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Fsh stimulates Leydig cell Wnt5a production, enriching zebrafish type A spermatogonia

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Abstract

Follicle-stimulating hormone (Fsh) modulates vertebrate spermatogenesis by regulating somatic cell functions in the testis. We have found previously that zebrafish Fsh stimulated the differentiating proliferation of type A undifferentiated spermatogonia (A_{und}) in an androgen-independent manner by regulating the production of growth factors and other signaling molecules in both Sertoli (SCs) and Leydig cells (LCs). For example, Fsh triggered the release of Igf3 that subsequently activated β-catenin signaling to promote the differentiating proliferation of A_{und}. In the present study, we report that Fsh moreover uses the non-canonical Wnt pathway to promote the proliferation and accumulation of A_{und}. Initially, we found that the stimulatory effect of Fsh on the proliferation activity of A_{und} was further strengthened when β-catenin signaling was inhibited, resulting in an accumulation of A_{und}. We then showed that this Fsh-induced accumulation of A_{und} was associated with increased transcript levels of the non-canonical Wnt ligand, *wnt5a. In situ* hybridization of *insl3* mRNA, a gene expressed in LCs, combined with Wnt5a immunocytochemistry identified LCs as the cellular source of Wnt5a in the adult zebrafish testis. Addition of an antagonist of Wnt5a to incubations with Fsh decreased both the proliferation activity and the relative section area occupied by A_{und}, while an agonist of Wnt5a increased these same parameters for A_{und}. Taken together, our data suggest that Fsh triggered LCs to release Wnt5a, which then promoted the proliferation and accumulation of A_{und}. Hence, Fsh uses non-canonical Wnt signaling to ensure the production of A_{und}, while also triggering β-catenin signaling via Igf3 to ensure spermatogonial differentiation.

Key Words

- Fsh
- Wnt
- spermatogonia
- proliferation
- differentiation

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Introduction

Spermatogenesis is a stem cell-based process that that requires communication between germ and somatic cells in the testis. When proliferating, the spermatogonial stem cells (SSCs) can either produce more SSCs or can produce differentiating daughter cells committed to develop into spermatozoa (De Rooij 2017). In fish, as in all vertebrates, SSCs form part of the population of type A_{und} spermatogonia (Lacerda *et al.* 2010, Nóbrega *et al.* 2010, Sato *et al.* 2017). In fish and amphibians, A_{und} are enveloped by Sertoli cells (SCs), thereby forming a spermatogenic cyst. These cysts are formed based on the production and hence proliferation of SCs, which associate with a type A_{und} spermatogonium to form a new spermatogenic cyst. SCs also proliferate while accompanying the development of a germ cell clone derived from a given A_{und} spermatogonium in its growing cyst (Schulz *et al.* 2010).

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The balance between self-renewal and differentiation is determined by the microenvironment the type A_{und} spermatogonium is exposed to in its cyst. For this microenvironment, the cyst-forming SCs are important contributors that are sensitive to reproductive hormones.

The pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (Fsh) are major modulators of vertebrate spermatogenesis. Upon gonadotropin signaling, somatic cells such as SCs, LCs and myoid cells produce paracrine factors modulating spermatogonial activity (Oatley *et al.* 2009, Ding *et al.* 2011, Chen *et al.* 2016, de Rooij 2017, Lord & Oatley 2017). In mammals, Fsh regulates SC activities to support spermatogenesis (Mullaney & Skinner 1992, Ding *et al.* 2011, Pitetti *et al.* 2013), while LH stimulates LC androgen production. Different from mammals, fish Fsh modulates the function of both SCs and LCs (Ohta *et al.* 2007, García-López *et al.* 2010), so that Fsh can regulate spermatogenesis in an androgen-dependent and -independent manner. With regard to androgen-independent regulation of spermatogenesis, zebrafish Fsh stimulated spermatogonial differentiation by modulating the release of inhibitory and stimulatory growth factors such as Amh and Igf3, respectively, from SCs (Skaar *et al.* 2011, Nóbrega *et al.* 2015), and by promoting the production of Insl3 in LCs (Assis *et al.* 2016, Crespo *et al.* 2016). Indeed, the androgen dependency of spermatogenesis in fish seems less prominent than that in mammals, considering that spermatogenesis, but not secondary sex characteristics, were normal after preventing the production of androgens by mutating the *cyp17* gene in medaka (Sato *et al.* 2008). Also, after loss of androgen receptor function, spermato- and spermiogenesis were still possible, though clearly compromised quantitatively (e.g. Crowder *et al.* 2018). Evidently, androgen-independent mechanisms promoting spermatogenesis function efficiently in fish, and information is available already on the role of a few signaling systems, such as Amh, Igf3 or Insl3 (see above) or Gsdf in trout (Sawatari *et al.* 2007). Nonetheless, other pathways mediating androgen-independent Fsh effects remain to be investigated. Notably, a recent study reported that close to 200 testicular transcripts responded to zebrafish Fsh, including genes belonging to the Wnt pathway (Crespo *et al.* 2016).

The Wnt signaling system is a conserved cell-to-cell communication system that consists of several Wnt ligands and receptors. This system operates in branches that differ in their intracellular signaling pathways. The canonical Wnt pathway relies on the availability of β-catenin (Salic *et al.* 2000), which interacts with transcription factors to modulate gene expression. The non-canonical Wnt pathway activates different intracellular pathways (e.g. calcium-dependent or Jun N-terminal kinases) in a β-catenin-independent manner (Van Amerongen 2012). In mice, recent studies have shown that the canonical pathway is relevant for modulating spermatogenesis. Takase & Nusse (2016) reported that the β-catenin-target gene *Axin2* is expressed specifically in A_{und} spermatogonia and *β-catenin* knockout in *Axin2*-positive spermatogonia reduced the number of differentiating type A spermatogonia (A_{diff}). Similarly, Tokue *et al.* (2017) found that activating the canonical Wnt pathway reduced the number of GFRa1-positive cells (a marker for SSCs) and that an inhibitor of the canonical Wnt pathway, *Shisa6*, maintains the number of GFRa1-positive cells. On the other hand, less is known on the function of the non-canonical Wnt pathways in spermatogenesis. An *in vitro* study showed that only A_{diff} spermatogonia responded to canonical Wnt signaling, while the SC-derived, non-canonical ligand WNT5a maintained the number of SSCs by reducing their apoptosis (Yeh *et al.* 2011). In addition, Tanaka *et al.* (2016) showed that LH-dependent androgen production downregulated *Wnt5a* transcript levels in SCs, which promoted SSC differentiation. In zebrafish, we have shown recently that β-catenin signaling is activated in germ cells through the SC-derived Igf3 in response to Fsh, thereby stimulating the differentiation of type A spermatogonia (Safian *et al.* 2018). However, information on the role, if any, of non-canonical Wnt signaling in zebrafish spermatogenesis is missing.

Here, we report that Fsh, via the non-canonical Wnt signaling system, triggers LC Wnt5a production, leading to an expansion of the population of type A_{und} spermatogonia.

