss of CRE implied a stronger reduction of luciferase activity compared with the loss of ATF-1-response element. Even though loss of CRE alone was effective, no further reduction was observed in the luciferase activity when ATF-1-response element and CRE were mutated simultaneously. On the other hand, in the shorter promoter sequences (Figure 4, D and F), the loss of CRE did not induce significant changes in the luciferase activity in comparison with the nonmutated sequence, whereas loss of the ATF-1-response element alone (Figure 4, D and F) or in combination with CRE (Figure 4F) strongly reduced luciferase activity, 1 variant of the promoter again showing a significant additional reduction in activity after losing both sites (Figure 4F). These results suggest that 1) the ATF-1 and CRE sites interact, and that 2) elements upstream of −1.0 kb interact with the CRE site or with the ATF-1/CRE interaction. Altogether, in silico and functional analysis of the *igf3* promoter suggests that the main pathway used by Fsh to regulate *igf3* expression is via the cAMP/PKA route.

Recombinant Igf3 stimulates zebrafish spermatogenesis

Using primary testis tissue cultures from adult males rendered androgen insufficient by exposure to E_2 in vivo for 3 weeks (39), we carried out a dose-finding experiment with increasing concentrations (10, 100, or 1000 ng/mL) of rzfIgf3. A clear stimulation of spermatogenesis was observed at a concentration of 100 ng/mL, as revealed by morphological and gene expression analyses (Figures 5 and 6, A–D). Examining the mRNA levels of the *dazl* gene, expressed in type B spermatogonia and leptotene/zygotene spermatocytes in zebrafish (42), or of the *piwil2* gene expressed in all germ cells except spermatozoa (41), we found 2- to 4-fold increases, respectively, in response to 100-ng/mL Igf3 (Figure 5). No significant changes were found for the mRNA level of a gene expressed in type A spermatogonia (*piwil1*) (31), in pachytene spermatocytes (*sycp3l*) (42), or in spermatids (*shippo1*) (43). Also the mRNA levels of 9 genes (preferentially) expressed in testicular somatic cells (*amh*, *ar*, *cyp17a1*, *fgf8*, *fshr*, *insl3*, *lhcgr*, *pgr*, and *star*) as well as *igf1ra* and *igf1rb* mRNA (expressed in somatic and germ cells) did not change in response to Igf3 (data not shown).

Morphological analysis of testis tissue incubated in the absence of Igf3 showed the typical appearance of androgen insufficiency of the estrogen exposure model: the tissue contains mainly type A_{und} and some type A_{diff} spermatogonia (Figure 6A). Tissue incubated with 100-ng/mL Igf3 showed a clearly different germ cell composition (Figure 6B). Type A_{diff} spermatogonia were more prominent, type B spermatogonia and also spermatocytes, both rare or absent from control samples, were regularly found after incubation with Igf3 (Figure 6 and Supplemental Figure 5). To examine whether the biological activity of Igf3 includes modulation of testicular androgen release in zebrafish, we quantified basal and Fsh-stimulated 11-KT release, both in the absence and presence of Igf3. We did not detect statistically significant changes in androgen release (Supplemental Figure 1B), which is in line with the absence on Igf3 effects on mRNA levels of genes involved in steroidogenesis (*star* and *cyp17a1*; see above). Thus, Igf3 directly stimulated the proliferation and differentiation of spermatogonia and entry into meiosis in tissue culture, which was not associated with a change of mRNA levels for several somatic genes (growth factors, receptors, genes related to steroidogenesis) or with a change in androgen release.

