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Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis

Diego Crespo [a], Luiz H.C. Assis [a], Tomasz Furmanek [b], Jan Bogerd [a], Rüdiger W. Schulz [a, b, *]

[a] Reproductive Biology Group, Division of Developmental Biology, Department of Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[b] Research Group Reproduction and Developmental Biology, Institute of Marine Research, Bergen, Norway

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ABSTRACT

Spermatogonial stem cells are quiescent, undergo self-renewal or differentiating divisions, thereby forming the cellular basis of spermatogenesis. This cellular development is orchestrated by follicle-stimulating hormone (FSH), through the production of Sertoli cell-derived factors, and by Leydig cell-released androgens. Here, we investigate the transcriptional events induced by Fsh in a steroid-independent manner on the restart of zebrafish (*Danio rerio*) spermatogenesis *ex vivo*, using testis from adult males where type A spermatogonia were enriched by estrogen treatment *in vivo*. Under these conditions, RNA sequencing preferentially detected differentially expressed genes in somatic/Sertoli cells. Fsh-stimulated spermatogonial proliferation was accompanied by modulating several signaling systems (i.e. Tgf-β, Hedgehog, Wnt and Notch pathways). *In silico* protein-protein interaction analysis indicated a role for Hedgehog family members potentially integrating signals from different pathways during fish spermatogenesis. Moreover, Fsh had a marked impact on metabolic genes, such as lactate and fatty acid metabolism, or on Sertoli cell barrier components. Fish Leydig cells express the Fsh receptor and one of the most robust Fsh-responsive genes was insulin-like 3 (*insl3*), a Leydig cell-derived growth factor. Follow-up work showed that recombinant zebrafish Insl3 mediated pro-differentiation effects of Fsh on spermatogonia in an androgen-independent manner. Our experimental approach allowed focusing on testicular somatic genes in zebrafish and showed that the activity of signaling systems known to be relevant in stem cells was modulated by Fsh, providing promising leads for future work, as exemplified by the studies on Insl3.

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1. Introduction

The pituitary gonadotropins, FSH (follicle-stimulating hormone) and LH (luteinizing hormone), are major regulators of gonadal reproductive functions in mammals. In the testis, FSH controls Sertoli cell functions, including the production of locally active factors such as GDNF, FGF2, IGF1, inhibin or activin (Barakat et al., 2008; Mullaney and Skinner, 1992; Nicholls et al., 2012; Pitetti et al., 2013; Tadokoro et al., 2002) that orchestrate germ cell development. LH controls Leydig cell testosterone production, which in turn regulates in Sertoli and peritubular myoid cells androgen receptor (AR) sensitive gene expression that is critical for spermatogenesis (De Gendt and Verhoeven, 2012; McLachlan et al., 2002). There is little cross activation of the FSH receptor (FSHR) by LH or of the LH receptor (LHCGR) by FSH and Leydig cells do not express the FSHR gene, while Sertoli cells do not express the LHCGR gene in mammals (Jia et al., 1991; Themmen and Huhtaniemi, 2000; Tilly et al., 1992). In mice, loss of LHCGR-mediated signaling is incompatible with fertility while loss of FSHR-mediated signaling results in a delayed puberty, smaller testes, and reduced sperm output but FSHR mutants are still fertile (Kumar, 2005).

This distribution of tasks between the two gonadotropins is different in teleost fish, considering that neither *lhb* nor *lhcgr* deficiency affected pubertal development or adult fertility in male zebrafish (Chu et al., 2014, 2015; Zhang et al., 2015a; Zhang et al., 2015b). This is probably related to the fact that in fish, Fsh is also a potent steroidogenic hormone (García-López et al., 2009, 2010; Mazon et al., 2014; Planas and Swanson, 1995; Van der Kraak et al., 1992), based on *fshr* gene expression not only by Sertoli but

* Corresponding author. Department Biology, Division Developmental Biology, Reproductive Biology Group, Kruyt Building, Room W606, Utrecht University, Science Faculty, Padualaan 8, 3584 CH, Utrecht, The Netherlands.
E-mail address: r.w.schulz@uu.nl (R.W. Schulz).

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also by Leydig cells (Chauvigne et al., 2012; García-López et al., 2009, 2010; Ohta et al., 2007). Hence, in fish effects of Fsh can be mediated by its steroidogenic activity via Leydig cells, or via Sertoli cells. Experimentally, the direct effects on Sertoli cells can be separated from steroid-mediated effects by applying a 3β-hydroxysteroid dehydrogenase inhibitor (trilostane; Nóbrega et al., 2015; Ohta et al., 2007; Sambroni et al., 2013), preventing the production of biologically active steroids, thus allowing to focus on direct Fsh effects on Sertoli and Leydig cells.

