



## Antagonistic regulation of spermatogonial differentiation in zebrafish (*Danio rerio*) by Igf3 and Amh



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### ABSTRACT

Fsh-mediated regulation of zebrafish spermatogenesis includes modulating the expression of testicular growth factors. Here, we study if and how two Sertoli cell-derived Fsh-responsive growth factors, anti-Müllerian hormone (Amh; inhibiting steroidogenesis and germ cell differentiation) and insulin-like growth factor 3 (Igf3; stimulating germ cell differentiation), cooperate in regulating spermatogonial development. In dose response and time course experiments with primary testis tissue cultures, Fsh up-regulated *igf3* transcript levels and down-regulated *amh* transcript levels; *igf3* transcript levels were more rapidly up-regulated and responded to lower Fsh concentrations than were required to decrease *amh* mRNA levels. Quantification of immunoreactive Amh and Igf3 on testis sections showed that Fsh increased slightly Igf3 staining but decreased clearly Amh staining. Studying the direct interaction of the two growth factors showed that Amh compromised Igf3-stimulated proliferation of type A (both undifferentiated [ $A_{und}$ ] and differentiating [ $A_{diff}$ ]) spermatogonia. Also the proliferation of those Sertoli cells associated with  $A_{und}$  spermatogonia was reduced by Amh. To gain more insight into how Amh inhibits germ cell development, we examined Amh-induced changes in testicular gene expression by RNA sequencing. The majority (69%) of the differentially expressed genes was down-regulated by Amh, including several stimulators of spermatogenesis, such as *igf3* and steroidogenesis-related genes. At the same time, Amh increased the expression of inhibitory signals, such as *inha* and *id3*, or facilitated prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) signaling. Evaluating one of the potentially inhibitory signals, we indeed found in tissue culture experiments that PGE<sub>2</sub> promoted the accumulation of  $A_{und}$  at the expense of  $A_{diff}$  and B spermatogonia. Our data suggest that an important aspect of Fsh bioactivity in stimulating spermatogenesis is implemented by restricting the different inhibitory effects of Amh and by counterbalancing them with stimulatory signals, such as Igf3.

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### 1. Introduction

Gonadotropins and sex steroids are reproductive hormones of

major relevance for the regulation of spermatogenesis in vertebrates. Among the gonadotropins, follicle-stimulating hormone (Fsh) is a more critical hormone than luteinizing hormone (Lh) in teleost fish, since both Leydig and Sertoli cells express the Fsh receptor (*fshr*) gene, rendering Fsh also a potent steroidogenic hormone (Chauvigne et al., 2012; García-López et al., 2010; Ohta et al., 2007). Moreover, loss-of-function studies showed that the luteinizing hormone/choriogonadotropin receptor (*lhcg*) gene is dispensable for male fertility; loss of the *fshr* gene delays the start of

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spermatogenesis in zebrafish (*Danio rerio*), while loss of both receptor genes resulted in male infertility (Chu et al., 2015; Zhang et al., 2015). Since androgens, in fish in particular 11-ketotestosterone (11-KT), stimulated spermatogenesis in Japanese eel (*Anguilla japonica*) and zebrafish testis tissue explants (Leal et al., 2009b; Miura et al., 1991), one mechanism to promote spermatogenesis is by stimulating androgen production by Fsh (Ohta et al., 2007). Also *in vivo*, recombinant Fsh, but not recombinant Lh, stimulated the onset of sea bass spermatogenesis and elevated circulating androgen levels (Mazón et al., 2011, 2014). Interestingly, Fsh also modulated testicular gene expression in rainbow trout (*Oncorhynchus mykiss*) at the beginning of pubertal development while inhibiting the production of biologically active steroids (Sambroni et al., 2013a), suggesting that non-steroidal signaling mediates part of the biological activity of Fsh. Also in adult zebrafish testis, Fsh modulates the expression of ~200 genes in a steroid-independent manner (Crespo et al., 2016).

More than 80% of the Fsh-modulated genes identified in the latter study were expressed by Sertoli cells or other somatic cells in the testis, including the secreted signaling factors anti-Müllerian hormone (*amh*) and insulin-like growth factor 3 (*igf3*). Testicular *amh* transcript levels decreased in response to Fsh, recombinant zebrafish Amh inhibited steroidogenesis and, independent of steroids, inhibited also the differentiation of type A spermatogonia (Skaar et al., 2011). Similar observations have been made before in Japanese eel (Miura et al., 2002), and a decrease of testicular *amh* expression was associated with the onset of pubertal spermatogenesis in various vertebrates, including several fish species (Johnsen et al., 2013; Maugars and Schmitz, 2008; Morinaga et al., 2007; Rey et al., 2009). Hence, by restricting *amh* transcript levels, Fsh controls Amh-mediated inhibition of androgen production and spermatogonial differentiation. On the other hand, Fsh increased *igf3* transcript levels in zebrafish and trout testes (Baudiffier et al., 2012; de Waal, 2009; Sambroni et al., 2013a); Igf3 is a fish-specific member of the Igf family that is predominately found in gonadal tissue (Wang et al., 2008). Further studies using recombinant zebrafish Igf3 showed that it mediated stimulatory effects of Fsh on the proliferation and differentiation of type A spermatogonia, without influencing sex steroid production (Nóbrega et al., 2015). This led us to develop a model, in which Fsh stimulates stimulators, and inhibits inhibitors, of spermatogenesis in zebrafish: the Fsh-stimulated androgen and Igf3 production would promote spermatogenesis, facilitated by the Fsh-mediated decrease of Amh, thereby restricting inhibitory effects on steroidogenesis and germ cell differentiation.

However, this model does not take into account the possibility that Amh and Igf3 directly influence each other's biological activity. Moreover, the timing and/or the dose-response effects of Fsh on the expression of the two growth factors genes may differ. Theoretically, this consideration could include androgens that might have effects on Amh and/or Igf3 actions, and conversely growth factor effects on androgen production. However, previous work in adult zebrafish has shown already that – different from juvenile Japanese eel (Miura et al., 2002) – 11-KT did not modulate testicular *amh* transcript levels, while Amh protein did inhibit gonadotropin-stimulated androgen production (Skaar et al., 2011). Igf3, on the other hand, did not modulate basal or gonadotropin-stimulated androgen production, while *igf3* transcript levels were up-regulated somewhat by androgens in adult zebrafish testis (Nóbrega et al., 2015). In the light of these data, we have designed the present experiments to investigate the Fsh-induced responses of *amh*/Amh and *igf3*/Igf3 transcript and protein levels in more detail (Fsh dose and time course experiments), and to examine if Amh influenced Igf3 effects or *vice versa*, while preventing the production of biologically active steroids. Moreover, considering

the broad inhibitory effects of Amh on zebrafish testis functions, we hypothesized that transcriptomic analysis of zebrafish tissue exposed to Amh may provide new leads for understanding how Amh exerts its inhibitory effects. Finally, results of the transcriptomic analyses triggered experiments to start evaluating the potential role(s) of prostaglandins (PGs) in zebrafish spermatogenesis.

## 2. Material and methods

### 2.1. Zebrafish stocks

Wild-type adult male zebrafish (*Danio rerio*, AB line) between 4 and 12 months of age were used in the present study. Animal housing and experimentation were consistent with the Dutch national regulations and the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands) has approved the protocols.

### 2.2. Testis tissue cultures

Using a previously established tissue culture system (Leal et al., 2009b), adult zebrafish testis tissue was incubated with recombinant zebrafish Fsh (García-López et al., 2010), at different doses (25, 50, 75, 100 and 1000 ng/mL), or with 75 ng/mL Fsh for different times (1, 3, 5 and 7 days) to quantify *igf3* and *amh* mRNA levels. To exclude effects on *igf3* transcript levels mediated by Fsh-stimulated androgen production (García-López et al., 2010; Nóbrega et al., 2015), we added trilostane (25 µg/mL; Chemos), an inhibitor of hydroxy-delta-5-steroid dehydrogenase, 3 beta, which blocks the production of biologically active steroids. Additional cultures for 7 days served to study if the proliferation activity of type A undifferentiated ( $A_{und}$ ), type A differentiating ( $A_{diff}$ ) and type B spermatogonia, stimulated by 100 ng/mL of recombinant zebrafish Igf3 (Nóbrega et al., 2015), was modulated by Amh (10 µg/mL; Skaar et al., 2011), using the proliferation marker BrdU (50 µg/mL) that was added to the culture medium for the last 6 h of incubation. Testis tissue was processed for BrdU immunodetection to determine the BrdU-labeling index of type  $A_{und}$ ,  $A_{diff}$  and B spermatogonia. To investigate if Amh modulates Igf3 effects on gene expression, zebrafish testes were incubated in the presence or absence of Amh (10 µg/mL) and Igf3 (100 ng/mL) for 5 days.