An Igf receptor inhibitor blocks Fsh-induced proliferation of type A spermatogonia

Next, we examined whether Igf3 mediated the stimulatory effect of Fsh on proliferation of type A spermatogonia. To this end, we quantified BrdU-incorporation into testis tissue cultures from males rendered androgen-insufficient. In line with previous experiments, we first showed that Fsh (500 ng/mL) led to an approximately 2-fold in-

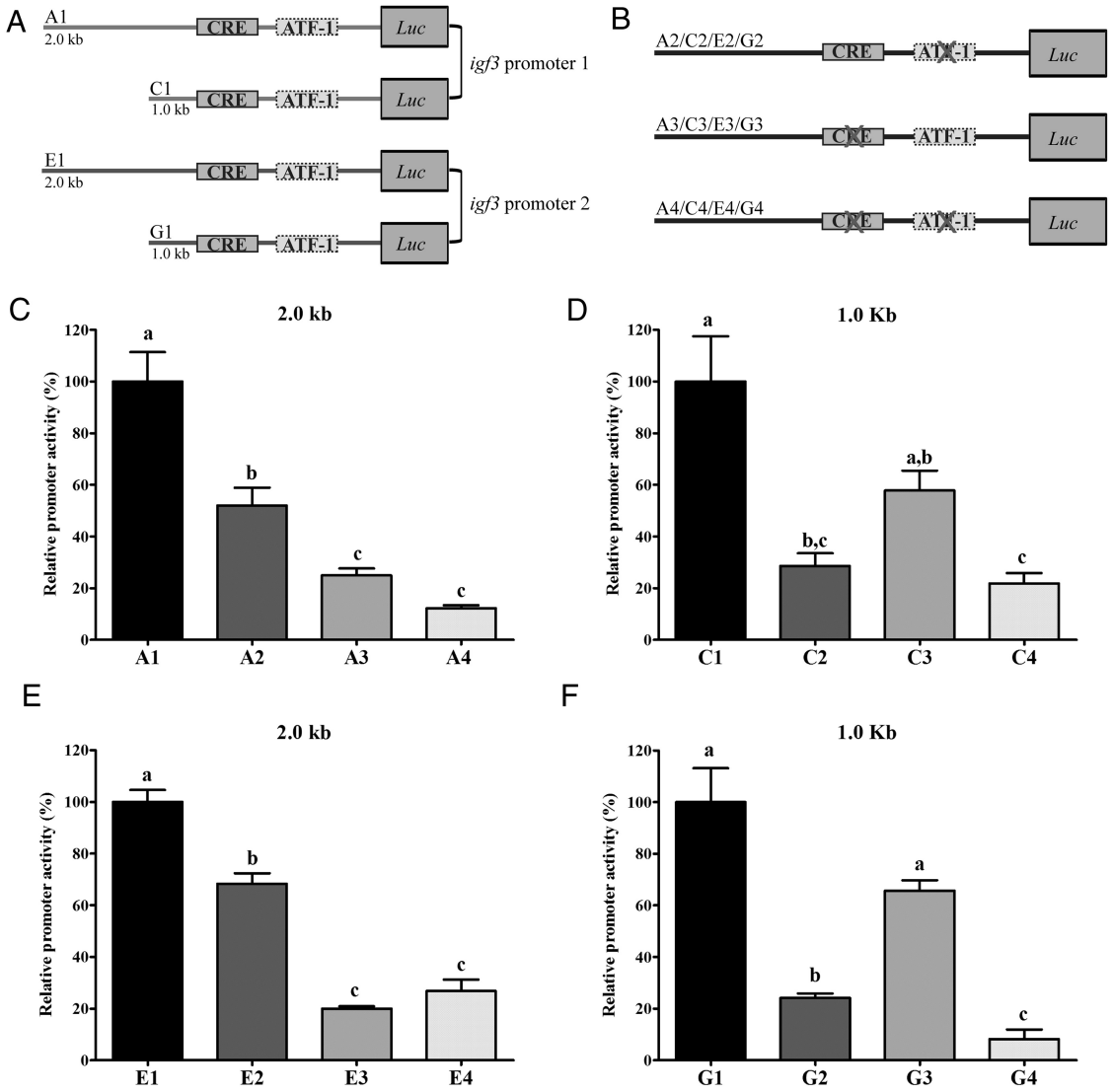

Figure 4. The *igf3* promoter activity in HEK 293T cells. A and B, Schematic representation of 2 different, probably polymorphic (A and C vs E and G) promoter regions of 2.0 kb (A1 and E1) and 1.0 kb (C1 and G1) of the zebrafish *igf3* gene, with presumed CRE-binding protein and ATF-1 binding sites indicated, as well as mutant promoter regions with inactivating point mutations in ATF-1 (A2, C2, E2, and G2), CRE-binding protein (A3, C3, E3, and G3), or both ATF-1 and CRE-binding protein (A4, C4, E4, and G4) binding sites. C–F, Luciferase activity of cells, transfected with the different promoter constructs, treated with or without 10μM forskolin. The columns are expressed as percentage of luciferase activity, normalized to the nonmutate *igf3* promoter sequences (mean ± SEM). Data were compiled from 6 independent transfections derived from 2 different cDNA preparations. Asterisks indicate significant differences ($P < .05$) between treated and control groups, different letters indicate significant differences ($P < .05$) between the different constructs.

crease of the mitotic index of type A_{und} spermatogonia (Figure 6D). Also Igf3 (100 ng/mL) resulted in a similar increase of the proliferation activity (Figure 6D) as found in previous experiments (Figure 6C and Supplemental Figure 5). An Igf receptor kinase inhibitor (NVP-AEW541) blocked this stimulatory effect of Igf3 (Figure 6D). Importantly, also the Fsh-mediated increase of the mitotic index of type A_{und} spermatogonia was blocked by the addition of NVP-AEW541 (Figure 6D). The Igf receptor inhibitor alone had no effect on the mitotic index (Figure 6D), suggesting that basal release of Igf3 (or of other Igf family members) is low or not associated with stimulating spermatogonial DNA synthesis.