A number of paracrine factors produced by Sertoli cells have already been identified as FSH targets in mammals (see above), but the rich transcriptional profile of Sertoli cells during mouse spermatogenesis (Zimmermann et al., 2015) suggests that additional signaling systems may be involved in mediating FSH effects as well (e.g. Hedgehog, WNT, and Notch [embryonic: Cool and Capel, 2009; Defalco et al., 2013; Garcia et al., 2013; Garcia and Hofmann, 2013; Ross and Capel, 2005; adult: Bitgood et al., 1996; Das et al., 2013; Murta et al., 2014; Tanwar et al., 2010]), while much is still to be learnt about the exact functioning of these signaling systems as regards Fsh-regulated somatic-germ cell communication. Our main interest is investigating the start of the spermatogenic process, in particular the questions how spermatogonial stem cell (SSC) proliferation activity (quiescence versus cell cycling) and mode (self-renewal versus differentiation) is regulated. In fish, anti-Müllerian hormone (Amh) restricts the proliferation activity to stem cell self-renewal divisions (Miura et al., 2002; Morinaga et al., 2007; Skaar et al., 2011), while another transforming growth factor β (Tgf-β) member (Gsdf; gonadal somatic cell derived factor) stimulated the proliferation of differentiating spermatogonia (Sawatari et al., 2007). Moreover, an inactivating mutation in the zebrafish *alk6b* gene (a type IB bone morphogenetic protein [Bmp] receptor) caused over-proliferation of undifferentiated spermatogonia resulting in testicular tumors (Neumann et al., 2011). On the other hand, insulin-like growth factor 3 (Igf3), a fish-specific member of the Igf family preferentially expressed in gonadal tissue, promoted the differentiating proliferation of spermatogonia (Nóbrega et al., 2015). Testis tissue culture studies in zebrafish showed that Fsh increased *igf3* but decreased *amh* transcript levels while information on Fsh effects on other paracrine signaling systems is largely missing in fish and is often incomplete also in mammals. Hence, Fsh is particularly interesting to study in fish because full male fertility is possible in the absence of Lh/Lhcgr in zebrafish (see above), i.e. seems guaranteed by the remaining gonadotropin Fsh. Its importance is further supported by data from salmonid fishes, where Lh, in contrast to Fsh, plasma levels are very low/undetectable during most of the pubertal or seasonal testis growth period (Oppen-Berntsen et al., 1994; Prat et al., 1996; Slater et al., 1994; Suzuki et al., 1988; Swanson et al., 1989). Finally, spermatogenesis can be studied *ex vivo* in fish and primary tissue culture systems have been developed that allow studying the complete spermatogenic process (e.g. Miura et al., 1991 - Japanese eel [*Anguilla japonica*]; or Leal et al., 2009 - zebrafish [*Danio rerio*]).

The current study intended primarily to identify, by RNA sequencing (RNAseq), testicular genes/pathways targeted by Fsh involved in regulating the initial steps of spermatogenesis in a paracrine manner via Sertoli cells, but potentially also via Leydig cells, though then in a steroid-independent manner. To this end, we used a previously established androgen-insufficiency model (de Waal et al., 2009), in which estrogen-mediated negative feedback on the brain-pituitary system *in vivo* interrupted adult spermatogenesis, and this was associated with an accumulation of type A spermatogonia. Using testis tissue from these males for subsequent *ex vivo* exposure to Fsh allowed investigating Fsh-effects on the restart of spermatogonial proliferation and differentiation in tissue culture. We find that the Fsh-induced, androgen-independent restart of spermatogonial proliferation and differentiation is associated with modulating a complex signaling network operating mainly in Sertoli cells. Moreover, next to steroidogenesis-related genes, the expression of Fsh receptors by fish Leydig cells also resulted in changes of Leydig cell growth factor expression, and we show that one of them, Insl3, promotes the differentiation of type A spermatogonia.

2. Material and methods

2.1. Animals and testis tissue culture

Adult male zebrafish were used for the experiments described in the present study. Handling and experimentation were consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands) approved the experimental protocols.