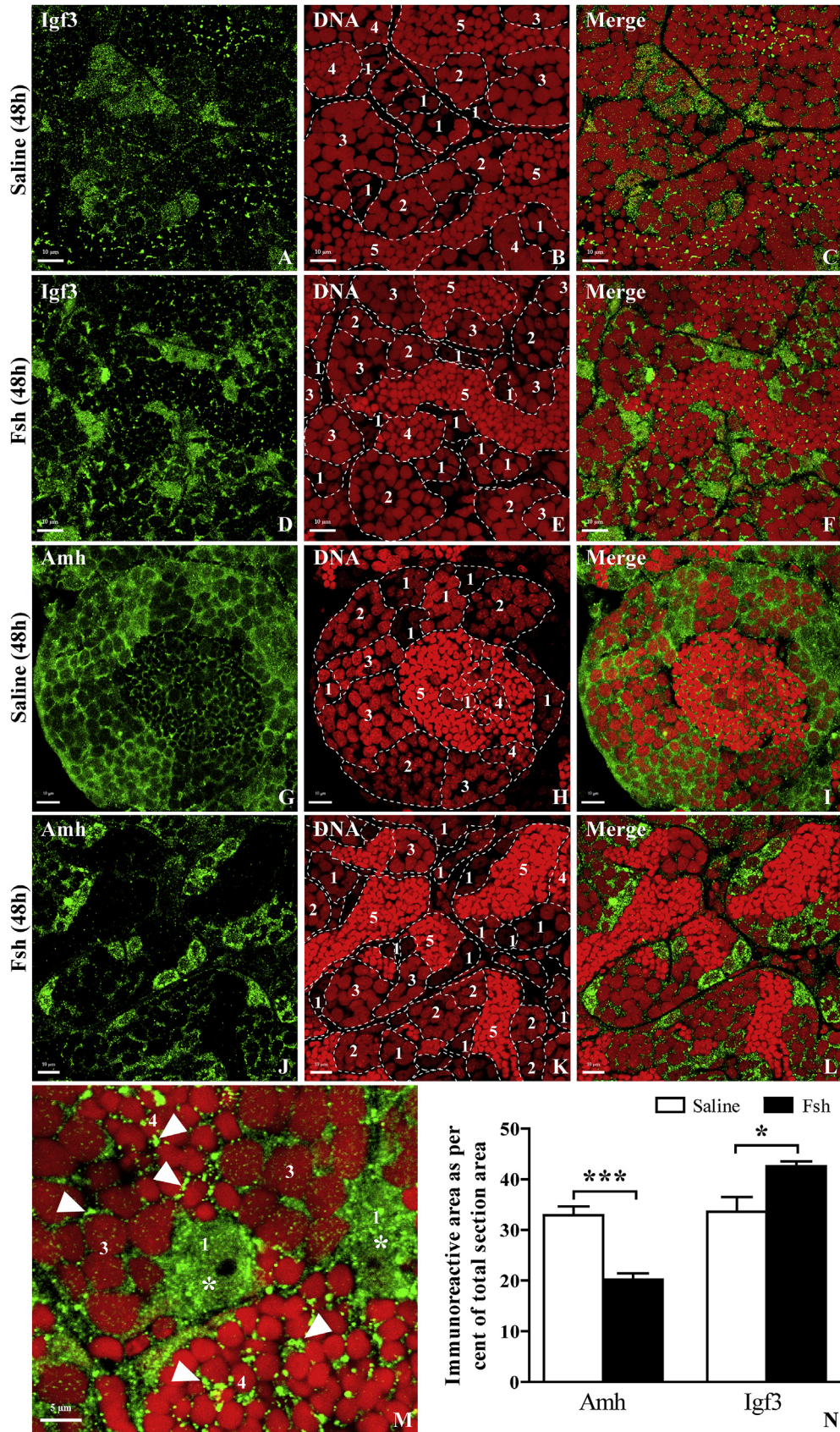
The inhibitory effect of Amh on zebrafish spermatogenesis becomes evident more readily when spermatogenic activity is stimulated above basal level, for example by androgens (Skaar et al., 2011). Therefore, testis tissue used for transcriptome analysis by RNAseq (see below) was incubated with medium containing 200 nM 11-KT for 3 days, in the absence or presence of Amh (10 µg/mL), as described previously (Skaar et al., 2011).

Components of the prostaglandin (PG) signaling system were retrieved in the RNAseq study, suggesting that PGE<sub>2</sub> may inhibit spermatogenesis. Therefore, we exposed zebrafish testis to 100 ng/mL Fsh for 4 days to stimulate spermatogenesis, and compared the expression of selected genes to testis tissue incubated with medium that contained, in addition to Fsh, 5 µM PGE<sub>2</sub> (Sigma-Aldrich; Feng et al., 2012; North et al., 2010). Finally, we incubated testis tissue for 5 days with medium containing Amh (10 µg/mL) or Igf3 (100 ng/mL), followed by examining testicular transcript levels of selected PG-related genes.

### 2.3. Testicular gene expression

After tissue culture, testis tissue was snap frozen in liquid nitrogen and stored at –80 °C until RNA isolation. Total RNA extraction, cDNA synthesis and real-time quantitative PCR (qPCR) were





**Fig. 1.** Localization and quantification of Igf3 and Amh protein in zebrafish testis following Fsh or saline injections. Sertoli cells and type A spermatogonia are Igf3-positive (green) in zebrafish testis 24 h after the second injection, i.e. 48 h after the start of treatment with saline (A-C) or Fsh (D-F). The Igf3 staining shows as a finely dispersed labeling covering larger areas in Sertoli cells contacting type A spermatogonia (as well as in the type A spermatogonia themselves) while a lower number of intensely green, somewhat larger structures were present in Sertoli cells contacting later stages of germ cell development (see higher magnification in M). We observed no major change in the Igf3 pattern in



carried out as described previously (Nóbrega et al., 2015). Data were normalized to *ef1a* (elongation factor 1a), a housekeeping control gene, which showed a constant expression pattern under the conditions analyzed without any statistically significant differences between control and treated groups (Supplemental Fig. 1). All qPCRs were performed in 20  $\mu$ L reactions and quantification cycle (Cq) values were obtained in a Step One Plus Real-Time PCR system (Applied Biosystems) using default settings. Relative mRNA levels were calculated as reported by Bogerd et al. (2001). The primers used in the qPCR analyses are listed in Supplemental Table 1.

#### 2.4. Morphometric and immunohistochemical (IHC) analyses

For BrdU immunodetection, zebrafish testes were fixed at 4 °C overnight in freshly prepared methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% acetic acid). The tissue was then dehydrated, embedded in Technovit 7100 (Kulzer), sectioned at 4  $\mu$ m thickness, and submitted to immunohistochemistry, as described previously by Leal et al. (2009b). Additional sections from the same experiments (i.e. Igf3 versus Amh + Igf3, and Fsh versus Fsh + PGE<sub>2</sub>) were counterstained with toluidine blue in order to quantify the proportions of section surface area occupied by type A<sub>und</sub>, type A<sub>diff</sub> or type B spermatogonia, as described previously (Assis et al., 2016).

For immunofluorescence analyses, adult zebrafish received two intracoelomic injections of Fsh (100 ng/g of body weight, n = 8) 24 h apart from each other. Testis tissue was collected 24 h after the second injection. The control group (n = 8) received two injections with saline. After fixation in 4% paraformaldehyde and embedding in paraplast, 4  $\mu$ m thick sections were prepared for fluorescent IHC according to Nóbrega et al. (2015), and antibodies raised against zebrafish Igf3 (2  $\mu$ g/mL; Nóbrega et al., 2015) or zebrafish Amh (100  $\mu$ g/mL; Skaar et al., 2011) were applied as described previously. Alexa Fluor 488 labeled anti-rabbit (1  $\mu$ g/mL; Life Technologies) was used as the secondary antibody. Propidium iodide (1  $\mu$ g/mL; Sigma-Aldrich) was used as nuclear counterstain. To quantify the Amh and Igf3 immunoreactive areas per testicular surface area, two images (at 200 $\times$  magnification) were taken at random from each of the 8 testes, such that 16 images were analyzed per condition (Fsh- and saline-injected fish) using a confocal laser scanning microscope (Zeiss LSM 700). The average of the two images per testis was used to perform statistical analysis (n = 8). Image J software (<http://imagej.nih.gov/ij/index.html>) served to quantify the immunoreactive area per section surface area, as exemplified in the Supplemental video.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2017.06.017>.