Materials and methods

Animals

Adult WT male zebrafish (AB strain) between 4 and 12 months of age were used in this study. All experiments carried out in this study followed the Dutch National regulations for animal care and all experimental protocols were approved by the Utrecht University Experimental Animal Committee (2013.III.06.045 and NVWA 10800).

Tissue culture

To study if Fsh uses the Wnt signaling system to regulate A_{und} spermatogonial fate in zebrafish, adult testes were

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dissected for tissue culture experiments using a previously described system (Leal *et al.* 2009), in which one testis was incubated under control conditions, the other under experimental conditions.

We have reported previously that 100 ng/mL Fsh-stimulated both self-renewal division of A_{und} and differentiating proliferation toward A_{diff} spermatogonia (Nóbrega *et al.* 2015, Safian *et al.* 2016), the latter by activating β-catenin signaling via Igf3 (Safian *et al.* 2018). To study the potential involvement of Wnt signaling in A_{und} proliferation and accumulation (i.e. self-renewal of A_{und} spermatogonia), zebrafish testes were incubated for 5 days in the presence of recombinant zebrafish Fsh (100 ng/mL) (García-López *et al.* 2010) with or without XAV939 (10 µM in dimethyl sulfoxide (0.01%); Sigma-Aldrich) (Safian *et al.* 2018), an inhibitor of the β-catenin-dependent pathway (Huang *et al.* 2009, Shimizu *et al.* 2012). In all experiments, incubation media for control and experimental groups contained the same final concentration of dimethyl sulfoxide.

To further study the role of the Wnt signaling system on Fsh-stimulated spermatogonial proliferation, zebrafish testes were incubated for 5 days in the presence of Fsh (100 ng/mL) with or without IWP-12 (50 µM in dimethyl sulfoxide (0.05%); Sigma-Aldrich) (Safian *et al.* 2018), an inhibitor of porcupine, a protein required to release Wnt ligands (Chen *et al.* 2009, Dodge *et al.* 2012) or in the presence of Fsh (100 ng/mL) and XAV939 (10 µM), with or without 50 µM IWP-12. Testes also were incubated in the absence or presence of XAV939 (10 µM; Sigma-Aldrich) or IWP-12 (50 µM; Sigma-Aldrich) for 5 days, to test for potential effects of these inhibitors alone.

To study changes in transcript levels of Wnt-related genes, testes were incubated in basal conditions with Fsh (100 ng/mL), recombinant zebrafish luteinizing hormone (Lh) (500 ng/mL) or with 11-ketotestosterone (11-KT) (200 nM in ethanol (0.01%); Sigma) (García-López *et al.* 2010) for 5 days. Based on results obtained with the above experiments, zebrafish testes were incubated for 5 days in the presence of Fsh (100 ng/mL) with or without Wnt antagonist III, Box5 (WAIIIB5; 100 µM, Merck). WAIIIB5 is a N-terminally butyloxycarbonyl (Boc) hexapeptide derived from the human WNT5a sequence that inhibits the effect of WNT5a and shares 100% identity with the zebrafish Wnt5a sequence (Jenei *et al.* 2009). In a parallel experiment, testes were incubated under basal conditions or in the presence of the Wnt5a agonist Foxy-5 (100 µM, Tocris), which is a modified WAIIIB5 (formyl instead of Boc in the N-terminal sequence) that mimics the effects of Wnt5a (Säfholm *et al.* 2008, Mehdawi *et al.* 2016).

The production of biologically active steroids was blocked by including trilostane (25 µg/mL in dimethyl sulfoxide (0.01%); Chemos), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, in all experiments with Fsh or Lh, both potent steroidogenic hormones in zebrafish (García-López *et al.* 2010).

At the end of the incubation period, testis tissues of the experiments described earlier was snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction and quantification of transcript levels of selected genes (see 'Transcript levels' section) or was fixed for morphological analysis (see 'Quantification of spermatogonial proliferation and of proportions of section surface areas' section).

Transcript levels

The relative transcript levels of germ cell markers and other genes of interest (Table 1) were analyzed by real-time, qPCR assays.

Total RNA was isolated from the tissue using an RNAqueous Micro kit (Ambion), according to the manufacturer's protocol. cDNA synthesis from total RNA and quantification of transcript levels were carried out as described previously (Bogerd *et al.* 2001). In brief, 2 µg of total RNA were reverse transcribed using 250 U of Superscript II RNase reverse transcriptase (Life Technologies). qPCRs were performed in SYBR Green assay mix (Applied Biosystems), specific qPCR primers (900 nM each) and 5 µL cDNA in a total volume of 20 µL. The quantification cycle (Cq) values were determined in aViiA7 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 (ΔΔCt method Livak & Schmittgen 2001)), as described in Bogerd *et al.* (2001). Transcript levels of the *elongation factor 1a* (*ef1a*) were stable and therefore, *ef1a* was used to normalize gene expression. All results were expressed as fold changes with respect to the control group.

Quantification of spermatogonial proliferation and of proportions of section surface areas

To quantify the proliferation activity of type A spermatogonia, 100 µg/mL of the proliferation marker 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was added to the tissue culture medium during the last 6 h of the incubation period. After fixation in methacarn (60% (v/v) absolute methanol, 30% chloroform and 10% acetic acid), the samples were dehydrated in graded ethanol (70, 96 and 100%), embedded in Technovit 7100 (Heraeus

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Table 1 Primers used for gene expression studies.

Target genes	Primers name	Sequence (5′-3′)	Gene information
elf1a	2476 (Fw)	GCCGTCCCACCGACAAG	Reference gene (Morais *et al.* 2013)
	2477 (Rv)	CCACACGACCCACAGGTACAG	
foxa2	5741 (Fw)	GTCAAAATGGAGGGACACGAAC	Potential marker for type A undifferentiated spermatogonia (Safian *et al.* 2017)
	5743 (Rv)	CATGTTGCTGACCGAGGTGTAA	
nanos2	4817 (Fw)	AAACGGAGAGACTGCGCAGAT	Expressed in type A_{und} spermatogonia (Beer & Draper 2013, Bellaiche *et al.* 2014)
	4818 (Rv)	CGTCCGTCCCTTGCCTTT	
piwil2	2994 (Fw)	TGATACCAGCAAGAAGAGCAGATCT	Expressed in all germ cell type except type A_{und} and spermatozoa (Houwing *et al.* 2008)
	2995 (Rv)	ATTTGGAAGGTCACCCTGGAGTA	
dazl	3104 (Fw)	AGTGCAGACTTTGCTAACCCTTATGTA	Expressed by B spermatogonia and primary spermatocytes (Chen *et al.* 2013)
	3105 (Rv)	GTCCACTGCTCCAAGTTGCTCT	
aldh1a2	4359 (Fw)	CGCTGGATGGGCAGATAAGA	Enzyme require for retinoic acid synthesis (Pradhan & Olsson 2015)
	4360 (Rv)	TCTGGTGAGGGTGAAAAATTCTC	
cyp26a1	4383 (Fw)	TGGGCTTGCCGTTCATTG	Enzyme involved in retinoic acid degradation (Feng *et al.* 2015)
	4384 (Rv)	CATGCGCAGAAACTTCCTTCTC	
wnt5a	5431 (Fw)	TGGAGATCGTGGACGCAAA	Non-canonical Wnt ligand (this paper)
	5432 (Rv)	CACTTCAGGAATCAGCAGAGGATT	
wnt11	5439 (Fw)	CTGAGCGTCATTTATCCATGCA	Non-canonical Wnt ligand (this paper)
	5440 (Rv)	ACGGAGCTCCCGTTTATCGT	

Fw, forward; Rv, reverse.