Using a previously established *ex vivo* culture system (Leal et al., 2009), adult zebrafish testis tissue was incubated for 48 h in the absence or presence of recombinant zebrafish Fsh (100 ng/mL), a concentration that led to a half-maximal stimulation of androgen release (García-López et al., 2010). Since the zebrafish Lhcgr is not responding even to very high concentrations of Fsh (So et al., 2005), we assume that 100 ng Fsh/mL did not cross activate the Lhcgr. Production of recombinant zebrafish Fsh in human embryonic kidney (HEK 293) cells, and its purification and characterization have been described previously (García-López et al., 2010). Since Fsh is a potent steroidogenic hormone in zebrafish (García-López et al., 2010) and our aim was to focus on the effects of Fsh not mediated by steroid hormones, these incubations were carried out in the presence of trilostane (Sigma-Aldrich; 25 μg/mL), an inhibitor of the 3β-hydroxysteroid dehydrogenase enzyme, which prevents the production of biologically active steroids. Moreover, before collecting testis tissue for *ex vivo* culture, the fish were exposed to 10 nM 17-β estradiol (E_2) *in vivo* for 3 weeks (illustrated in Fig. 1A), with a daily change of the E_2-containing water, as described previously (de Waal et al., 2009). The resulting androgen insufficiency interrupted spermatogenesis such that type A spermatogonia accumulate while the testes become depleted of type B spermatogonia, spermatocytes and spermatids (de Waal et al., 2009). Under these conditions, the effects of Fsh on type A spermatogonia and Sertoli cell proliferation activity were investigated by studying the incorporation of the S-phase marker bromodeoxyuridine (BrdU, Sigma-Aldrich; 50 μg/mL), which was added to the medium during the last 6 h of the 48 h culture period. To quantify proliferation of type A spermatogonia, the mitotic index was determined by examining at least 100 germ cells/cysts, differentiating between BrdU-labeled and unlabeled cells. To evaluate Sertoli cell proliferation, 10 randomly selected fields per sample were photographed at ×400 magnification and BrdU-labeled cells were quantified. In independent experiments, RNA was isolated from 6 testes incubated in the absence and from 6 testes incubated in the presence of Fsh under otherwise similar conditions (i.e. following estrogen exposure *in vivo* and incubated with trilostane-containing medium). Libraries were generated for RNA sequencing (as described below), and incubation medium was collected to quantify testicular androgen (11-ketotestosterone; 11-KT) release as described previously (García-López et al., 2010).

2.2. Transcriptomic analysis by RNAseq

Total RNA was isolated from testis tissue using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA integrity was checked with an Agilent Bio-analyzer 2100 total RNA Nano series II chip (Agilent). Only samples with a RNA integrity

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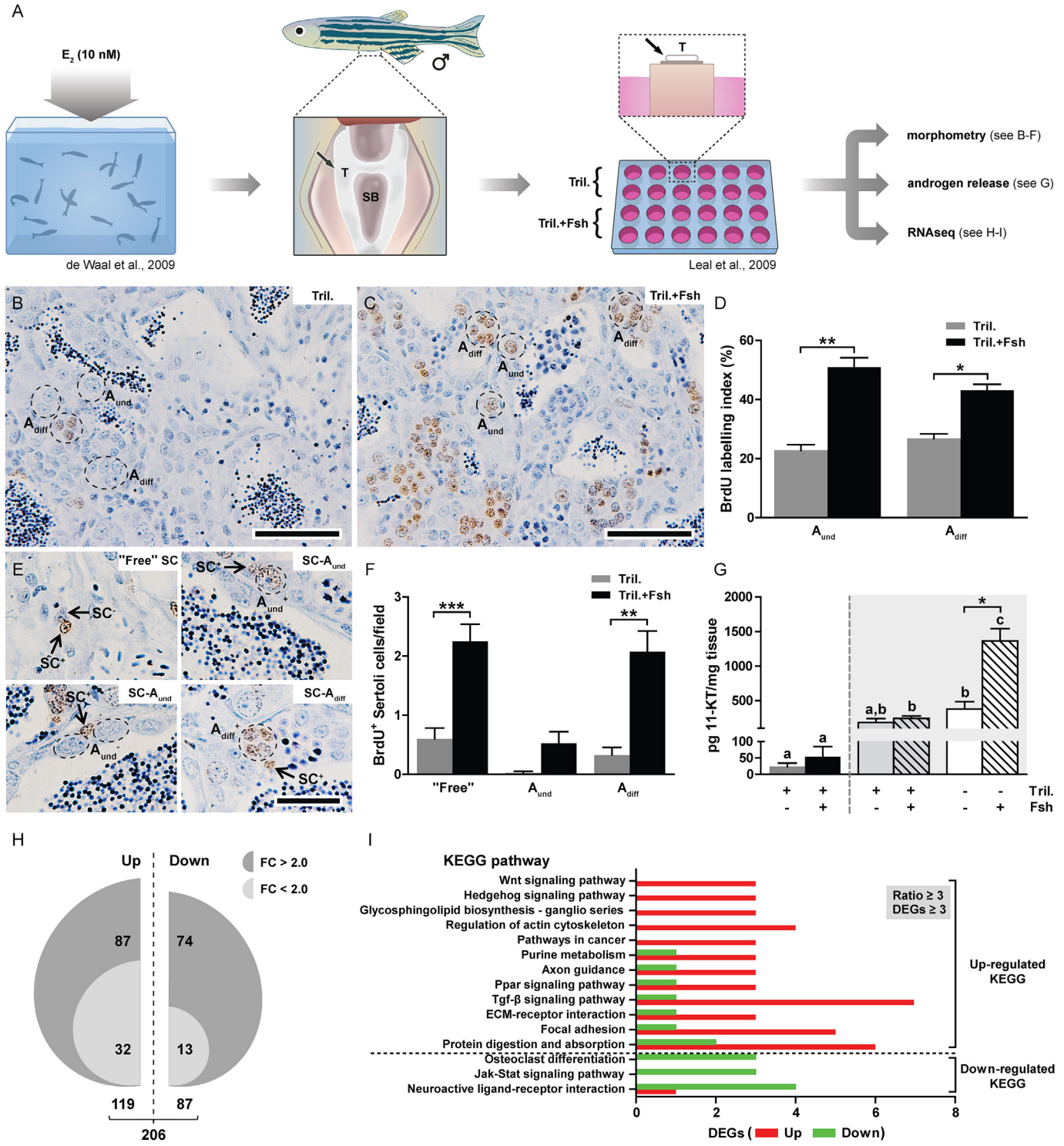