Discussion

Our data show that Fsh stimulates in a cAMP-dependent manner Sertoli cell production of Igf3, which then stimulates via Igf receptor signaling but independent of androgen release, the proliferation and differentiation of spermatogonia and their entry into meiosis in the adult zebrafish testis. Although Fsh could still effectively stimulate *igf3* transcript levels when androgen production was blocked, androgens also exerted a moderate stimulatory effect on *igf3* transcript levels.

The pituitary gonadotropins control testicular development and function by regulating the activity of local

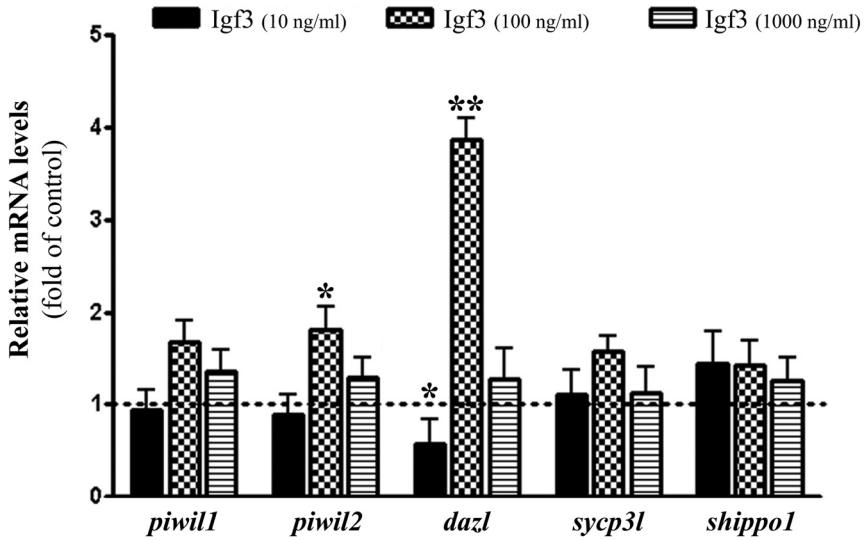

Figure 5. Gene expression in response to different concentrations of Igf3. Transcript levels of selected genes in zebrafish testis tissue cultured for 7 days in the presence of different concentrations of rzfIgf3 (10, 100, and 1000 ng/mL). The columns represent the fold change of the relative mRNA levels in the treated group over its respective control (mean ± SEM). Asterisks indicate significant differences ($P < .05$) between treated and control groups.

signaling systems in vertebrates in general, but evolution seems to have taken a different path in teleost fish with respect to the gonadotropic hormones and their biological activities. Fsh is the main gonadotropin driving the initial stages of spermatogenesis in fish (33, 34, 44–48), by regulating both Leydig and Sertoli cell activities until spawning. Further support for this conclusion is provided by the observation that loss-of-function of the *lhcgr* (49) or *lhb* (49, 50) gene in zebrafish results in female but not in male infertility (49, 50), suggesting that Lh bioactivity is not required for spermatogenesis in zebrafish whereas loss-of-function of the *fshb* gene in male zebrafish delayed puberty (50). However, male infertility was observed when both *fshr* and *lhcgr* genes were knocked out (51). For the present study, we focused our experimental approach on the Sertoli cell-mediated effects of Fsh by neutralizing its steroidogenic activity.