Kulzer) and sectioned at a thickness of 4 µm. To determine the proliferation activity, one set of sections was used to localize BrdU as described previously (Leal *et al.* 2009). The BrdU-labeling index was determined by analyzing 100 cysts (A_{diff} spermatogonia) or 100 A_{und} cells, discriminating between BrdU-positive and -negative cysts and cells, respectively. Furthermore, for one set of experiments, the number of BrdU-positive free SCs (apparently not associated with germ cells), SCs contacting BrdU-positive or -negative A_{und}, and SCs contacting BrdU-positive or -negative A_{diff} was determined using ten randomly chosen, non-overlapping fields at ×400 magnification.

To quantify the proportion of section area occupied by A_{und} and A_{diff} spermatogonia, another set of sections was stained with toluidine blue and ten randomly chosen, non-overlapping fields were photographed at ×400 magnification with a digital camera. The images were analyzed quantitatively based on the number of points counted over the germ cell types investigated (A_{und} and A_{diff}), using the ImageJ freeware (National Institute of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij) with a 540-point grid, i.e. 5400 data points were analyzed per individual.

Wnt5a quantification by Western blot

To study if Fsh stimulates Wnt5a protein production, zebrafish testes were incubated under basal conditions or in the presence of Fsh (100 ng/mL) for 5 days. After the incubation period, tissue was processed for Western blot according to Safian *et al.* (2018). Briefly, total proteins were extracted and resolved in 10% SDS-PAGE. Primary Wnt5a antibody incubation was carried out overnight at 4°C (2 µg/mL; AS-55879, Anaspec). After PBS washes, secondary horseradish peroxidase-conjugated goat anti-rabbit (0.2 µg/mL; 111-035-003, Jackson ImmunoResearch Laboratories Inc.) was incubated for 1 h at room temperature. Protein detection was performed using Pierce ECL Plus Substrate (Thermo Scientific) according to the manufacturer's instructions. Films were scanned and densitometric analysis of the bands was performed with ImageJ freeware. Wnt5a analysis was normalized to Coomassie blue staining of total protein (Fuentes *et al.* 2013).

Immunofluorescence on paraffin sections

The cell types responsible for Wnt5a production in adult zebrafish testis were identified by immunofluorescence. After fixation in phosphate-buffered 4% paraformaldehyde at 4°C overnight, testis tissue was dehydrated and embedded in paraplast (Sigma-Aldrich). Sections of 5 µm thickness were dewaxed and rehydrated, according to conventional techniques. After antigen retrieval (10 mM sodium citrate, 0.05% Tween 20, pH 6.0; at 98°C for 10 min) nonspecific antibody binding

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was blocked by pre-incubating slides with 5% normal goat serum (Sigma-Aldrich) and 1% acetylated bovine serum albumin (BSA) (Sigma-Aldrich) for 30 min at room temperature. Then, sections were incubated with a primary antibody raised in rabbit against zebrafish Wnt5a (4 µg/mL; AS-55879, Anaspec) in PBS containing 1% BSA overnight at 4°C. After PBS washes, sections were incubated with secondary goat anti-rabbit Alexa fluor 488 (8 µg/mL, A11034, Sigma-Aldrich) containing 10% normal horse serum (Sigma-Aldrich) for 90 min at room temperature. After PBS washes, the sections were incubated for 3 min with propidium iodide (1 µg/mL, Sigma-Aldrich). Sections were mounted in Vectashield (H-1000, Vector) and images were taken using a confocal laser scanning microscope (LSM 700, Zeiss). As negative control for the anti-Wnt5a staining, 4 µg/mL of primary antibody was pre-incubated with 20 µg/mL Wnt5a blocking peptide (AS-55879P, Anaspec) (volume ratio antibody:blocking peptide of 1:5) for 2 h at room temperature in PBS containing 1% BSA.

Testis whole mount *in situ* hybridization of *insl3* and immunofluorescent detection of Wnt5a

To further characterize the cell type(s) showing immunocytochemical labeling for Wnt5a, zebrafish testis was used to localize Wnt5a by immunofluorescence in combination with localizing transcripts of the LC-specific *insl3* gene by fluorescent *in situ* hybridization. Zebrafish *insl3* DIG-riboprobes were generated as described previously (Good-Ávila *et al.* 2009). We followed previously described methods (Draper 2017) with minor modifications. In brief, tissue was treated with proteinase K (20 µg/mL; Sigma-Aldrich) for 20 min at 37°C and fixed in 4% paraformaldehyde for 20 min. Hybridization with sense and antisense DIG riboprobes was performed overnight at 70°C. After hybridization, tissue was incubated in PBT containing 5% normal goat serum (Sigma-Aldrich) and 1% acetylated BSA (Sigma-Aldrich) for 1 h. Then, tissue was incubated with rabbit anti-zebrafish Wnt5a antibody (see above) and anti-DIG conjugated to alkaline phosphatase (1:2000; Roche). After PBT washes, testis was incubated in 5% normal horse serum (Sigma-Aldrich) for 1 h, followed by secondary goat anti-rabbit Alexa fluor 488 (8 µg/mL, A11034, Sigma-Aldrich) containing 5% normal horse serum (Sigma-Aldrich) overnight at 4°C. After PBT washes, *in situ* hybridization was developed using HNPP/Fast Red (Roche) according to the manufacturer's protocol. Images were taken using a confocal laser scanning microscope (LSM 700, Zeiss).

Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 5 software package. Since our tissue culture system compares experiments the two testes of a given fish incubated under control versus experimental conditions, we applied Student's *t*-test for paired observation to estimate the statistical significance of fold changes between treated and control conditions. These data are presented as fold of basal (mean ± standard error of the mean (S.E.M.)). We also compared all conditions, so that results were also processed statistically using one-way ANOVA, followed by Tukey's *post-hoc* test.

Results

Fsh-stimulated proliferation and accumulation of A_{und} is further enhanced when blocking the β-catenin-dependent pathway

We reported that Fsh stimulated both self-renewal and differentiating proliferation of type A spermatogonia in zebrafish (Nóbrega *et al.* 2015, Safian *et al.* 2016) and that Fsh stimulated the release of Igf3 that in turn activated β-catenin signaling to promote the differentiation of type A spermatogonia (Safian *et al.* 2018). To study if the Wnt signaling system is involved in Fsh-stimulated self-renewal of A_{und}, zebrafish testes were exposed to Fsh with or without XAV939, an inhibitor of the β-catenin-dependent pathway. For A_{und} spermatogonia, both the proliferation activity and the proportion of section surface area increased while these parameters decreased for A_{diff} (Fig. 1A and B and Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). In parallel experiments, we quantified the transcript levels of selected genes and found increased mRNA levels of *foxa2* and *nanos2*, markers for type A spermatogonia (Beer & Draper 2013, Bellaiche *et al.* 2014, Safian *et al.* 2017), and decreased transcript levels of *dazl,* expressed by B spermatogonia and primary spermatocytes (Chen *et al.* 2013) in response to Fsh and XAV939 (Fig. 1C). These experiments suggest that blocking β-catenin signaling reduced the Fsh-stimulated production of type A_{diff} spermatogonia, while strengthening Fsh effects on the proliferation and accumulation of A_{und} spermatogonia.