Fig. 1. Proliferation and transcriptomic response of zebrafish testis to Fsh. **(A)** Schematic representation of the experimental set-up used in this study. Testis tissue was collected from adult males exposed to 10 nM estradiol (E_2) *in vivo* for 21 days, to induce androgen insufficiency. Testicular explants were cultured for 48 h in the absence or presence of 100 ng/mL Fsh, co-incubated with 25 μg/mL trilostane to prevent the production of 3-keto,delta-4 steroids, and then used for different purposes. **(B–C)** Immunocytochemical detection of BrdU incorporation served to quantify germ cell proliferation activity. **(D)** Quantification of BrdU labeling indices of type A_{und} and type A_{diff} spermatogonia (n = 4). **(E–F)** Immunocytochemical detection of BrdU in Sertoli cells (E) and quantification of BrdU-positive Sertoli cells per section surface area (F) (n = 4). **(G)** Quantification of testicular androgen (11-KT) production to evaluate the effects of E_2-pretreatment and/or trilostane. Males were pre-exposed (white background) or not (grey background) to 10 nM E_2 *in vivo* for 21 days and testes were then incubated in the absence or presence of 100 ng/mL Fsh with medium that did or did not contain 25 μg/mL of trilostane (n = 11–12). Incubation medium was collected after 48 h of culture and 11-KT levels determined by RIA. **(H)** Total numbers of up- (left) and down-regulated genes (right) identified by RNAseq. Numbers of differentially expressed genes (DEGs; 1-P_{NOI} < 0.05) are shown in two categories after fold change (FC) selection (FC > 2.0, dark grey background; or < 2.0, light grey). **(I)** KEGG pathways modulated in Fsh-treated testes. Each pathway shown is represented by at least 3 DEGs (1-P_{NOI} < 0.05) and has a ratio of regulated genes (up-/down-, or *vice versa*) higher than 3. DEGs are highlighted with red (up-) or green (down-regulated) background. In D, F and G, data are expressed as mean ± SEM and asterisks indicate significant differences between treated and control groups ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). In G, bars with different letters are significantly different. A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; SB, swim bladder; SC, Sertoli cell; T, testis; Tril., trilostane. Scale bar represents 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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number > 8 were used for library preparation. Illumina RNAseq libraries were prepared from 2 μg 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 × 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 17 and 26 million reads per sample and mapping efficiency for uniquely mapped reads was between 45.19 and 78.72% (see Supplemental Table 1), with an average of 70.09%. RNAseq-derived reads were mapped to the zebrafish genome (Zv9) with STAR v2.3 (Dobin et al., 2012) using standard parameters for single-ended reads. The read counts were extracted using the Python package HTSeq (http://www-huber.embl.de/HTSeq/doc/overview.html/; Anders et al., 2015), and normalized using the totally mapped reads per library. Differential expression analysis was performed using the R/Bioconductor package NOISeq ($1\text{-}P_{NOI} < 0.05$, equivalent to false discovery rate [FDR] adjusted *P*-value (Tarazona et al., 2011, 2015)). The raw RNAseq data of the 12 samples sequenced (6 biological replicates per condition) have been deposited in the NCBI GEO database with accession number GSE84436.

Regulated KEGG pathways in Fsh-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 3 were considered for the analysis.

Functional enrichment analyses were carried out using a plugin available at http://www.baderlab.org/Software/EnrichmentMap/ (Merico et al., 2010) for the Cytoscape network environment (Shannon et al., 2003). 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 grouped according to their function. DAVID Bioinformatics Resources 6.7 (http://david.ncifcrf.gov/; Huang et al., 2008) was used to retrieve GO terms from the list of DEGs and exported as the input for each functional enrichment analysis.

In the absence of information on testicular cell type-specific gene expression in zebrafish, we took a side-step to mouse (*Mus musculus*) data to estimate the DEG-fraction expressed by Sertoli cells. To this end, IDs for Fsh-modulated genes were first converted to mouse Ensembl IDs by using the g:Orth tool (g:Profiler; http://biit.cs.ut.ee/gprofiler/). Retrieved mouse orthologues were then tested against published data sets describing the Sertoli cell transcriptome. The data set of DEGs obtained in the present study was compared also with a zebrafish data set (Crespo et al., unpublished results) obtained by analyzing testis samples, in which Sertoli cells were highly enriched following treatment of zebrafish with a cytostatic agent, so that more than 90% of the spermatogenic tubules showed Sertoli cells only (Nóbrega et al., 2010; Crespo et al., unpublished results).