In rodents, FSH can modulate the production of Sertoli cell growth factors that are relevant either for SSC self-renewal (eg, GDNF [5], basic fibroblast growth factor [52]) or differentiation (eg, activin [9]). We became interested in the insulin/Igf signaling system in this regard in zebrafish because this system appears to have an evolutionarily conserved role in regulating male germ line stem cell proliferation.

In *Caenorhabditis elegans*, expression of insulin/Igf ligand occurs in somatic cells, whereas receptor signaling is crucial in germ cells (53, 54). In *Drosophila melanogaster*, receptor signaling is relevant in both germ and somatic cells (55). In mice, receptors are also expressed in germinal and somatic compartments of the testis, but receptor ablation is only dispensable in the germ cell compartment, whereas Sertoli cell-specific loss of the IGF receptor (or of IGF and insulin receptors), compromised Sertoli cell proliferation and hence adult testis size and sperm output (56). Also in trout (57) and seabream (58), Igf receptors were found in somatic and germ cells, which is consistent with our analysis of Igf receptor gene expression in wild-type and germ cell-free mutant zebrafish. Genetic evidence as regards the cell type-specific effects of receptor gene ablation is missing in zebrafish, but recent work showed that thyroid hormone-induced Sertoli cell proliferation involved elevated *igf3* transcript levels, and that this proliferation was compromised (but not fully blocked) by an Igf receptor antagonist (59). One way of understanding the results on Igf3-stimulated germ cell development presented here is assuming a direct stimulatory effect of Igf3 on germ cells, as has been shown for Igf1 in primary spermatogonial cell culture studies of trout (60), and possibly also an autocrine stimulation of Sertoli cell proliferation to support the survival of newly formed spermatogonia type A. In the eel, on the other hand, Igf does not have stimulatory effects on germ cell proliferation per se but is required as permissive factor for androgen-mediated stimulation of spermatogenesis (39), so that in eel Igf signaling might regulate Sertoli cell functions that facilitate androgen action.

In mammals, Igf ligands can reach the testes from extratesticular sites via the circulation but can also be derived from different testicular cell types, such as Sertoli and Leydig cells in mice (61) or different germ cell types in stallion (62). Both somatic (Leydig and Sertoli cells) and germ cells (mainly spermatogonia and spermatocytes) have been identified as Igf1 sources also in fish (57, 58, 63, 64). In tilapia, Igf3 protein was found in the interstitial tissue (65), using an antiserum raised against the complete Igf3 sequence, so that a cross-reaction with Igf1, previously localized to Leydig cells (64) seems possible. In zebrafish, we detected both mRNA and protein exclusively in Sertoli cells.

In this study, we have demonstrated that *igf3* is the main *igf* family member responding to Fsh in zebrafish testis, according to the next evidence. First, *igf2a/b* transcript levels did not change or were reduced in response to Fsh or androgens. Secondly, comparing the cycle thresh-