Fsh uses a β-catenin-independent Wnt ligand to promote A_{und} proliferation

Considering that the non-canonical ligand WNT5A stimulated self-renewal divisions of A_{und} spermatogonia

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in mice (Tanaka *et al.* 2016, Tokue *et al.* 2017) and the results described earlier, we examined if non-canonical Wnt ligand transcript levels responded to Fsh while blocking β-catenin signaling with XAV939. We found that *wnt5a* and *wnt11* expression was upregulated (Fig. 2A); the transcript levels of three other, non-canonical Wnt ligands expressed in the zebrafish testis (*wnt4a* and *b*, *wnt5b*) did not change significantly (data not shown). This opened the possibility that a β-catenin-independent pathway mediated Fsh effects on the proliferation activity of A_{und}. We tested this hypothesis with the following experiments. As expected, Fsh stimulated the proliferation of both A_{und} and A_{diff} spermatogonia (Fig. 2B and C). In the additional presence of XAV939, the proliferation of A_{und} was further stimulated, while the proliferation activity of A_{diff} was reduced (Fig. 2B and Supplementary Fig. 1A and compare to B). An inhibitor of Wnt ligand release (IWP-12) reduced the BrdU-labeling index of A_{und}, irrespective of the presence of XAV939 (Fig. 2B and Supplementary Fig. 1A and B compare to C and D), whereas A_{diff} proliferation did not change (Fig. 2C). These experiments suggest that Fsh can stimulate the production of certain non-canonical Wnt ligands and that the Fsh-triggered effect on the proliferation activity of A_{und} depends on the release of Wnt ligands. Neither XAV939 nor IWP-12 alone modulated the proliferation of A_{und} and A_{diff} (Fig. 2B, C and H), suggesting there was little basal Wnt release as well as little basal activity of β-catenin-dependent signaling.

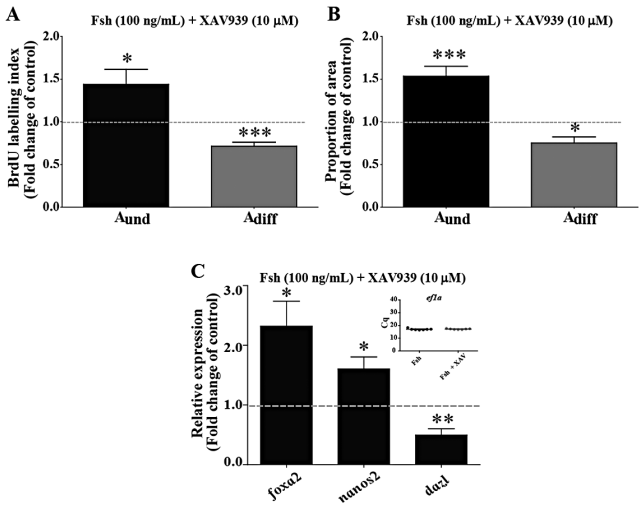

Figure 1
Inhibiting canonical β-catenin signaling increases Fsh-stimulated self-renewal of A_{und} spermatogonia but compromises the production of A_{diff} spermatogonia. (A) BrdU-labeling indices of type A_{und} and type A_{diff} spermatogonia in the presence of Fsh alone (100 ng/mL; dotted line; control condition) or in combination with 10 µM XAV393, an inhibitor of β-catenin signaling (represented by bars; $n=7$). (B) Proportion of section surface area occupied by cysts containing type A_{und} and type A_{diff} spermatogonia in the presence of Fsh alone (100 ng/mL; dotted line; control condition) or in combination with 10 µM XAV393 (represented by bars; $n=7$). (C) Gene expression analysis of germ cell markers in adult zebrafish testis after 5 days of tissue culture in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with 10 µM XAV393 (represented by bars; $n=7$). The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the insert. Results are presented as fold changes with respect to the control group (100 ng/mL Fsh). Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$; ***$P<0.001$) between groups.

Fsh but neither Lh nor androgen modulate Wnt5a production in LCs

To test if Fsh stimulates the transcript levels of non-canonical Wnt ligands also in the absence of XAV939, the mRNA levels of *wnt5a* and *wnt11* were analyzed in the absence and presence of Fsh (100 ng/mL) for 5 days. In addition, the transcripts of *wnt5a* and *wnt11* were also analyzed in testes incubated under basal conditions or in the presence of the androgen 11 KT (200 nM) or Lh (500 ng/mL) for 5 days. Only the mRNA levels of *wnt5a* but not of *wnt11* (and neither of *wnt4a*, *wnt4b* and *wnt5b*; data not shown) transcript levels increased in response to Fsh (Fig. 3A). Since transcript levels of *wnt5a* were induced by Fsh, we also analyzed if Fsh stimulates the production of Wn5a protein. Western blot and densitometric analysis revealed that 100 ng/mL Fsh increased the protein levels of Wnt5a in zebrafish testis after 5 days of incubation (Fig. 3B and C).

Cellular localization of Wnt5a production

Immunocytochemical studies localized Wnt5a protein to the interstitial compartment of the zebrafish testis (Fig. 3D, E, F, G, H and I). To further characterize the source of Wnt5a production, we performed a double fluorescent-labeling approach combining Wnt5a immunocytochemistry with *in situ* hybridization of the LC marker, *insl3* mRNA (Assis *et al.* 2016). This analysis showed that Wnt5a protein and *insl3* transcript were co-localized in LCs (Supplementary Fig. 1F, G, H and I).