STRING database v10 (http://string-db.org/; Szklarczyk et al., 2015) was used for protein-protein interaction analysis among identified genes, which groups proteins based on pairwise similarities in relevant descriptor variables, resulting in functionally connected network sets. A FDR test was performed to determine if the protein list was enriched in interactions. Hierarchical clustering (i.e. mean linkage clustering; MLC) was applied to groups of proteins, each highlighted by a different color.

2.3. Studies on candidate gene expression and validation

Additional *ex vivo* experiments were carried out using testes from zebrafish not pre-treated with E_2 to investigate the expression of genes identified by RNAseq, in response to different experimental conditions: testicular explants were incubated for 48 h in the absence or presence of Fsh (100 ng/mL) in medium containing trilostane (25 μg/mL) or not. Also medium was collected for RIA quantification of androgen release. Moreover, testis tissue was cultured in the absence or presence of 11-KT (200 nM; Sigma-Aldrich) for 48 h to determine mRNA abundance of selected genes.

Candidate gene expression was also investigated *in vivo*, in testes collected 4 h after intraperitoneal (i.p.) injection of Fsh (100 ng/g body weight) and expressed relative to the control condition (i.p. injection of phosphate-buffered saline, PBS).

To validate RNAseq results, aliquots of the sample set used for RNAseq were also analyzed by qPCR for a set of 16 selected genes.

For all other experiments, total RNA was isolated using the RNAqueous Kit (Ambion) following the manufacturer's instructions. Relative mRNA levels of candidate genes were analyzed by real-time, quantitative PCR (qPCR; see Supplemental Table 2 for detailed primer information) as previously described (Bogerd et al., 2001; García-López et al., 2010). *18S* rRNA was used as housekeeping control gene due to its constant expression under the conditions analyzed (Supplemental Fig. 1).

Finally, using *in situ* hybridization (ISH) with digoxigenin-labeled sense and antisense cRNA probes (Supplemental Table 3), the cellular localization of candidate genes (i.e. LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase [*lfng*], desert hedgehog [*dhh*], prostaglandin E synthase 3b (cytosolic) [*ptges3b*], lefty1 [*lft1*] and inhibin, beta Ab [*inhbab*]) was investigated as previously described (Assis et al., 2016).

2.4. Recombinant zebrafish Insl3 production and determination of its biological activity in the adult male gonad

One of the Fsh-target genes, insulin-like 3 (*insl3*) was selected for functional studies. Recombinant zebrafish Insl3 was produced, following a strategy as described by Park et al. (2008). Complementary DNA sequences encoding the mature B and A chains of zebrafish Insl3 (Good-Avila et al., 2009) were appended with a cystatin signal peptide for secretion at the N terminus. In addition, the B chain was tagged with C-terminal Strep II tags (GSAWSHPQFEKGGGSGGGSGGSAWSHPQFEKGG; each Strep II tag underlined), while the B and A chains were connected by a mini-C domain linker of eight amino acids (SLSQEDAL) flanked by convertase cleavage sites (KR and RSRR, respectively) and cloned into a proprietary mammalian expression vector (U-Protein Express B.V.). The tagging with the two Strep II tag epitopes allowed efficient purification of recombinant peptide without affecting the bioactivity of the Insl3 peptide. For efficient processing and secretion of the mature zebrafish Insl3 peptide, HEK293-EBNA cells were routinely co-transfected with the expression construct and a one-tenth aliquot of a mouse prohormone convertase 1 expression vector (Marriott et al., 1992). Expression and purification of recombinant zebrafish Insl3 was essentially the same as described for recombinant zebrafish Igf3 (Nóbrega et al., 2015).

The biological activity of zebrafish Insl3 recombinant peptide was examined using the above-mentioned testis tissue culture system (Leal et al., 2009). We analyzed Insl3 effects on the proliferation activity of type A spermatogonia, quantifying BrdU incorporation, as described above. Additional experiments evaluated Insl3 effects on the proportions of section surface area occupied by different germ cell types, as previously described (Assis et al., 2016), as well as by quantifying candidate gene expression by qPCR.

2.5. Statistical analysis

GraphPad Prism 5.0 package (GraphPad Software, Inc.) was used

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for statistical analysis. Significant differences between groups were identified using Student's *t*-test (paired or unpaired, as appropriate) or the non-parametric Kruskal-Wallis test followed by Dunn's test for multiple group comparisons (Fig. 1G). A significance level of $P < 0.05$ was applied in all analyses. Data are represented as mean ± SEM.