#### 2.5. Transcriptome analysis by RNA sequencing (RNAseq)

From testis tissue incubated with 11-KT in the presence or absence of Amh for 3 days (see point 2.2), RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA integrity was verified on an Agilent Bio-analyzer 2100 total RNA Nano series II chip (Agilent). Only samples with a

RNA integrity number >8 were used for library preparation. Illumina RNAseq libraries were prepared from 2  $\mu$ g total RNA using the Illumina TruSeq RNA Sample Prep Kit v2 (Illumina, Inc.) according to the manufacturer's instructions. The resulting RNAseq libraries were sequenced on an Illumina HiSeq2500 sequencer (Illumina, Inc.) as 1  $\times$  50 nucleotide reads. Image analysis and base calling were done by the Illumina pipeline. Quality control of the obtained reads was performed using FastQC suite (v0.10.1; default parameters). The sequencing yield ranged between ~14 and ~23 million reads per sample and mapping efficiency for uniquely mapped reads was between 62.2 and 68.8% (see Supplemental Table 2). Reads were aligned to the zebrafish genome (Zv9) using TopHat (v2.0.5; Trapnell et al., 2009). The resulting files were filtered using SAMtools (v0.1.18; Li et al., 2009), and the read counts extracted using the Python package HTSeq (<http://www-huber.embl.de/HTSeq/doc/overview.html/>; Anders et al., 2014). Data analysis was performed using the R/Bioconductor package DESeq (p < 0.05; Anders and Huber, 2010). The raw RNAseq data of the 10 samples sequenced (5 biological replicates per condition) have been deposited in the NCBI GEO database with accession number GSE86944. Regulated KEGG pathways in Amh-treated testis tissue were determined using the KEGG Mapper tool (Wang et al., 2014). KEGG pathways represented by at least 3 differentially expressed genes (DEGs) and by the ratios of regulated genes (up-/down-, and vice versa) higher than 2 were considered for the analysis.

For functional enrichment analysis, we used a freely available plugin (<http://www.baderlab.org/Software/EnrichmentMap/>; Isserlin et al., 2014; Merico et al., 2010) for the Cytoscape network environment (Shannon et al., 2013). The Enrichment Map plugin calculates over-representation of genes involved in closely related Gene Ontology (GO) categories (Ashburner et al., 2000), resulting in a network composed of gene sets related and grouped according to their function. DAVID Bioinformatics Resources 6.7 (Huang et al., 2008) was used to retrieve GO terms from the list of differentially expressed genes (p < 0.05) and exported as input for the functional enrichment analysis. To determine biological enrichment, a false discovery rate (FDR) q-value of 15% was applied. In addition, the overlap coefficient used to connect related GO terms was set at 0.7.

#### 2.6. Cellular localization of inhibin a (*inha*) mRNA in zebrafish testis

One of the few transcripts up-regulated by Amh was *inha*, which was localized in zebrafish testis by *in situ* hybridization (ISH), as described previously (Assis et al., 2016). We used 10  $\mu$ m thick cryosections prepared from 4% paraformaldehyde-fixed testis tissue from adult zebrafish. DIG-labeled sense and antisense cRNAs were synthesized from an *inha*-specific PCR product generated with primers 5146–5147 (Supplemental Table 1).

#### 2.7. Statistical analyses

Values are presented as mean  $\pm$  SEM, and data were analyzed using a paired *t*-test when comparing the two testes of males, or one-way ANOVA followed by the Student–Newman–Keuls test for three or more groups using GraphPad Prism (v4; GraphPad

response to Fsh. Sertoli cells are Amh-positive (green) in zebrafish testis 24 h after the second injection with saline (G-I) or Fsh (J-L). Fsh treatment resulted in a more restricted staining pattern: while Sertoli cells contacting type A spermatogonia kept an intense label, the signal became weaker and covered less area in Sertoli cells contacting all later germ cell stages. Fsh treatment also reduced the staining among the spermatozoa in the tubular lumen. M) Higher magnification of testis from Fsh-treated male, showing Sertoli cells contacting type A spermatogonia (asterisks) with a finely dispersed staining, while larger spots (arrowheads) are more typical of Sertoli cells contacting germ cells in more advanced stages of differentiation. N) Quantification of Amh and Igf3 immunoreactive material per surface area of zebrafish testis sections in response to two Fsh (2  $\times$  100 ng/g bw) or saline injections. Testis tissue was fixed 24 h after the second Fsh or saline injection. Data are expressed as mean  $\pm$  SEM (n = 8) percentage immunoreactive material, determined as indicated in the Supplemental video. Asterisks indicate significant differences between treatments (unpaired *t*-test, \*\*\*p < 0.001; \*p < 0.05). 1, type A spermatogonia (including type A<sub>und</sub> and A<sub>diff</sub> spermatogonia); 2, type B spermatogonia; 3, spermatocytes; 4, spermatids; and 5, spermatozoa. Propidium iodide (red) was used as DNA counterstaining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Software). The significance level ( $p$ ) was fixed at 0.05 in both cases.

### 3. Results and discussion

#### 3.1. Fsh led to changes in both *Igf3* and *Amh* protein and transcript levels

To study the effects of Fsh on *Amh* and *Igf3* protein localization patterns in adult zebrafish testis, males received Fsh or saline injections. In the control group, *Igf3* was found in Sertoli cells contacting germ cells in all stages of development as well as in type A spermatogonia (Fig. 1A–C). The staining pattern in Sertoli cells varied with the developmental stage of the germ cells. The *Igf3* signal was finely dispersed initially in Sertoli cells contacting type A spermatogonia but became more aggregated, forming intensely stained spots, in Sertoli cells contacting type B spermatogonia, spermatocytes and spermatids (Fig. 1A–C; M). The detection of *Igf3* being restricted to the intratubular compartment in the zebrafish testis differs from the situation in tilapia (*Oreochromis niloticus*), where *Igf3* protein was also localized to interstitial cells and modulated the expression of steroidogenesis-related genes (Li et al., 2012). In zebrafish, *Igf3* neither changed basal nor gonadotropin-stimulated testicular androgen release (Nóbrega et al., 2015) so that the profile of biological activities of *Igf3* may show species-specific differences.

Following Fsh treatment, the *Igf3* protein expression pattern did not differ clearly from the control situation except for an increased intensity (Fig. 1D–F). Quantifying the surface area of *Igf3* immunoreactive material revealed an increase of about 25% in response to Fsh (Fig. 1N). Previously, we detected *igf3* mRNA by ISH in Sertoli cells contacting germ cells from type A spermatogonia to spermatocytes, and the biological activity of *Igf3* included stimulating the proliferation of spermatogonia and their entry into meiosis (Nóbrega et al., 2015). Low *igf3* mRNA level in Sertoli cells associated with spermatids (Nóbrega et al., 2015) suggests that *igf3* gene expression fades out when spermiogenesis starts while *Igf3* protein remains present. While detection of *Igf3* protein in Sertoli cells associated with spermatids might indicate a yet unidentified function of *Igf3* during spermiogenesis, it may also reflect the presence of residual *Igf3* and future work will show if the appearance of intensely stained *Igf3* aggregates is a step towards intracellular breakdown. The presence of *Igf3* in type A spermatogonia is intriguing and may be part of an autocrine loop to support spermatogonial development. However, it could also be involved in germ to Sertoli cell signaling, such as in the *Igf3*-stimulated Sertoli proliferation that we have reported previously in zebrafish (Morais et al., 2013).