Fsh-regulated Wnt5a stimulated A_{und} self-renewal

The results presented so far suggest that Fsh-stimulated Wnt5a release from LC increased the proliferation activity of type A_{und} spermatogonia. Considering that WNT5a promoted self-renewal division of mouse SSCs (Tanaka *et al.* 2016), we decided to obtain more direct evidence on

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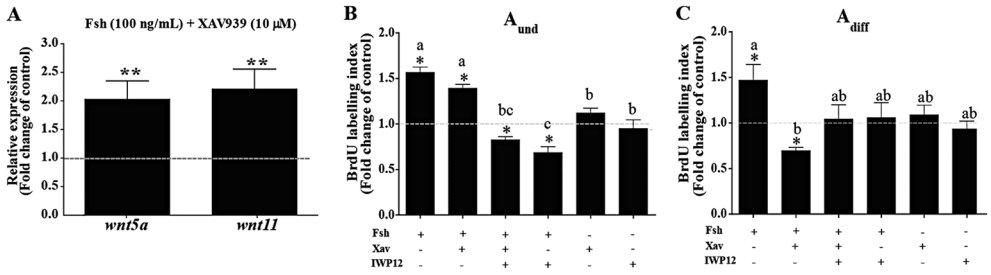

Figure 2

Fsh modulates the proliferation activity of type A spermatogonia via non-canonical Wnt ligands. (A) Gene expression analysis in adult zebrafish testis after 5 days of tissue culture in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with 10 µM XAV393 (represented by bars; $n=7$). (B) BrdU-labeling indices of type A_{und} under control (dotted line) or experimental (represented by a bars) conditions after 5 days of incubation: Fsh ($n=8$); Fsh+XAV ($n=7$); Fsh + XAV + IWP-12 ($n=8$); Fsh + IWP-12 ($n=8$); XAV ($n=5$); IWP-12 ($n=6$). (C) BrdU-labeling indices of type A_{diff} under control (dotted line) or experimental (represented by a bars) conditions after 5 days of incubation: Fsh ($n=8$); Fsh + XAV ($n=7$); Fsh + XAV + IWP-12 ($n=8$); Fsh + IWP-12 ($n=8$); XAV ($n=5$); IWP-12 ($n=6$). Results are presented as fold changes with respect to the control group (see 'Material and methods' section). Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) between groups and different letters indicate significant differences ($P<0.05$) between groups.

the biological activity of Wnt5a. We analyzed the effects of an antagonist of Wnt5a (WAIIIB5) on Fsh-stimulated spermatogenesis. The proliferation activity and proportion of section surface area occupied by A_{und} spermatogonia in the presence of Fsh (100 ng/mL) were reduced when also WAIIIB5 (100 µM) was present (Fig. 4A and B). Furthermore, the transcript levels of *piwil2* (expressed by all germ cells except A_{und} and spermatozoa) (Chen *et al.* 2013) and *dazl* increased (Fig. 4C). We also studied the effects of the Wnt5a agonist Foxy-5. The proliferation activity and proportion of area of A_{und} increased in response to Foxy-5 (100 µM) after 5 days of tissue culture while these parameters did not change for A_{diff} (Fig. 4D and E). Moreover, the transcript levels of *foxa2* increased (Fig. 4F) in the presence of Foxy-5 while those of *piwil2* and *dazl* decreased (Fig. 4F). Collectively, these results

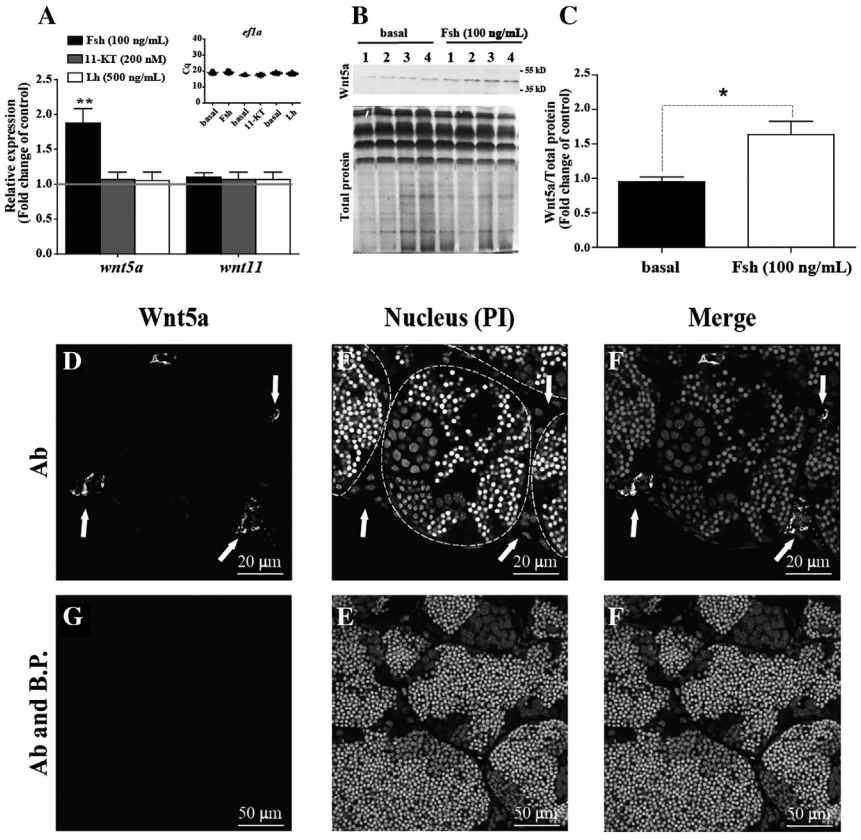

Figure 3

Endocrine regulation of Wnt5a protein production and cellular localization of Wnt5a. (A) Transcript levels of *wnt5a* and *wnt11* in basal conditions (dotted line; control condition) or in the presence of Fsh (100 ng/mL; $n=8$; represented by black bars), 11-KT (200 nM; $n=6$; represented by gray bars) or Lh (500 ng/mL; $n=9$; represented by white bars) in adult zebrafish testis. The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the insert. Results are presented as fold changes with respect to the control group (basal). (B) Western blot and (C) densitometric analysis of Wnt5a in zebrafish testis under basal conditions or in response to Fsh (100 ng/mL) after 5 days of incubation ($n=4$). Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) between basal or control and experimental group. (D, E, F, G, H and I) Immunofluorescent detection of Wnt5a in paraffin sections of zebrafish testis. White dotted lines delimitate the spermatogenic tubules compartment. White arrows indicate the interstitial compartment. (G, H and I) Wnt5a antibody (Ab) preabsorbed with blocking peptide (B.P.) in paraffin sections of zebrafish testis.

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suggest that Wnt5a signaling increased the number of A_{und} spermatogonia by stimulating their proliferation while no change was observed among A_{diff} spermatogonia.

Wnt5a antagonist and agonist modulates the transcript levels of retinoic acid-related genes

To further study the function of Wnt5a in zebrafish testis, the transcript levels of selected genes know to be important for spermatogenesis were analyzed in response to Fsh (100 ng/mL) and WAIIIB5 (100 µM) or in the presence of the Wnt5a agonist, Foxy-5 (100 µM). Wnt5a/Foxy-5 seemed to target mainly undifferentiated spermatogonia. We therefore selected a number of genes known to regulate the differentiation of type A_{und} spermatogonia. However, neither growth factor (*amh*, *insl3*, *igf3*) nor growth factor-binding protein (*igfbp1a*, *-3* and *-6a*) transcript levels changed significantly in response to Fsh (100 ng/mL) and WAIIIB5 (100 µM) or in response to the Wnt5a agonist, and neither did the transcript levels of two steroidogenesis-related genes (*star* and *cyp17a1*; data not shown). On the other hand, WAIIIB5 (100 µM) decreased the transcript levels of *cyp26a1*, an enzyme involved in retinoic acid degradation (Feng *et al.* 2015) in a Fsh-stimulated spermatogenesis (Fig. 5). In contrast, Foxy-5 (100 µM) increased *cyp26a1* mRNA levels (Fig. 5). The transcript levels of *aldh1a2,* an enzyme required for retinoic acid synthesis (Pradhan & Olsson 2015), did not responsd to either Fsh and WAIIIB5 or Foxy-5 (Fig. 5).