3. Results

3.1. Fsh rapidly induces early spermatogonia proliferation independently of androgen signaling

Previous work showed that Fsh induced spermatogonial proliferation after 7 days of *ex vivo* culture (Nóbrega et al., 2015). Since we aimed at examining Fsh-modulated gene expression during initial stages of spermatogenesis, we first determined the effects of Fsh on spermatogonial proliferation. Immunocytochemical detection of BrdU showed a clear stimulatory effect of Fsh after 2 days of *ex vivo* culture. The mitotic indices of type A undifferentiated (A_{und}; single cells with a large nucleus of ~9 μm diameter) and type A differentiating spermatogonia (A_{diff}; pairs or groups of cells with a nuclear diameter of ~5–7 μm) increased significantly (Fig. 1B–D). Fsh also promoted the proliferation of "free" Sertoli cells (i.e. not associated with germ cells) and of Sertoli cells contacting type A_{diff} spermatogonia (Fig. 1E and F).

Androgen levels were measured to evaluate E_2 and trilostane effects on steroid production. In the presence of trilostane, Fsh was unable to induce significant increases in androgen release. Exposure to E_2 *in vivo* for 3 weeks further reduced androgen release to less than 10% of the amounts found without exposure to E_2 (Fig. 1G). As expected, Fsh stimulated 11-KT release from testis tissue of males not exposed to E_2 and incubated in the absence of trilostane (Fig. 1G), confirming the biological activity of the recombinant zebrafish Fsh that has been produced and characterized previously (García-López et al., 2010).

3.2. Fsh effects on testicular gene expression

To better understand the global effects of Fsh on the expression of factors produced by testicular somatic cells potentially regulating spermatogenesis in a paracrine manner, an RNAseq approach was implemented. Our experimental model excluded steroid-mediated effects of Fsh and focused on initial stages of spermatogenesis as we used testis tissue enriched in type A spermatogonia.

Total numbers of significantly ($1\text{-}P_{NOI} < 0.05$) up- and down-regulated genes in response to Fsh *ex vivo* treatment are shown in Fig. 1H (see Supplemental Table 4 for complete gene set information). The expression of slightly more genes increased (119 or ~58%) than decreased (87 or ~42%) in response to Fsh, respectively, while the proportion of DEGs that reached a more than 2-fold change in expression was higher for the Fsh-inhibited genes (74 or 85%; Fig. 1H). Very similar effects were observed after applying less strict selection criteria (Supplemental Fig. 2B and Supplemental Table 4). KEGG analysis revealed that Fsh modulated a number of pathways involved in different developmental processes (such as Wnt, Hedgehog and Tgf-β signaling pathways; Fig. 1I). The majority of pathways significantly affected by Fsh were up-regulated (Fig. 1I), and included genes encoding secreted factors (e.g. *dhh*, *bmp7b*, *amh*, *gsdf*, *inha* and *inhbab*), as well as transcription factors (e.g. *epas1a* and *relb*) (Supplemental Fig. 2A).

To identify biological functions enriched among Fsh-regulated genes, DEGs were first used as input to retrieve GO term information (DAVID database) and subsequently tested and mapped for functional enrichment ($P < 0.05$, FDR < 0.1). The analysis showed numerous overlapping gene sets, i.e. genes belonging to GO terms closely related, grouped mainly in 5 clusters (labeled as development, extracellular matrix, cell adhesion, cellular metabolic process and oxidative stress; Fig. 2). Terms related to reproduction were part of the development cluster (in grey; Fig. 2). A total of 39 DEGs belong to this gene set (26 up- and 13 down-regulated, respectively; selected genes are shown in the red and green circles in Fig. 2) and, among those genes, testicular expression and known/potential functions for their putative homologs in mammalian species have been demonstrated (e.g. *lfng*, *cftr*, *ren*, *sepp1a* and *zbtb32* (Austin et al., 2012; Chen et al., 2012; Furu and Klungland, 2013; Hahn et al., 2009; Olson et al., 2005)). In addition, genes previously identified by KEGG analysis (Fig. 1I) were also retrieved in this gene set (e.g. *amh*, *bmp7b*, *dhh*, *inha*, *lrp2a* and *socs1b*).

Also genes involved in metabolic processes were enriched in response to Fsh (cellular metabolic process cluster; Fig. 2). Solute carriers, acyl-CoA dehydrogenases and lactate and fatty acid metabolism-related enzymes were observed. Moreover, we found genes associated to oxidative stress (such as *epas1a*, *nqo1*and *gpx3*) and a few genes related to aldehyde dehydrogenase activity (Fig. 2). Significant enrichment for the response to steroid hormone stimulus GO category was identified (Fig. 2), although no steroidogenic enzymes were found within the gene list. Gene sets also regulated by Fsh were response to vitamin D and negative regulation of B cell differentiation (Fig. 2). At the cellular component level, Fsh caused a clear modulation in the expression of genes grouped into the extracellular matrix and cell adhesion clusters (Fig. 2), suggesting a role for Fsh in testis tissue remodeling. Finally, the enriched plasma membrane GO term, consisting of 28 up- and 15 down-regulated genes, showed no clear connection to one of the other gene sets retrieved in the analysis (Fig. 2).