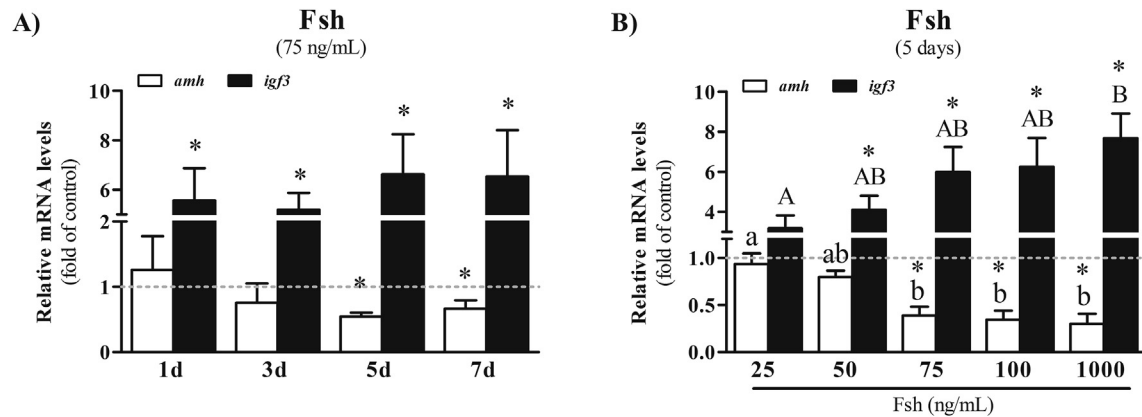
As pointed out earlier (de Waal, 2009; Nóbrega et al., 2015; Sambroni et al., 2013b), testicular *igf3* transcript levels increased in response to Fsh in zebrafish and trout. However, Fsh dose- and time-response characteristics had not been studied yet. Fsh increased *igf3* mRNA levels at all times analyzed and at most doses used, except for the lowest one (25 ng/mL; Fig. 2A–B). The relatively small (1.3-fold; Fig. 1N) increase in *Igf3*-positive section surface area as observed by IHC does not seem to fit to 6- to 8-fold increases in *igf3* transcript levels. In this regard, it is important to recall that there is very little constitutive or basal release of *Igf3* (Safian et al., 2016) and that Fsh-stimulated spermatogonial proliferation and differentiation depended on functional *Igf* receptors (Nóbrega et al., 2015), i.e. on acutely stimulated *Igf3* release. Since samples for immunocytochemistry were collected 48 h after the start of Fsh injection, a treatment triggering *Igf3* release, we can assume a decrease of the cellular content of *Igf3*. At the same time, however, the Fsh-induced increase in *igf3* transcript levels probably resulted in *Igf3* *de novo* synthesis. It therefore seems possible that

Fsh triggered two processes changing cellular *Igf3* levels into opposite directions, such that a comparatively small net change in the cellular *Igf3* protein content took place. In addition, it cannot be excluded that mechanisms regulating the use of *igf3* mRNA, potentially involving micro RNAs (Hale et al., 2014) or long, non-coding RNAs (Chen and Zhang, 2016), contribute to the smaller protein than mRNA amplitude.

Previous work localized *amh* transcripts to Sertoli cells in zebrafish, in particular to those associated with type A spermatogonia (Rodríguez-Mari et al., 2005). This seems in line with the observation that one effect of *Amh* was to inhibit the differentiation of type A spermatogonia (Skaar et al., 2011). Still, *Amh* protein was also found in Sertoli cells associated with germ cells in later stages of differentiation, although the staining appeared less intense than in Sertoli cells associated with type A spermatogonia (Skaar et al., 2011). However, the previously applied combination of fixation, counterstaining and IHC technique made identification of germ cell types difficult and was suboptimal as regards the sensitivity of *Amh* detection. Therefore, we switched to fluorescent IHC in combination with confocal laser scanning microscopy to increase the sensitivity of *Amh* detection and to improve cell type identification by staining the nuclei with propidium iodide, which allowed measuring the nuclear diameter of the germ cell types, in turn facilitating germ cell identification based on previous nuclear diameter measurements (Leal et al., 2009a). Moreover, no information was available on the response of the *Amh* protein expression pattern to a challenge with Fsh and if the response to Fsh would depend on the germ cell type the Sertoli cell was associated with. Analyzing *Amh* expression in the control group, we found *Amh* protein in Sertoli cells contacting germ cells in all stages of development; signal was also found among spermatozoa in the lumen (Fig. 1G–I). In testes of Fsh-treated fish, the *Amh* staining was lost from Sertoli cells contacting spermatids and from the lumen among spermatozoa (Fig. 1J–L), while the signal remained strong in Sertoli cells contacting type A spermatogonia. Also Sertoli cells associated with type B spermatogonia and spermatocytes remained *Amh*-positive, although the signal appeared weaker following Fsh treatment (Fig. 1J–L). Quantification of the *Amh*-immunoreactive area on testis sections revealed a reduction by approximately 40% in response to Fsh (Fig. 1N).

We confirmed previous findings that *amh* mRNA levels decreased in response to Fsh (García-López et al., 2010; Skaar et al., 2011). Moreover, we show that exposure to Fsh (75 ng/mL) for more than 3 days (Fig. 2A), and to doses higher than 50 ng/mL (Fig. 2B) were required to down-regulate *amh* transcript levels. The IHC data, on the other hand, showed that Fsh reduced the *Amh* protein staining and distribution in the zebrafish testis already after 2 days (Fig. 1). One possibility to explain this discrepancy is an Fsh-induced *Amh* degradation. Moreover, one of the Fsh functions in the pubertal mammalian testis is to stimulate Sertoli cell fluid production (Grover et al., 2004) that later on is mainly regulated by androgens (Sharpe, 1994; de Gendt et al., 2004). Assuming a similar function for Fsh and/or Fsh-triggered androgen production in the zebrafish testis, it seems possible that reduced staining for *Amh* in the tubular lumen is a consequence of increased tubular fluid flow.

In addition to the antagonism between *Igf3* that stimulated (Nóbrega et al., 2015) and *Amh* that inhibited (Skaar et al., 2011) proliferation and differentiation of type A spermatogonia, the present results suggest that the different response patterns of the two growth factors' transcripts to increasing levels of Fsh, provides an option for a graded control of the stimulation of spermatogenesis: a weak Fsh stimulation would only elevate *igf3* levels while a strong Fsh stimulation would further elevate *igf3* levels but also reduce *amh* levels. Moreover, Fsh stimulation would increase Leydig cell production of androgens (García-López et al., 2010) and



**Fig. 2.** Time-course (A; 1–7 days) and dose-response effects (B; 25–1000 ng/mL) of Fsh stimulation *ex vivo* on *amh* (open columns) and *igf3* (black columns) transcript levels in adult zebrafish testis tissue. The medium contained 25  $\mu$ g/mL trilostane to prevent the production of biologically active steroids that can modulate *igf3* transcript levels (Nóbrega et al., 2015). Bars marked with an asterisk are significantly different from the respective controls (paired *t*-test,  $p < 0.05$ , comparing the two testes of an individual;  $n = 6–8$  per time period or Fsh concentration) while different letters indicate statistical differences among the Fsh doses used (ANOVA,  $p < 0.05$ ). Data are expressed as mean  $\pm$  SEM fold change in gene expression relative to the control condition, which is set at 1 (dashed line).

insulin-like peptide 3 (InsI3; Assis et al., 2016) that both stimulate germ cell differentiation, while fading Amh signaling would alleviate its inhibitory effects on androgen and *insl3* production (Skaar et al., 2011).

### 3.2. Amh inhibited Igf3-induced proliferation of type $A_{und}$ and type $A_{diff}$ spermatogonia

Considering the data presented above, it appears that shortly after the beginning of an Fsh stimulus or when an Fsh stimulus is not very strong, both Amh and Igf3 are present simultaneously. Therefore, we studied if Amh compromises the stimulatory effects of Igf3. Type  $A_{und}$  spermatogonia showed the expected (Nóbrega et al., 2015) high mitotic index in the presence of Igf3, which was clearly reduced when Amh was present (Fig. 3 A). Amh also reduced the mitotic index of type  $A_{diff}$  but not the one of type B spermatogonia (Fig. 3 A). Skaar et al. (2011) reported that Amh inhibited differentiation, rather than proliferation, of type  $A_{und}$  spermatogonia, i.e. it was mainly self-renewal proliferation that these early spermatogonia carried out in the presence of Amh, resulting in an accumulation of type  $A_{und}$  spermatogonia in response to Amh exposure, while further differentiated germ cell types became depleted. Since analyzing only the proliferation activity does not provide information on the question if spermatogonia are heading for a self-renewal or a differentiating division, we also quantified the proportion of the section surface area occupied by different spermatogonial types. Amh reduced the proportion of type  $A_{diff}$  spermatogonia cysts, while the proportions of type  $A_{und}$  and of B spermatogonia remained unaltered (Fig. 3 B). Hence, in contrast to androgen-stimulated spermatogenesis (Skaar et al., 2011), Amh did not induce an accumulation of type  $A_{und}$  or a depletion of type B spermatogonia when Igf3 was used to stimulate spermatogenesis. Taken together, the proliferation and surface proportion data show that Amh compromised the Igf3-induced differentiating divisions of type  $A_{und}$  spermatogonia and the further proliferation of type  $A_{diff}$  germ cells. However, it is not clear if the difference in the behavior of type  $A_{und}$  spermatogonia is based on a balanced reduction of the differentiating and of the self-renewal divisions of type  $A_{und}$  germ cells, or rather on a higher rate of differentiation of  $A_{und}$  germ cells, despite the presence of Amh, when Igf3 instead of androgen drives spermatogenesis.