Effects of Fsh on Sertoli cell proliferation

SC proliferation is required for fertility in the adult fish testis (De França *et al.* 2015), either for the production of new spermatogenic cysts to accommodate A_{und} derived from self-renewal divisions or for the growth of existing cysts to accommodate the growing germ cell clone. Therefore, we have also studied the number of BrdU-positive SCs

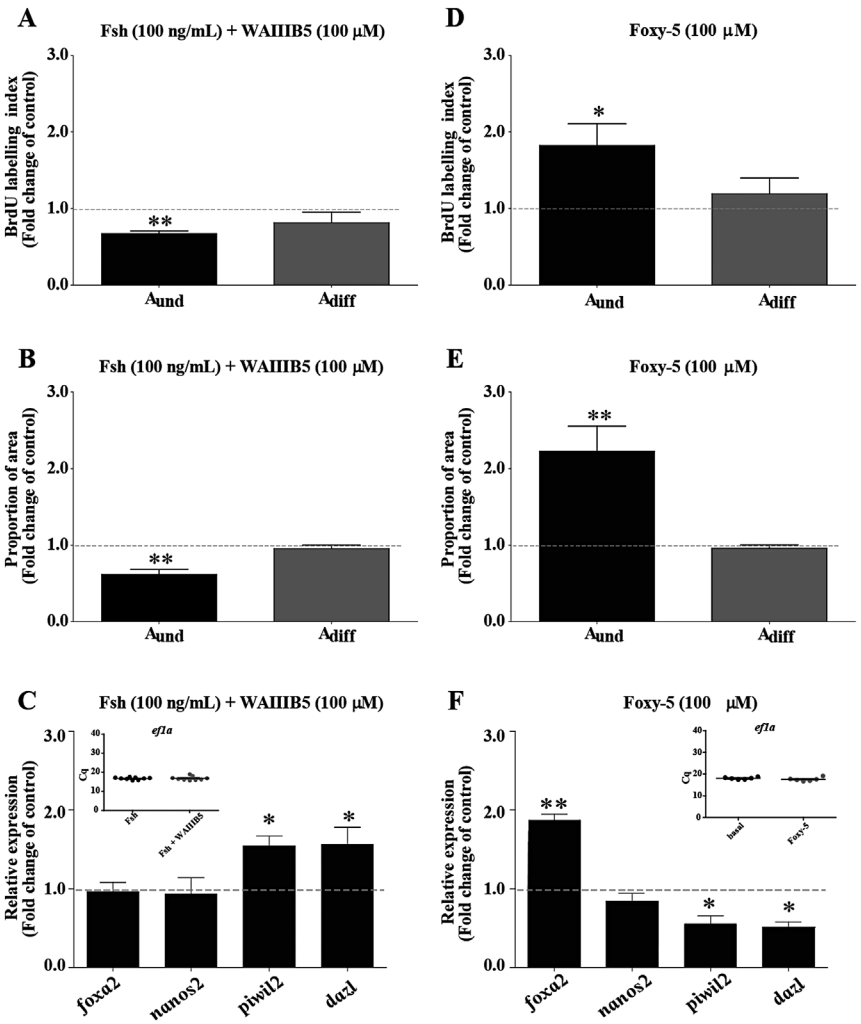

Figure 4
Effects of the Wnt5a antagonist (WAIIIB5) and the Wnt5a agonist (Foxy-5) on type A spermatogonia. (A) BrdU-labeling indices of type A_{und} and type A_{diff} spermatogonia in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with WAIIIB5 (100 µM; $n=7$; represented by bars). (B) Proportion of section surface area occupied by cysts containing type A_{und} and type A_{diff} spermatogonia in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with WAIIIB5 (100 µM; $n=7$; represented by bars). (C) Gene expression analysis of selected genes in adult zebrafish testis after 5 days of tissue culture in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with WAIIIB5 (100 µM; $n=9$; represented by bars). The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the insert. (D) BrdU-labeling indices of type A_{und} and type A_{diff} spermatogonia under basal conditions (dotted line; control condition) or in the presence of Foxy-5 (100 µM; $n=5$; represented by bars). (E) Proportion of section surface area occupied by cysts containing type A_{und} and type A_{diff} spermatogonia under basal conditions (dotted line; control condition) or in the presence of Foxy-5 (100 µM; $n=5$; represented by bars). (F) Gene expression analysis of selected genes in adult zebrafish testis after 5 days of tissue culture under basal conditions (dotted line; control condition) or in the presence of Foxy-5 (100 µM; represented by bars; $n=7$). The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the insert. Results are presented as fold changes with respect to the control group (basal). Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) between control and experimental group.

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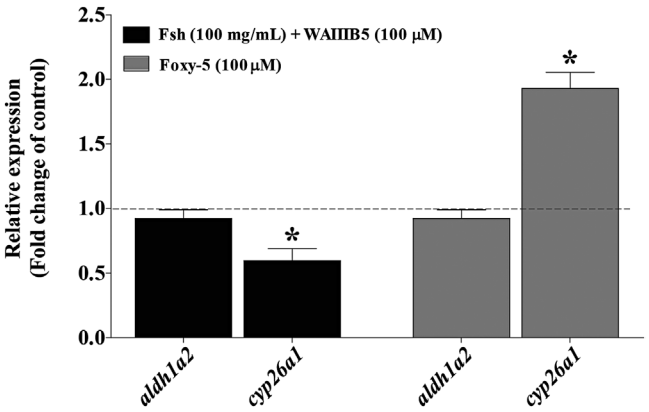

Figure 5
Transcript levels of retinoic acid-related genes in response to Fsh and the Wnt5a antagonist (WAIIIB5) or in response to the Wnt5a agonist (Foxy-5) after 5 days of incubation. Transcript levels are expressed as fold-change compared to control condition represented by a dotted line (100 ng/mL Fsh or basal conditions), as induced by Fsh (100 ng/mL) and WAIIIB5 (100 µM; $n=8$; represented by bars) or Foxy-5 (100 µM; $n=7$; represented by bars). Asterisks indicate significant differences (*$P<0.05$) between basal and experimental group.

that were free (i.e. apparently not in contact with germ cells) or that contacted A_{und} or A_{diff} spermatogonia. This was examined under basal conditions, in the presence of Fsh (100 ng/mL) alone or in the additional presence of XAV939 (10 µM), inhibiting β-catenin-signaling, the Wnt5a antagonist WAIIIB (100 µM) or of the Wnt5a agonist (Foxy-5, 100 µM). In addition, SC proliferation was also studied in the presence of Fsh (100 ng/mL) with or without either XAV939 (10 µM), inhibiting β-catenin-signaling, or the Wnt5a antagonist WAIIIB (100 µM). Fsh increased the number of proliferating SCs contacting BrdU-positive A_{und} and A_{diff} (Fig. 6A). In the additional presence of the β-catenin-pathway inhibitor, proliferation of free SCs increased, but proliferation of SCs contacting BrdU-positive A_{und} decreased (Fig. 6B). The BrdU-positive free SC and proliferating SCs contacting BrdU-negative A_{und} decreased in response to Fsh (100 ng/mL) and the Wnt5a inhibitor WAIIIB (100 µM) while the proliferation of free SC increased in the presence of Foxy-5 (Fig. 6C and D).