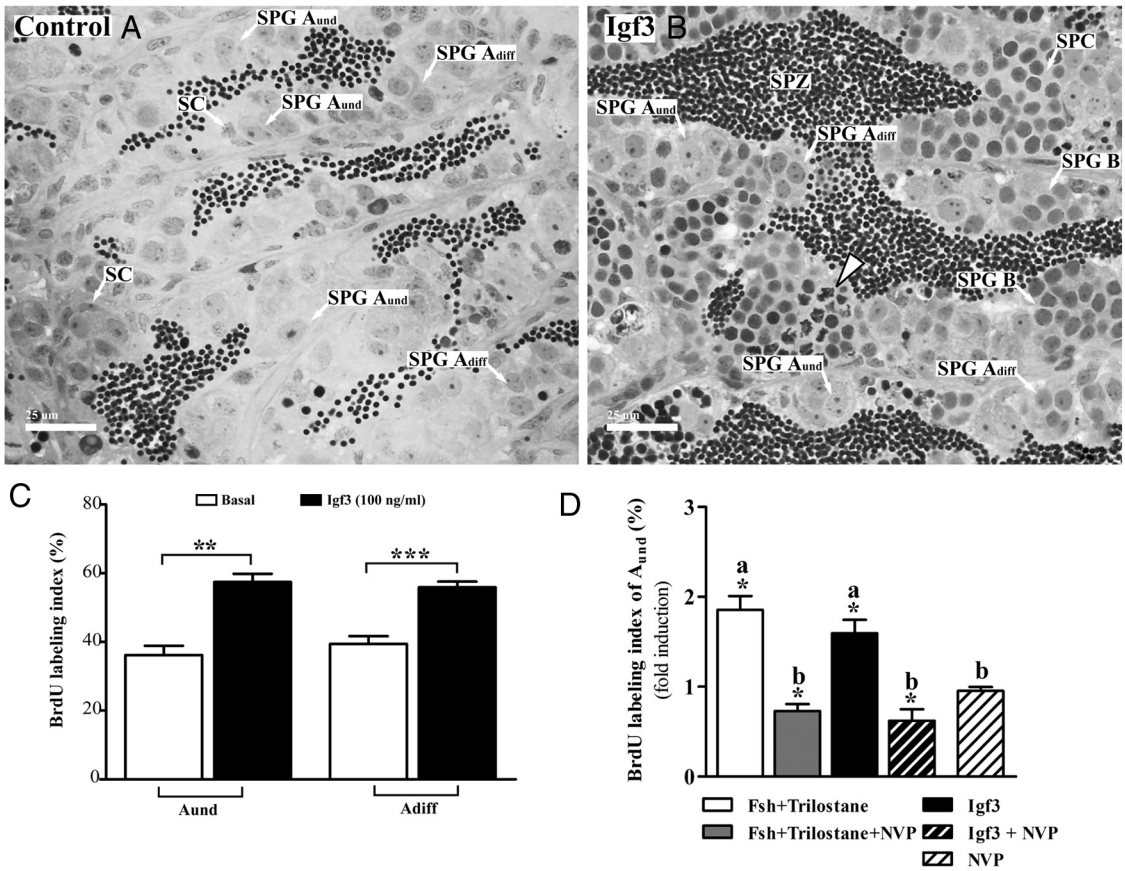

Figure 6. Effects in vitro and in vivo of rzfIgf3 in the zebrafish testis. A and B, Morphological analysis of zebrafish testis, after 7 days in culture in the absence or presence of rzfIgf3 (100 ng/mL), demonstrating Igf3-induced recovery of spermatogenesis after its interruption by exposure to 10nM E_2 in vivo. C, BrdU labeling index of A_{und} and A_{diff} in zebrafish testis, preexposed to 10nM E_2 in vivo for 21 days, and cultured for 7 days in the absence or presence of rzfIgf3 (100 ng/mL; n = 8). D, BrdU labeling index of A_{und} spermatogonia under different conditions: Fsh+Tril, Fsh+Tril+NVP, Igf3, Igf3+NVP, and NVP. In the presence of the Igf1R inhibitor (NVP), Igf3-, but also Fsh-, induced proliferation of A_{und} was suppressed. SC, Sertoli cells; SPG A_{und}, A_{und} spermatogonia; SPG A_{diff}, type A differentiated spermatogonia; SPG B, type B spermatogonia; SPC, spermatocyte; SPZ, spermatozoa; NVP, Igf receptor inhibitor NVP-AEW541. Bars marked with *, $P < .05$ are significantly different from control condition, and different letters denote statistical significant differences among the different treatments.