To obtain additional information in this regard, we have quantified Sertoli cell proliferation, reasoning that a change (reduction)

in the proliferation of Sertoli cells associated with differentiating spermatogonia would reflect a reduction of differentiating germ cell divisions (i.e. a reduced rate of spermatogenic cyst growth), while a reduced proliferation of Sertoli cells associated with single type  $A_{und}$  spermatogonia would indicate a reduced self-renewal activity, i.e. a reduced generation of new niche space (de França et al., 2015; Leal et al., 2009a). Since only Sertoli cells associated with type  $A_{und}$  spermatogonia showed a reduced mitotic index (Fig. 3 C), reduced self-renewal divisions would represent the main contribution to the Amh-induced decrease of the mitotic indices of type A spermatogonia. Androgen-driven transition of type  $A_{diff}$  into type B spermatogonia was reported to be compromised by Amh (Skaar et al., 2011), but was not affected in this experiment when using Igf3 to stimulate spermatogenesis. A possible explanation for the stronger inhibitory effect of Amh on androgen- than on Igf3-induced spermatogonial development may be related to the fact that both androgens and Amh exert their effects on Sertoli cells, while Igf receptors are also expressed by germ cells (Nóbrega et al., 2015), where Amh cannot interfere directly, since Amh receptor expression has not been reported for germ cells in vertebrates including fish (Kluver et al., 2007).

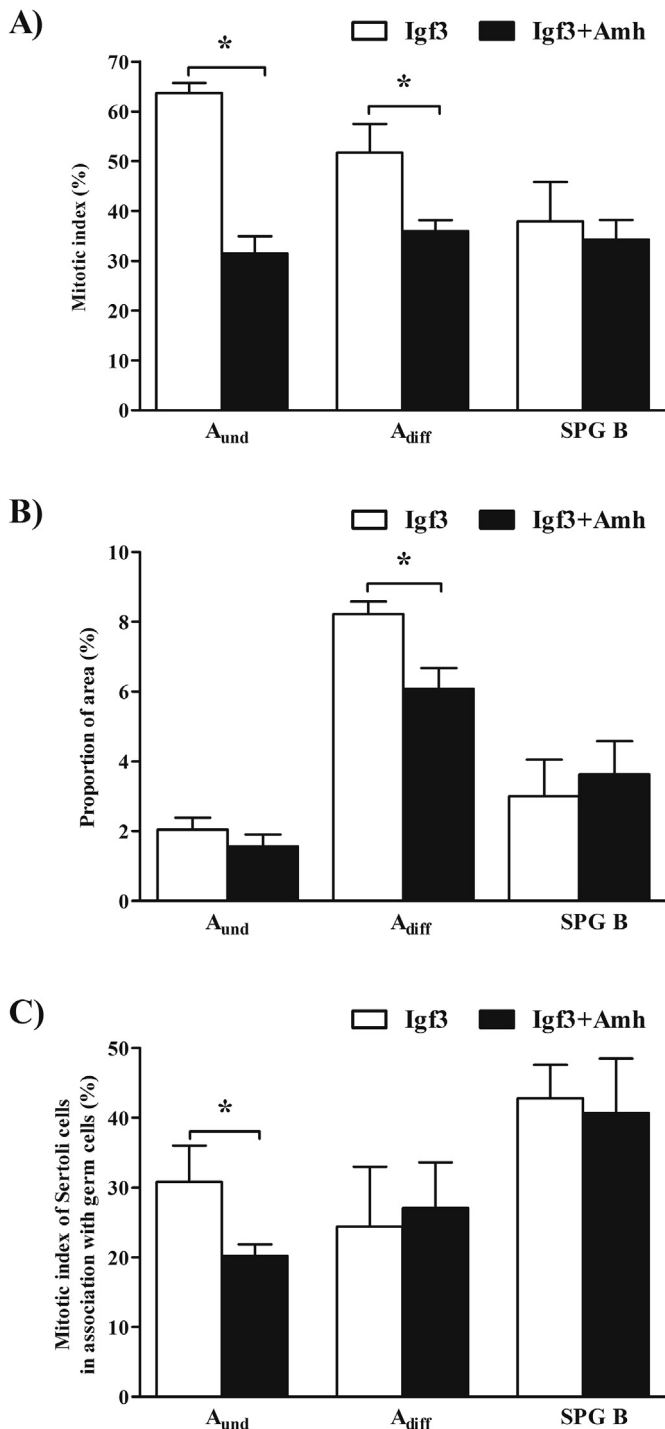
We have shown that Amh restricts Igf3 effects but considering that also androgen production and *insl3* transcript levels were reduced (Skaar et al., 2011), Amh seems to exert a range of inhibitory effects. We therefore studied the effect of Amh on testicular gene expression by RNAseq, also because there is little information available in this regard in vertebrates in general.

### 3.3. Amh-induced changes in testicular gene expression

To this end, we have used the zebrafish testis tissue culture approach and, as reported previously (Skaar et al., 2011), we have carried out the experiments in androgen-containing medium to support spermatogenesis as the control condition, versus androgen-containing medium in combination with Amh as the experimental condition.

Gene expression profiling of adult zebrafish testis tissue *ex vivo* revealed that Amh caused a significant modulation in the expression of 236 genes compared with the control condition (Supplemental Table 3; in total, 33,737 unique genes included in the zebrafish genome [Zv9] were considered for the analysis). The majority of the DEGs ( $p < 0.05$ ; 163, 69.07% of the total) was down-regulated by Amh (Fig. 4A; Supplemental Table 3). Similar





**Fig. 3. Quantitative morphological analysis of zebrafish testis tissue after *ex vivo* exposure to Igf3 (100 ng/mL) in the absence or presence of Amh (10 µg/mL).** A) Mitotic indices of type A undifferentiated (A<sub>und</sub>), type A differentiating (A<sub>diff</sub>) and type B (SPG B) spermatogonia. B) Proportion of section surface area containing cysts with type A<sub>und</sub>, A<sub>diff</sub> or B spermatogonia. C) Mitotic indices of Sertoli cells contacting type A<sub>und</sub>, type A<sub>diff</sub> or B spermatogonia. An asterisk indicates a significant difference between treatments (paired *t*-test, *p* < 0.05, comparing the two testes of an individual; *n* = 6).

proportions were observed after applying less strict selection criteria (Supplemental Fig. 2A), so that Amh mainly inhibited gene expression. This was also observed in the mammalian ovary challenged by AMH (Nilsson et al., 2007), although different from the

latter study, our control situation included androgens. KEGG pathway analyses confirmed this Amh-associated expression pattern in the zebrafish testis since all the pathways identified were classified as down-regulated (Fig. 4 B).

We selected 6 down- and 4 up-regulated genes in order to confirm differential gene expression by qPCR analysis (Supplemental Fig. 2B). In all cases, qPCR analysis confirmed the RNAseq results and no significant differences were found between the two techniques.

In the next experiment, we asked if androgens modulate Amh effects. We selected 4 growth factors, 3 intracellular proteins, and 3 steroidogenesis-related genes to study this question. In all but one case (*kitlgb*), there were no statistically significant differences in transcript levels comparing the absence and presence of androgen (Fig. 5), suggesting that the RNAseq data indeed mainly reflect Amh effects on testicular gene expression. Among the genes analyzed, *igf3* transcript levels were down-regulated by Amh (Fig. 5), while the expression of *id3* was up-regulated (Fig. 5), a gene involved in the maintenance of self-renewal, pluripotency and the undifferentiated state of progenitor cells (Carroll et al., 2011; Hong et al., 2011), providing potentially important clues for mechanisms by which Amh exerts its effects in the zebrafish testis.