Discussion

Fsh promotes spermatogenesis by modulating LC (fish-specific, in context with the presence of the Fshr on LCs) and SC (in all vertebrates, in context with the presence of the Fshr on SCs) functions. A previous study showed that zebrafish Fsh activated, in an androgen-independent manner, the differentiating proliferation of type A spermatogonia, but did not change the proportion of A_{und} while increasing the one for A_{diff} spermatogonia (Safian *et al.* 2016). However, it was not known how Fsh acts to prevent depletion of type A_{und} spermatogonia. Here, we found that the effects of Fsh on A_{und} self-renewal and differentiating divisions were altered significantly when inhibiting β-catenin signaling, viz. the reduced proliferation activity and proportion of A_{diff} spermatogonia while these parameters were further increased for A_{und} spermatogonia. These morphometric results were supported by respective changes in germ cell marker gene transcript levels that suggested increases in type A spermatogonia at the expense of more differentiated germ cell types. We conclude that blocking β-catenin restricted Fsh effects from activating both self-renewal and differentiating divisions of type A spermatogonia toward preferentially facilitating the proliferation and accumulation of A_{und} spermatogonia in the zebrafish testis. Inhibition of these β-catenin-dependent pro-differentiation effects of Fsh is probably related to a downstream mediator of Fsh, Igf3, which activated β-catenin signaling independent of Wnt ligands to stimulate the differentiation of type A spermatogonia (Safian *et al.* 2018).

The above results also demonstrate that Fsh does not require β-catenin activity to stimulate the proliferation activity of A_{und} spermatogonia. This is in line with findings of Yeh *et al.* (2011) who reported that murine SSCs did not respond to canonical Wnt signaling. However, the findings that Fsh stimulated the proliferation activity and accumulation of zebrafish A_{und} (i) depended on Wnt ligand release and (ii) that Fsh triggered the production of Wnt5a by LCs jointly suggested an involvement of non-canonical Wnt signaling in regulating the activity of A_{und} spermatogonia. The relevance of this signaling was supported further by experiments with agonists and antagonists of Wnt5a. A recent study in mice also showed that WNT5A induced an accumulation of type A_{und} spermatogonia (Tanaka *et al.* 2016). Intriguingly, the regulation of Wnt5a is quite different in these two models: Tanaka and colleagues found that LH, but not Fsh, downregulated *Wnt5a* transcript levels in SCs indirectly by stimulating LC androgen production. In zebrafish, on the other hand, neither Lh nor androgen modulated testicular transcript levels of *wnt5a* while Fsh increased LC *wnt5a* transcript levels and probably also Wnt5a release. Regarding receptors potentially responding to WNT5a in testis, Yeh *et al.* (2011) found that *in vitro*, SSCs express the non-canonical signaling frizzled receptors (FZD) 3, 5 and 7 and also the alternative receptor Ror2. RNA sequencing data from previous experiments showed

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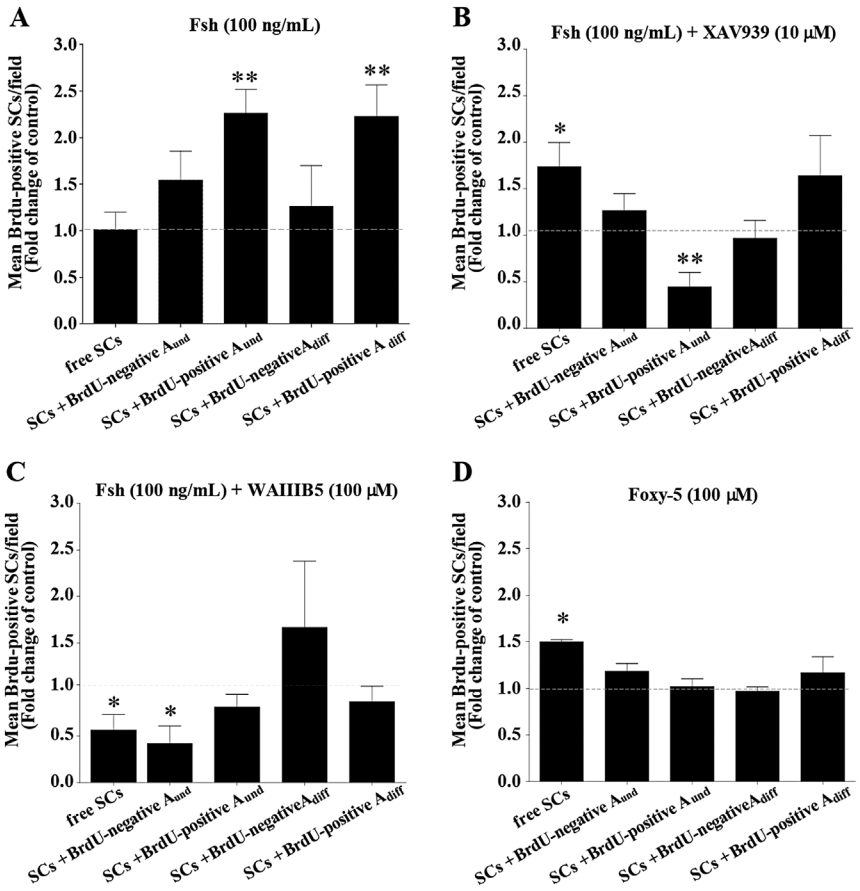

Figure 6
Effect of Fsh in basal conditions or in the presence of inhibitors or Foxy-5 on Sertoli cell proliferation contacting type A spermatogonia. Number of BrdU-positive 'free' Sertoli cells, Sertoli cells contacting BrdU-negative and BrdU-positive A_{und}, and Sertoli cells contacting BrdU-negative and BrdU-positive A_{diff} in the presence of: (A) 25 µg/mL trilostane (dotted line; control condition) or in the presence of Fsh (100 ng/mL; $n=8$; represented by bars), (B) in the presence of Fsh (100 ng/mL; dotted line; control condition) or in combination with XAV393 (10 µM; $n=7$; represented by bars), (C) in the presence of Fsh (100 ng/mL, dotted line; control condition) or in combination with WAIIIB5 (100 µM; $n=7$; represented by bars) and (D) in basal conditions or in the presence of Foxy-5 (100 µM; $n=5$). Results are presented as fold changes with respect to the control group. Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) between control and experimental group.

that zebrafish testis tissue also expresses several non-canonical Wnt receptors (Crespo *et al.* 2016, Morais *et al.* 2017). However, no information is available on the question which receptor specifically responds to WNT5a in mice or zebrafish testis tissue. Collectively, the data from the experiments shown in Figs 3 and 4 indicate that the role of Wnt5a in promoting self-renewal divisions of undifferentiated spermatogonia is conserved between zebrafish and mice, while the endocrine regulation and cellular site of expression of this factor differs between the two species.