In order to validate RNAseq results by an independent method, the transcript levels of 16 genes were quantified by qPCR, analyzing the same sample set used for library preparation (Supplemental Fig. 3). Fsh-induced changes in the expression of 11 genes was confirmed, although in qPCR quantification, statistical significance was not reached for *ptges3b* and *inhbab* (Supplemental Fig. 3). The 5 genes not significantly affected according to RNAseq ($1\text{-}P_{NOI} > 0.05$) were added to this analysis due to the clear change in read numbers upon Fsh treatment. Among those genes, statistical significance was reached for *nfkb2* and *ccl25b* transcript levels by qPCR analysis ($P < 0.05$; Supplemental Fig. 3).

Next, we searched for information on the cellular site of gene expression for the 206 DEGs identified. Since databases for testicular cell type-specific gene expression are not available for zebrafish, we searched for murine orthologous and identified 116, of which 52 (44.83%) have been reported to be expressed by Sertoli cells (Fig. 3). The same set of 206 DEGs was compared with a RNAseq data set (Crespo et al., unpublished results) obtained from adult zebrafish testes, in which most germ cells were depleted by a cytostatic agent (busulfan; as described in Nóbrega et al., 2010), so that more than 90% of the spermatogenic tubuli showed a Sertoli cell only appearance. Hence, this data set represents Sertoli cell-enriched expression, although other testicular somatic cells were present. From the 206 DEGs of the present study, 168 (81.55%) were found in the Sertoli cell-enriched data set. Importantly, nearly all (50 of 52) genes identified as Sertoli cell genes in the murine data set were also found in the Sertoli cell-enriched zebrafish data set (Fig. 3). Hence, we find that in total 82.52% of the 206 DEGs show a somatic, preferentially Sertoli cell, expression.

Next, we investigated potential protein-protein interactions using as input a data set of 65 DEGs (50 previously identified by KEGG and GO analyses plus 15 selected as candidates; Fig. 4). *In silico* analysis revealed a protein network significantly enriched in interactions ($P < 0.01$), showing that 27 proteins encoded by their corresponding genes (41.54% of total; bold font in Fig. 4) were

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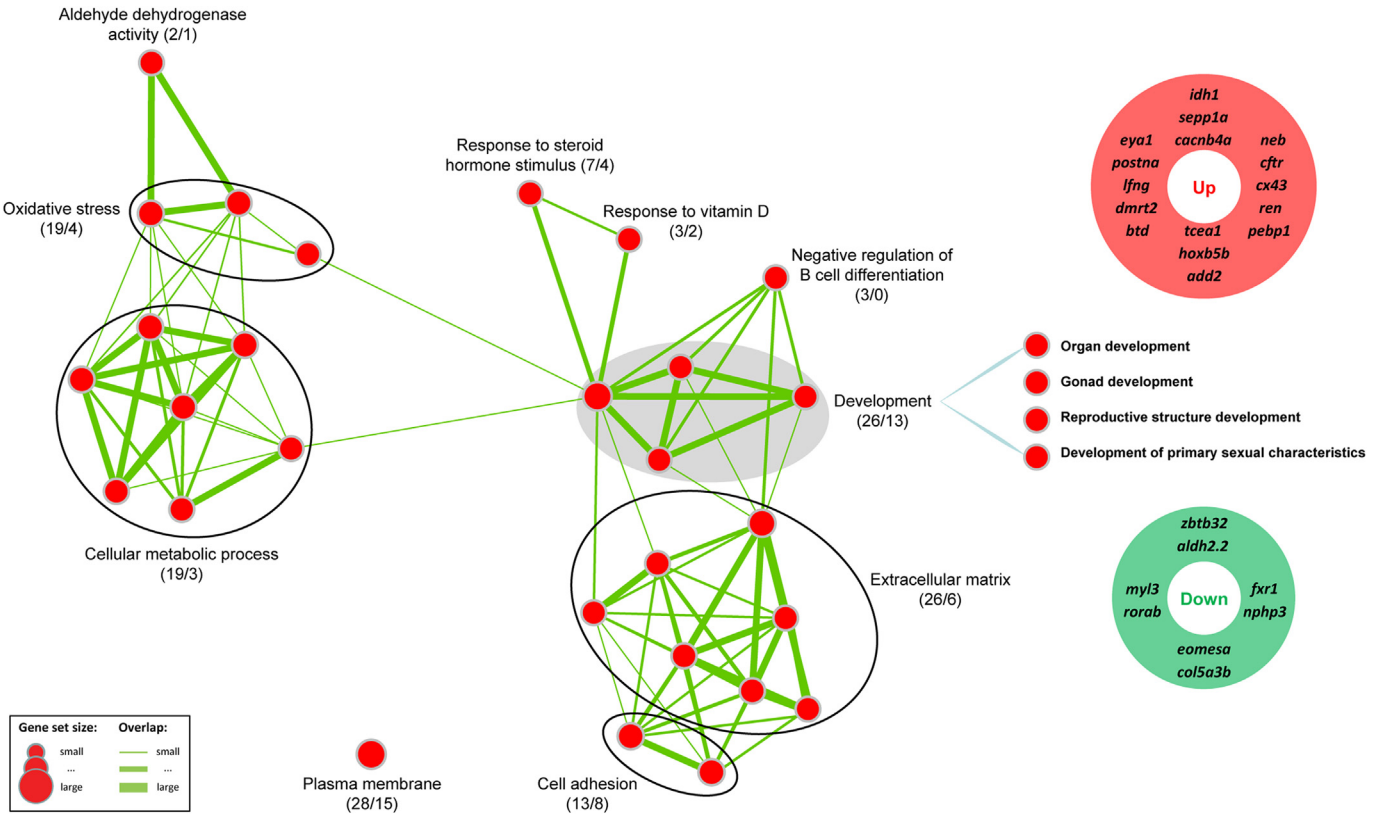