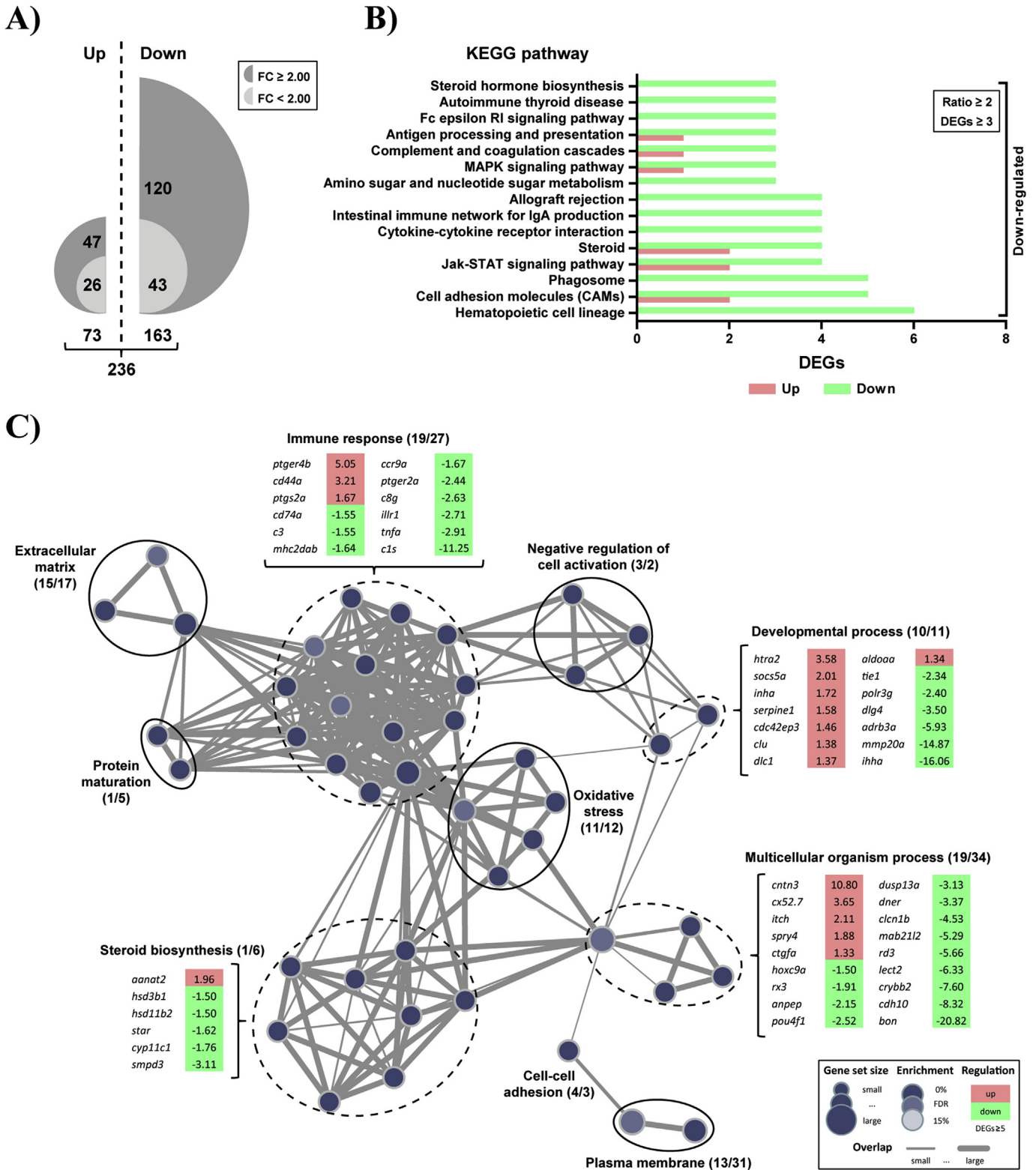
### 3.3.1. Steroidogenesis-related genes

To further analyze Amh effects, DEGs were tested and mapped for functional enrichment (see Material and methods section). Among the genes affected by Amh, several functionally related gene clusters emerged that were characterized by a high number of overlapping genes (Fig. 4C; Supplemental Table 4). Amh treatment caused a clear inhibition of genes involved in steroidogenesis (*steroid biosynthesis* cluster), including *star*, *hsd3b1*, *hsd11b2* and *cyp11c1* (Fig. 4C). KEGG analyses similarly reported steroid as well as steroid hormone biosynthesis pathway as down-regulated (Fig. 4B). These observations add to previous findings that Amh suppressed Fsh-stimulated increases in *cyp17a1*, *star*, and *insl3* gene expression and androgen release in adult zebrafish (Skaar et al., 2011). AMH-mediated inhibition of Leydig cell steroidogenesis is known from studies in mammals (Teixeira et al., 1999), suggesting that an evolutionary conserved aspect of the inhibitory effects of Amh resides in compromising Leydig cell function.

### 3.3.2. Tgf-β family members

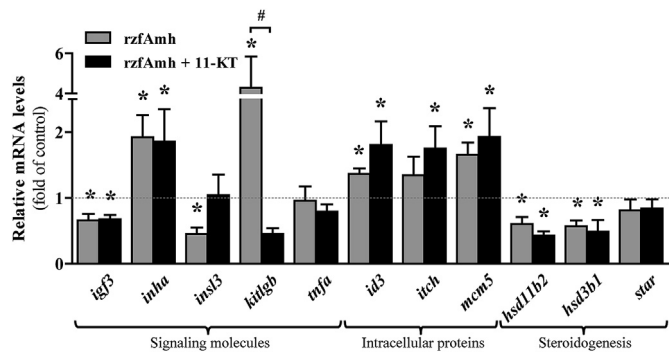
A total of 21 DEGs (10 up- and 11 down-regulated) were mapped in the cluster *developmental process* (Fig. 4C). This included factors regulating the spermatogenetic process in vertebrates, such as *htra2* (Hayashi et al., 2006; Tain et al., 2009), *clu* (Plotton et al., 2006) and *inha* (Hedger and Winnall, 2012; Nalam et al., 2010). Considering *inha*, studies in immature rainbow trout reported that androgen treatment *in vivo* decreased (Rolland et al., 2013) while androgen treatment *ex vivo* increased (Sambroni et al., 2013a) testicular *inha* transcript levels. This seems to be different in adult zebrafish, since androgens did not influence the stimulatory effects of Amh on *inha* levels (Fig. 5). Amh also increased follistatin-like 4 (*fstl4*) transcript levels (Supplemental Table 3). Follistatin binds and neutralizes TGF-β family members, in particular Activin (Ueno et al., 1987; Welt et al., 2002). In conjunction with the Amh-induced up-regulation of *inha* and the fact that Inhibin and Activin use the same type II receptor, we propose that Amh shifted this branch of Tgf-β signaling from Activin to Inhibin.

Recent work in mammals identified an intratesticular role for Inhibin in balancing Activin signaling, which stimulates Sertoli cell proliferation and regulates the integrity of the blood-testis barrier (Itman et al., 2015; Nicholls et al., 2012). Therefore, it is possible that Inhibin may mediate inhibitory effects of Amh also in zebrafish spermatogenesis by reducing Sertoli cell proliferation. There are



**Fig. 4. Testicular gene expression in response to Amh treatment.** **A)** Total numbers of up- and down-regulated genes identified by RNAseq ( $p < 0.05$ ). **B)** Regulated KEGG pathways in Amh-treated testis were determined using the KEGG Mapper tool (Wang et al., 2014). KEGG pathways by at least 3 DEGs and ratio of regulated genes higher than 2 were considered for the analysis (using these criteria only down-regulated pathways were found). **C)** RNAseq data were mapped into functionally-related gene sets forming enrichment clusters. Nodes represent statistically significant GO terms ( $p < 0.05$ ; FDR 15%, enrichment significance represented as a color gradient) and links (grey lines) represent the number of overlapping genes (indicated by their thickness) between connected sets. Solid black lines encircle groups of closely related GO terms into functional clusters (total number of up- and down-regulated genes, respectively, is shown numerically between brackets). Clusters of particular interest are highlighted by a dashed black line, with selected genes listed next to the cluster. Only clusters represented by a minimum number of 5 DEGs were considered for enrichment analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)





**Fig. 5. Effects of androgen on Amh-modulated transcript levels of selected genes.** Testicular gene expression analysis after *ex vivo* exposure for three days to Amh (10 µg/mL), comparing basal medium (grey columns) and medium containing 200 nM 11-ketotestosterone (11-KT, black columns). Asterisks indicate significant differences ( $p < 0.05$ ; unpaired *t*-test) compared with the respective controls that were incubated in medium without Amh. A hashtag indicates a significant difference between the absence and presence of androgen. Data are expressed as mean  $\pm$  SEM fold change ( $n = 5-8$ ) in gene expression, relative to the control condition, which is set at 1 (dashed line).