While this Wnt5a function may be conserved in vertebrates, no information is available so far on signaling mechanisms potentially targeted by Wnt5a. Our initial studies do not provide evidence for an involvement of Amh, Insl3, Igf3 or androgens. However, retinoic acid signaling may be involved. Studies in mice have shown that a diet deficient in the retinoic acid precursor vitamin A resulted in the accumulation of undifferentiated spermatogonia (Van Pelt & de Rooij 1990, Li *et al.* 2011). Interestingly, it was found recently that somatic cells protect SSC from exogenous retinoic acid while allowing further differentiated cells to respond to retinoic acid in mice (Lord *et al.* 2018). In zebrafish, exposure to a diet low in vitamin D and also to an inhibitor of Aldh1a2 resulted in lower sperm count and fecundity after 3 weeks (Pradhan & Olsson 2015). In addition, mutation of *aldh1a2* delayed the entry into meiosis while disrupting the *cyp26a1* gene (encoding the enzyme degrading retinoic acid), accelerated the entry into meiosis in Nile tilapia (Feng *et al.* 2015). As *cyp26a1* expression has been localized in LCs in the adult zebrafish testis (Rodríguez-Marí *et al.* 2013), it will be interesting to investigate which germ cell types respond to RA signaling in zebrafish. It therefore seems possible that one of the consequences of Wnt5a action in the zebrafish testis is to reduce retinoic acid signaling, thereby favoring the accumulation of A_{und} spermatogonia.

In addition to the mechanism described in the present study (Fsh-stimulated Wnt5a of LC origin promotes the production of undifferentiated spermatogonia), other mechanisms have alike effects in zebrafish: Amh inhibits the differentiation of A_{und}, promoting their accumulation, (Skaar *et al.* 2011) and T_3 stimulated the generation of new cysts by increasing the proliferation of A_{und} spermatogonia and SCs in adult zebrafish testis (Morais *et al.* 2013).

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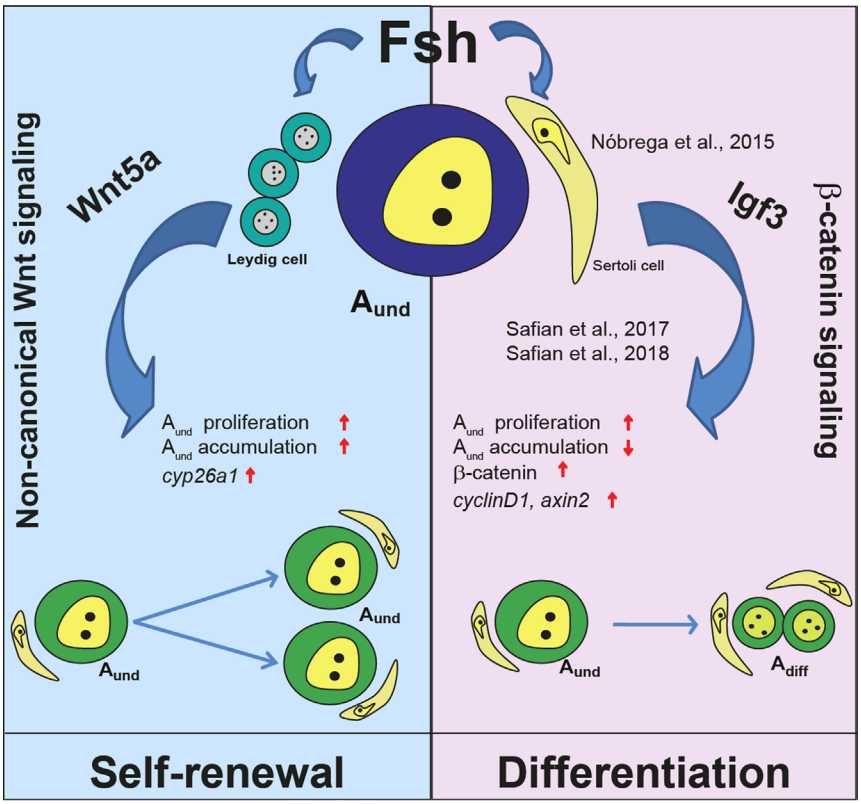

Figure 7
Schematic representation of the effects of Fsh on spermatogenesis by modulating canonical and β-catenin signaling. Fsh stimulates the production of the β-catenin-independent ligand Wnt5a in LCs, which modulates the transcription of genes important for spermatogenesis, to stimulate the accumulation of type A_{und} spermatogonia and the production of new cysts (left). Fsh also stimulates differentiating divisions of spermatogonia by increasing Igf3 release from Sertoli cells (Nóbrega *et al.* 2015), which activates β-catenin-dependent signaling (Safian *et al.* 2018). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0447.

It appears that different, independent mechanisms, making use of distinct hormones and growth factors, operate to ensure that a sufficient number of spermatogenic cysts with a single A_{und} enveloped by SCs are available to form the basis of the spermatogenic process.

As mentioned earlier, the generation of new spermatogenic cysts and the growth and differentiation of existing cysts requires SC proliferation in the postpubertal fish testis (Schulz *et al.* 2005, De França *et al.* 2015). Therefore, we have also studied SC proliferation. In line with previous results (Safian *et al.* 2018), Fsh increased the proliferation activity of SCs contacting BrdU-positive A_{und} and A_{diff} in the presence of β-catenin signaling inhibitor, indicating a change in the Fsh effects from supporting the further development of existing cysts toward facilitating the formation of new spermatogenic cyst. Inhibiting and stimulating Wnt5a signaling by using a Wnt5a antagonist and agonist, respectively, revealed that Fsh-stimulated Wnt5a supports the formation of new cyst by increasing the proliferation of free SCs, and by supporting the proliferation of SCs associated with non-proliferating A_{und}. Taken together, these observations suggest that part of the Fsh effect that promotes the expansion of the A_{und} spermatogonial population includes the production of additional SCs that can then support newly formed A_{und}, thereby forming new spermatogenic cysts.

In summary, the pituitary hormone Fsh promoted the formation of A_{und} spermatogonia and SCs by stimulating LC production of the non-canonical Wnt ligand Wnt5a. This Fsh effect takes place in parallel to Fsh-triggered Igf3/β-catenin (Safian *et al.* 2018), Insl3 (Assis *et al.* 2016) and androgen signaling (García-López *et al.* 2010, Crowder *et al.* 2018) that all stimulate germ cell differentiation. We propose that the capacity of Fsh to induce a balanced stimulation of the spermatogenic process involves both the production of new spermatogenic cysts containing A_{und} via the non-canonical Wnt pathway and LC-derived Wnt5a, while spermatogonial differentiation is triggered via Igf3/β-catenin signaling (Safian *et al.* 2018) (Fig. 7). Additional pro-differentiation routes have been identified already as mentioned earlier, and other signaling pathways may be involved in promoting the production of A_{und}.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0447.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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