old values for *igf1* and *igf3* transcripts indicated that under basal and certainly under Fsh/androgen stimulation, *igf3* transcripts were more abundant. We have moreover interrogated a recently completed RNA sequencing experiment for normalized RNA read numbers for *igf1* and *igf3*, respectively. In testis tissue from adults analyzed directly after dissection and not subjected to any treatment, *igf3* transcripts were 6.2-fold more abundant than *igf1* transcripts. Finally, the response of *igf1* transcript levels was transient (visible at 2 h but not after 2 d), whereas *igf3* mRNA levels show a quick (at 2 h) and sustained (clearly elevated at 2 and 7 d) response. Therefore, we dismissed *igf1* and *igf2a/b* from our further analysis and considered *igf3* as the main *igf* gene family member that was stimulated by Fsh in zebrafish testis.

Further analysis demonstrated that rzfFsh increased testicular *igf3* transcript levels through cAMP/PKA pathway. Similarly, human chorionic gonadotropin increased via cAMP/PKA *igf3* transcript levels in tilapia (65) and zebrafish ovarian tissue (66, 67). Analysis of the *igf3* promoter after transfection into HEK 293T cells demonstrated that the 2 cAMP-sensitive elements, CRE and ATF-1, were relevant for the activation of the luciferase reporter gene, because 80%–90% of the promoter activity was lost when both transcription factor binding sites were mutated in the *igf3* gene promoter. Moreover, the results suggested that ATF-1/CRE interaction and efficiency in the transcriptional activation of *igf3* promoter are modulated by elements present upstream −1.0 kb of the promoter sequence.

Igf3 mediated gonadotropin action also in the zebrafish ovary, but in females, the final stages of oocyte development were the target of Igf3 action and Lh rather than Fsh appeared to be responsible for regulating *igf3* gene expression (68). Also purified Chinook salmon Fsh increased *igf3* transcript levels in rainbow trout testes, whereas a somewhat puzzling aspect of the trout dataset is the observation that androgens inhibited basal and Fsh-

stimulated increases in *igf3* transcript levels although Fsh strongly stimulated androgen production (69, 70). The Fsh-induced increase of *igf3* transcript levels in the zebrafish testis, on the contrary, was slightly reduced when the production of biologically active androgens was blocked by trilostane, suggesting a mild, positive influence of androgens on *igf3* transcript levels that was also confirmed experimentally (Figure 1E). The reason for this discrepancy between trout and zebrafish is not clear at present but might be related to the fact that trout testis was studied at the beginning of pubertal development, whereas we used testis tissue from adult males. Also in adult tilapia, androgen insufficiency after estrogen exposure led to reduced *igf3* transcript levels (64). Altogether, these data suggest that Fsh is a major stimulator of testicular *igf3* transcript levels.

Considering that Gh increased testicular *igf1* transcript levels in trout Sertoli cells, spermatogonia, and spermatocytes (57), that Gh receptors were found in trout testis tissue, in particular in Sertoli cell-enriched fractions (44), and that recombinant human IGF-1 stimulated the proliferation of trout spermatogonia in primary cell culture experiments (60), it seemed possible that piscine Gh may have an effect on testicular *igf3* transcript levels as well. However, because Gh did not change *igf3* mRNA levels in zebrafish ovarian tissue (67), and because overexpression of Gh had no effect on *igf3* transcript levels in tilapia testes (71), we did not study this aspect in adult zebrafish testis.

The present results on the localization and effects of Igf3 suggest autocrine stimulation of Sertoli cell proliferation (59) and paracrine stimulation of the proliferation of type A and type B spermatogonia as well as their entry into meiosis. Future studies will have to show what germ cell stage(s) can respond directly to Igf3, eg, by studying Igf receptor localization. Moreover, Igf-binding proteins might modulate Igf effects (72), and ongoing studies in our laboratory show that several Igf-binding proteins are expressed in the zebrafish testis, so that further studies are needed to understand the complex Igf signaling system in the zebrafish testis.

Although rzfIgf3 showed clear biological activity at 100 ng/mL, we noted a bell-shaped dose-response curve in some experiments (Figure 5). It is possible that the reduced biological activity at high Igf3 concentrations is related to observations made at high insulin concentrations when ligand dimerization compromised receptor signaling (73), or to high IGF concentrations that induced a negative cooperativity at the IGF receptor (74).