two modes of Sertoli cell proliferation in fish. Mode 1 provides “free” Sertoli cells that increase spermatogonial stem cell niche space and are the basis for the production of new spermatogenic cysts, and mode 2, the proliferation of Sertoli cells that are associated with already differentiating spermatogenic cysts and where Sertoli cell proliferation creates the space required for the differentiating and hence expanding germ cell clone (de França et al., 2015). Examining Sertoli cell proliferation demonstrated that Amh (Fig. 3C) specifically decreased the mitotic index of Sertoli cells contacting type  $A_{und}$  spermatogonia, suggesting that (i) Sertoli cell proliferation accompanying the growth of existing cysts (i.e. Sertoli cells contacting type  $A_{diff}$  and B spermatogonia) is regulated differently from Sertoli cell proliferation to generate new cysts, and that (ii) Amh reduced divisions from type  $A_{und}$  to  $A_{diff}$  spermatogonia. Future work will have to show if this is indeed an Inhibin-mediated effect sensitive to the Inhibin/Activin balance in the zebrafish testis. Inhibin-mediated interference with Activin signaling may also be relevant for the further development of  $A_{diff}$  spermatogonia, for example, that showed reduced mitotic activity in response to Amh. In eel, human Activin induced differentiating proliferation of spermatogonia in primary tissue cultures from immature testis (Miura et al., 1995). Finally, in addition to intratesticular Inhibin effects discussed so far, *inha* transcript was localized to Sertoli cells in the zebrafish testis (Supplemental Fig. 3), similar to other vertebrates (Barakat et al., 2008; Tada et al., 2002). The cystic type of spermatogenesis allowed pinpointing *inha* expression to Sertoli cells contacting type B spermatogonia and spermatocytes (Supplemental Fig. 3). Beyond intratesticular effects, another aspect of the Amh-mediated up-regulation of testicular Inhibin production may be the potential reduction of pituitary Fsh release (Poon et al., 2009; Sambroni et al., 2013b), although direct experimental evidence for Inhibin-mediated suppression of pituitary Fsh release is missing so far for fish.

### 3.3.3. Immune and prostaglandin-related genes

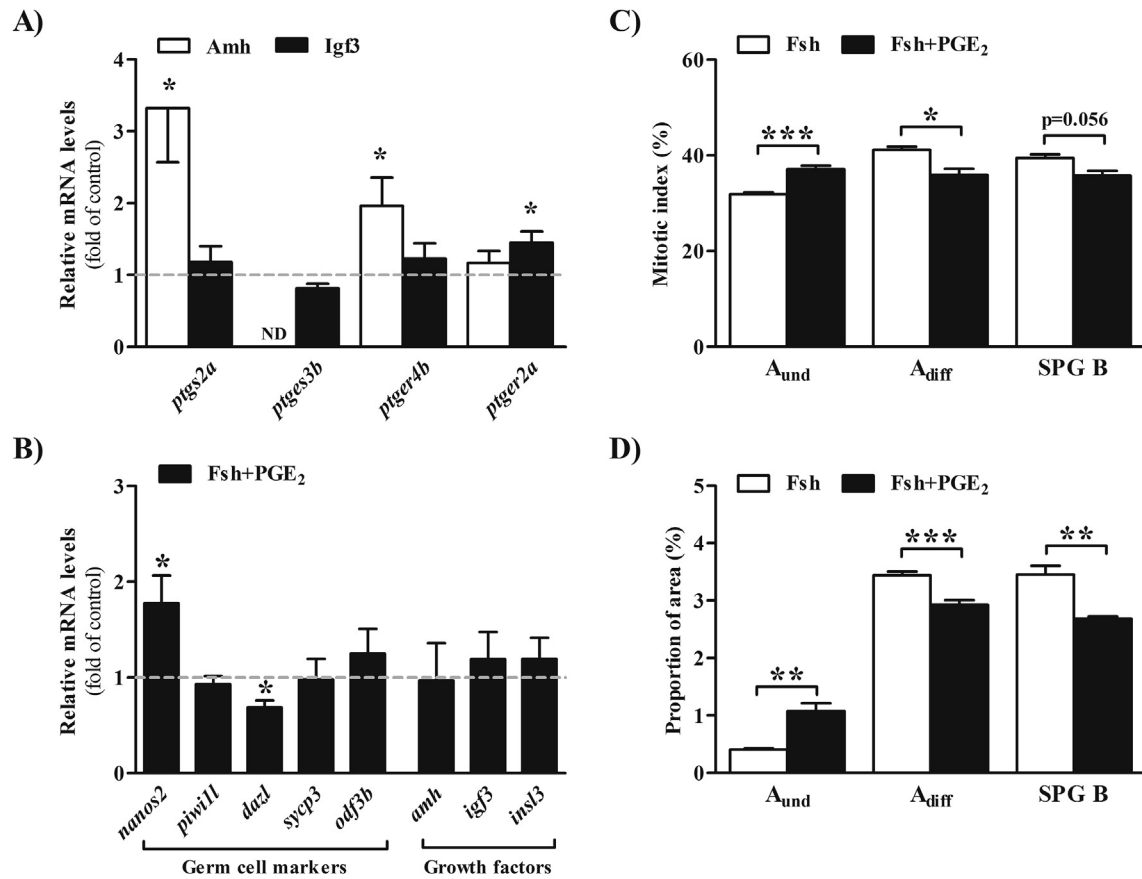
Several studies have identified expression of genes related to the immune system in the testis (Gong et al., 2013; Martinovic-Weigelt et al., 2011; Rolland et al., 2009; Ryser et al., 2011), suggesting that some of these molecules may also have functions in spermatogenesis. A large number of genes involved in immune responses were also observed in the present study (immune response cluster; Fig. 4C; Supplemental Table 4). Among those genes, different

members of the complement system (such as *c1s*, *c3* and *c8g*) were down-regulated by Amh. Complement components such as C1 (Scully et al., 2015; Sumida et al., 2015) and C3 (Maurer et al., 2015; Rutkowski et al., 2010), acting in wound healing and regeneration, stimulate proliferation and might do so also in the testis, a process inhibited by Amh in type  $A_{und}$  spermatogonia (Skaar et al., 2011). A particular group of genes that is usually categorized in the immune response cluster, the PG-related genes, caught our interest. The PGE<sub>2</sub> receptors *ptger4a* and *ptger2a* and the PG synthase *ptgs2a* were modulated by Amh (Fig. 4C). D-type PGs keep spermatogonia in an undifferentiated state in the embryonic mouse testis (Moniot et al., 2014), PGs are involved in zebrafish gonadal sex differentiation (Pradhan and Olsson, 2014), and also in the proliferation/differentiation behavior of bladder cancer stem cells (Kurtova et al., 2015). Exploring the potential role of PGs in zebrafish spermatogenesis, and guided by the differentially expressed genes retrieved in the RNA sequencing study, we analyzed the expression of selected PG-related genes in zebrafish testis cultured in the presence of Amh or Igf3 (Fig. 6A). Amh up-regulated the expression of *ptgs2a* and *ptger4b* genes while Igf3 up-regulated the receptor *ptger2a* and down-regulated another PG synthase gene, the *ptgs3b* (Fig. 6A). A much stronger (26.4-fold) down-regulation of *ptgs3b* transcript levels was reported in response to Fsh (Crespo et al., 2016). Considering that Fsh seems to reduce PGE<sub>2</sub> synthesis while Amh may increase Ptger4b-mediated sensitivity to PGE<sub>2</sub> and promote its production, we speculated that PGE<sub>2</sub> may inhibit spermatogonial differentiation. Using *nanos2* transcript levels as a marker for type  $A_{und}$  spermatogonia (Beer and Draper, 2013; Bellaiche et al., 2014; Draper, 2017), we stimulated spermatogonial differentiation in an experiments with Fsh (100 ng/mL; control condition) and examined if the additional presence of PGE<sub>2</sub> (5 µM; experimental condition) changed *nanos2* transcript abundance. In the presence of PGE<sub>2</sub>, *nanos2* transcript levels increased significantly, while those of *dazl* – expressed in type B spermatogonia and spermatocytes in zebrafish (Chen et al., 2013) – were reduced (Fig. 6B). In addition, PGE<sub>2</sub> increased the mitotic index of  $A_{und}$  spermatogonia as well as the area occupied by this cell type (Fig. 6C–D). On the other hand, PGE<sub>2</sub> inhibited the proliferation activity and reduced the number of  $A_{diff}$  and B spermatogonia (Fig. 6C–D). The PGE<sub>2</sub>-mediated up-regulation of *nanos2* transcript levels may block further germ cell differentiation by suppressing target genes required for differentiation, such as *Dazl* (Kato et al., 2016). Since Fsh also regulates the expression of growth factors able to modulate spermatogonial proliferation and differentiation, we also examined if PGE<sub>2</sub> modulated *amh*, *igf3*, or *insl3* expression. This, however, was not the case (Fig. 6B). Taken together, these observations suggest that PG signaling participates in regulating spermatogonial development in adult zebrafish testis, although it would rather be the E-type than the D-type PG that were reported to function in the embryonic mouse testis (Moniot et al., 2014).

### 3.3.4. Insulin/Igf/relaxin family members

Examining selected testicular transcript levels after incubation with Amh in the absence of androgens (Fig. 5) showed that *insl3* was down-regulated. Skaar et al. (2011) reported that Amh inhibited Fsh-induced increases in *insl3* mRNA levels in the zebrafish testis. Since *Insl3* promoted differentiating mitoses of type  $A_{und}$  spermatogonia in adult zebrafish testis tissue in primary culture (Assis et al., 2016; Crespo et al., 2016), yet another mechanism by which Amh can inhibit spermatogonial differentiation is to weaken the pro-differentiation effects of *Insl3*.

Having shown that Amh modulated the expression of several genes, including *igf3* (Fig. 5), we wondered if Igf3 in turn would modulate transcript levels of *amh* and a number of other genes discussed here, namely *inha*, *insl3*, *kitlgb*, *tnfa*, *hsd11b2*, *hsd3b1*, *star*,



**Fig. 6. Potential role of PGE<sub>2</sub> in mediating inhibitory effects on spermatogenesis.** A) Relative mRNA levels of selected PG-related genes in testicular explants incubated for 5 days with Amh (10  $\mu$ g/mL; open columns) or Igf3 (100 ng/mL; black columns) compared with its respective controls. B) Testicular gene expression after *ex vivo* exposure for 4 days to Fsh and PGE<sub>2</sub> (100 ng/mL and 5  $\mu$ M, respectively) compared to Fsh alone. Asterisks indicate significant differences induced by exposure to PGE<sub>2</sub> ( $p < 0.05$ ). Data are expressed as mean  $\pm$  SEM fold change ( $n = 5-8$ ) in gene expression, relative to the control condition, which is set at 1 (dashed line). C-D) Quantitative determination of mitotic indices (C) and volumetric proportion of cysts (D) of type *A*<sub>und</sub>, type *A*<sub>diff</sub> and B spermatogonia in zebrafish testes cultured for 4 days and exposed to Fsh and PGE<sub>2</sub> (100 ng/mL and 5  $\mu$ M, respectively) compared with Fsh only. Data are shown as mean  $\pm$  SEM ( $n = 7$ ). ND, not determined. Paired *t*-test, \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ .

*id3*, *itch* and *mcm5*. Quantifying these transcripts after incubating testis tissue in the presence of Igf3 for 5 days did not reveal significant changes among these genes (data not shown). However, with respect to Amh, this differed when increasing Igf3 bioactivity by pharmacological blocking of Igf binding proteins (Safian et al., 2016).

### 3.3.5. Miscellaneous

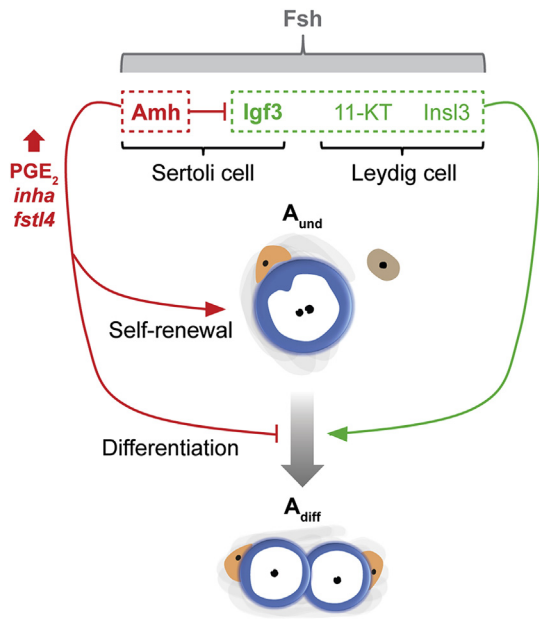
We also identified a significant enrichment in gene sets belonging to the *multicellular organism process* cluster (highest number of DEGs mapped, 53; Fig. 4C). Here, genes involved in the regulation of cell growth and proliferation in different tissues were mainly down-regulated in response to Amh (e.g. *pou4f1*, *crybb2*, *spry4*, *mab21l2*, and *itch*). For instance, loss of *crybb2*, mainly expressed in spermatogonia, reduced fertility as a result of the disordered proliferation and increased apoptosis of germ cells in mice (Xiang et al., 2012). The expression of an E3 ubiquitin protein ligase (*itch*) is required for embryonic stem cell self-renewal (Liao et al., 2013) and was increased by Amh. Interestingly, previous work has shown that Amh increased the number of single spermatogonia type *A*<sub>und</sub> (Skaar et al., 2011), a cell population containing the spermatogonial stem cells (SSCs; Nóbrega et al., 2010). Support of stem cell self-renewal by Amh is also in line with the observations that neutralization of endogenous Amh in the Japanese eel testis induced differentiating spermatogonial proliferation

(Miura et al., 2002). This suggests that Itch-mediated ubiquitination is important for the stemness of spermatogonial cells in zebrafish. Other genes with yet ill-defined functions in mammalian reproduction, but expressed in the testis, were found up-regulated (i.e. *aldoaa*, *cd44a*, *ctgfa*).

Functional enrichment results also included gene sets related to a variety of different cellular processes (such as cell-cell adhesion, negative regulation of cell activation and oxidative stress; Fig. 4C). In terms of site of expression, many DEGs identified in response to Amh are related to the extracellular matrix and plasma membrane compartments (Fig. 4C). The decrease in spermatogenic activity following Amh treatment and the associated morphological changes in the germinal epithelium may involve remodeling of extracellular matrix components. In this regard, it is interesting to note that androgen-stimulated spermatogenesis was accompanied by elevated expression of extracellular matrix proteins (Rolland et al., 2009).

## 4. Conclusions

Amh compromised the stimulatory effect of Igf3 on spermatogonial proliferation and differentiation (see Fig. 7). Moreover, Amh reduced *igf3* transcript levels, both in the absence and presence of androgens, so that Amh counteracted the biological activity of Igf3 and reduced its production. Amh also inhibited androgen



**Fig. 7. Schematic representation of the endocrine and paracrine regulation of zebrafish spermatogenesis.** Fsh stimulated Leydig cell insulin-like 3 (Insl3; Crespo et al., 2016) and androgen production (García-López et al., 2010), factors promoting germ cell differentiation. Fsh also reduced Sertoli cell expression of anti-Müllerian hormone (Amh; Skaar et al., 2011) that would otherwise exert diverse inhibitory effects on Leydig cells as well as on spermatogonial differentiation. Fsh furthermore stimulated the expression of insulin-like growth factor 3 (Igf3; Nóbrega et al., 2015), which promotes germ cell differentiation. In the present study, we show that Amh inhibits both *igf3* expression as well as Igf3-induced proliferation and differentiation of spermatogonia. In addition, Amh stimulates the expression of Tgf beta family members with potentially inhibitory effects on spermatogenesis (e.g. *inha*, *fstl4*), and we report that PGE<sub>2</sub> prevents germ cell differentiation.

production and *insl3* gene expression, two other stimulators of germ cell differentiation, while increasing inhibitory signals, such as *inha*, *fstl4*, *id3* and PG-related genes. The importance of Amh for the regulation of early stages of germ cell development is also exemplified in medaka (*Oryzias latipes*), where loss of the Amh receptor II resulted in an over-proliferation of undifferentiated spermatogonia (Nakamura et al., 2012). It therefore appears that Amh plays a central, inhibitory role (by inhibiting stimulators and stimulating inhibitors), suggesting that an important aspect of the biological activity of Fsh to stimulate spermatogenesis involves controlling the inhibitory influence of Amh (Fig. 7).

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2017.06.017>.

### Disclosure statement

The authors have nothing to disclose.

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