In experiments with recombinant Fsh, we noted that not only spermatogonial and meiotic stages were stimulated but that, different from effects observed after incubations with recombinant Igf3, also postmeiotic stages were found (Figure 2H), whereas the production of biologically active steroids was blocked by trilostane. This is remarkable in 2 ways. First, previous work using immature Japanese eel (*Anguilla japonica*) testis tissue suggested that the stimulatory effect of Fsh on spermatogenesis is explained by the Fsh-triggered production of androgens in Leydig cells (46). This is different in adult zebrafish testes, where the stimulatory effects of Fsh are clearly visible despite inhibiting androgen production, but are compromised by an inhibitor of Igf receptor signaling. As discussed above, this might reflect species-specific differences in the cellular site of Igf action that, in eel, seems to be of a permissive, potentially autocrine manner on Sertoli cells to facilitate androgen action. Secondly, Fsh seems to have a broader range of stimulatory actions on spermatogenesis (eg, including stimulation of postmeiotic stages) than Igf3, suggesting that other, nonsteroidal mediators of Fsh action may be responsible for the transition through meiosis and entrance into spermiogenesis. In this regard, zebrafish Fsh shows a broader range of biological activity than in rodents where androgen rather than FSH action is required for the completion of meiosis and spermiogenesis (17, 21). Several experiments on Fsh effects were carried out using E_2-pretreated fish. This pretreatment altered Leydig cell function and resulted in an androgen insufficiency that in turn down-regulated spermatogenic activity

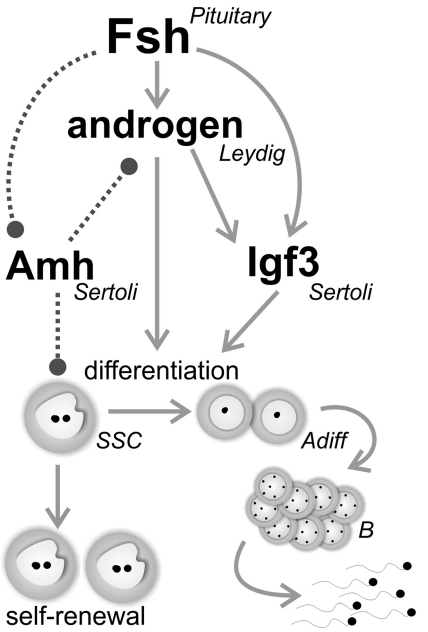

Figure 7. Schematic representation of Fsh bioactivity on zebrafish spermatogenesis. Fsh stimulates Leydig cell androgen production, which in turn promotes germ cell differentiation. At the same time, Fsh reduces (stippled line) Sertoli cell expression of Amh that would otherwise inhibit Leydig androgen production and differentiation of spermatogonia. Fsh also stimulates Sertoli cell expression of Igf3, which promotes germ cell differentiation; androgens also (weakly) stimulate Igf3 production.

(32). However, comparing results from proliferation (this work) and gene expression (34) with and without E_2 pretreatment (Supplemental Figure 6), similar results were obtained, except that the amplitude of the response was greater in E_2-pretreated males. It therefore appears that Fsh effects are not compromised but rather magnified in the androgen-insufficiency model.

Upon recovering from a cytostatic insult, type A_{und} spermatogonia became activated in zebrafish and transcript levels of *igf3* were up-regulated, whereas those of *amh* were down-regulated (31). Moreover, other experiments showed that Fsh suppressed testicular *amh* transcript levels in adult zebrafish, thereby preventing inhibitory effects Amh would otherwise have exerted on androgen production, and also preventing inhibitory effects of Amh on proliferation and differentiation of type A spermatogonia (3). It therefore appears that Fsh stimulates zebrafish spermatogenesis by controlling the balance between inhibitory (eg, Amh) and stimulatory (eg, Igf3, sex steroids) signals (Figure 7). However, additional, yet to be described factors and components, including those mediating stimulatory effects on meiotic and postmeiotic stages are probably involved in mediating Fsh action on spermatogenesis. Transcriptomic analyses are ongoing to identify additional testicular Fsh target genes in zebrafish.

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