Fig. 2. Functional enrichment of Fsh-induced gene expression in the zebrafish testis. Data obtained by RNAseq analysis were mapped (after Gene Ontology [GO] enrichment analysis) resulting in a network of functionally related gene sets (red nodes) that form enrichment groups. Nodes represent statistically significant GO terms ($P < 0.05$, FDR < 0.1), links (green lines) represent the number of overlapping genes (indicated by their thickness) between connected sets. Groups of closely related GO terms are encircled with solid lines and labeled (numbers of up- and down-regulated genes are shown, respectively). The group labeled as development is highlighted with a grey background and its individual GO terms are shown, as well as selected genes that were either up- (red) or down-regulated (green) and identified in this set. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

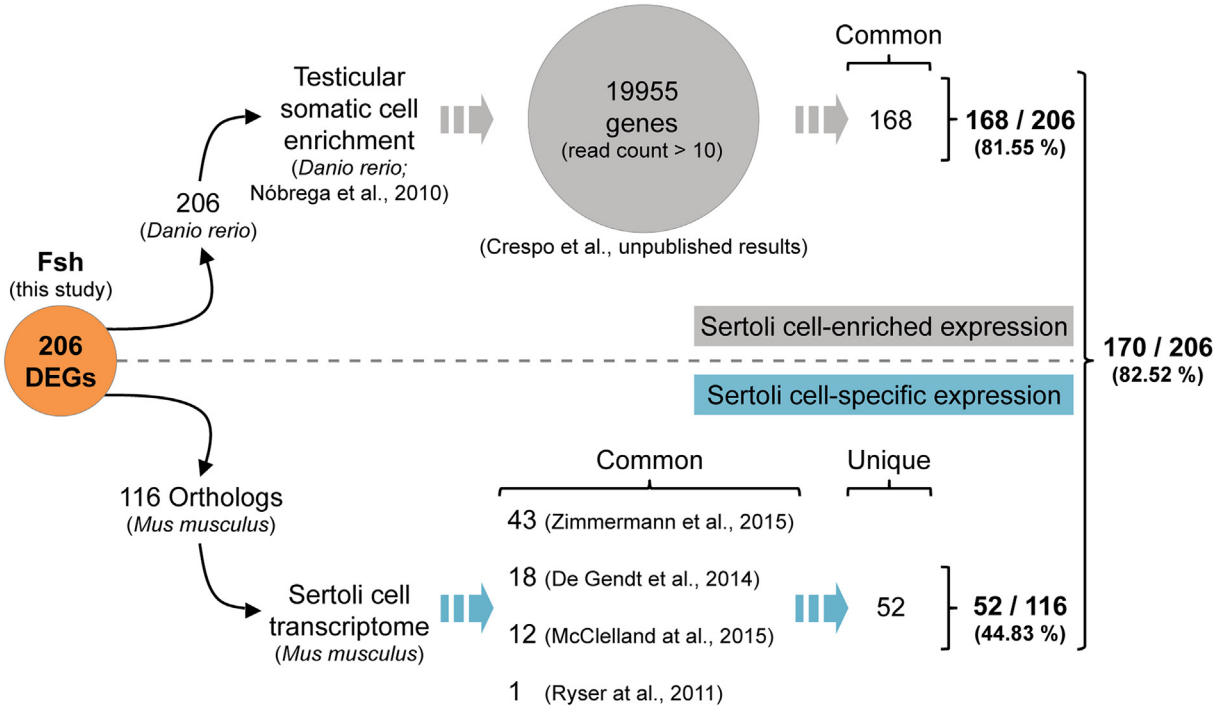

Fig. 3. Estimation of somatic/Sertoli cell associated gene expression. Fsh-modulated genes were tested against Sertoli cell transcriptome data sets available in mice (*Mus musculus*; after ortholog conversion) and zebrafish (*Danio rerio*). Specific and combined common gene numbers are indicated. DEGs, differentially expressed genes.

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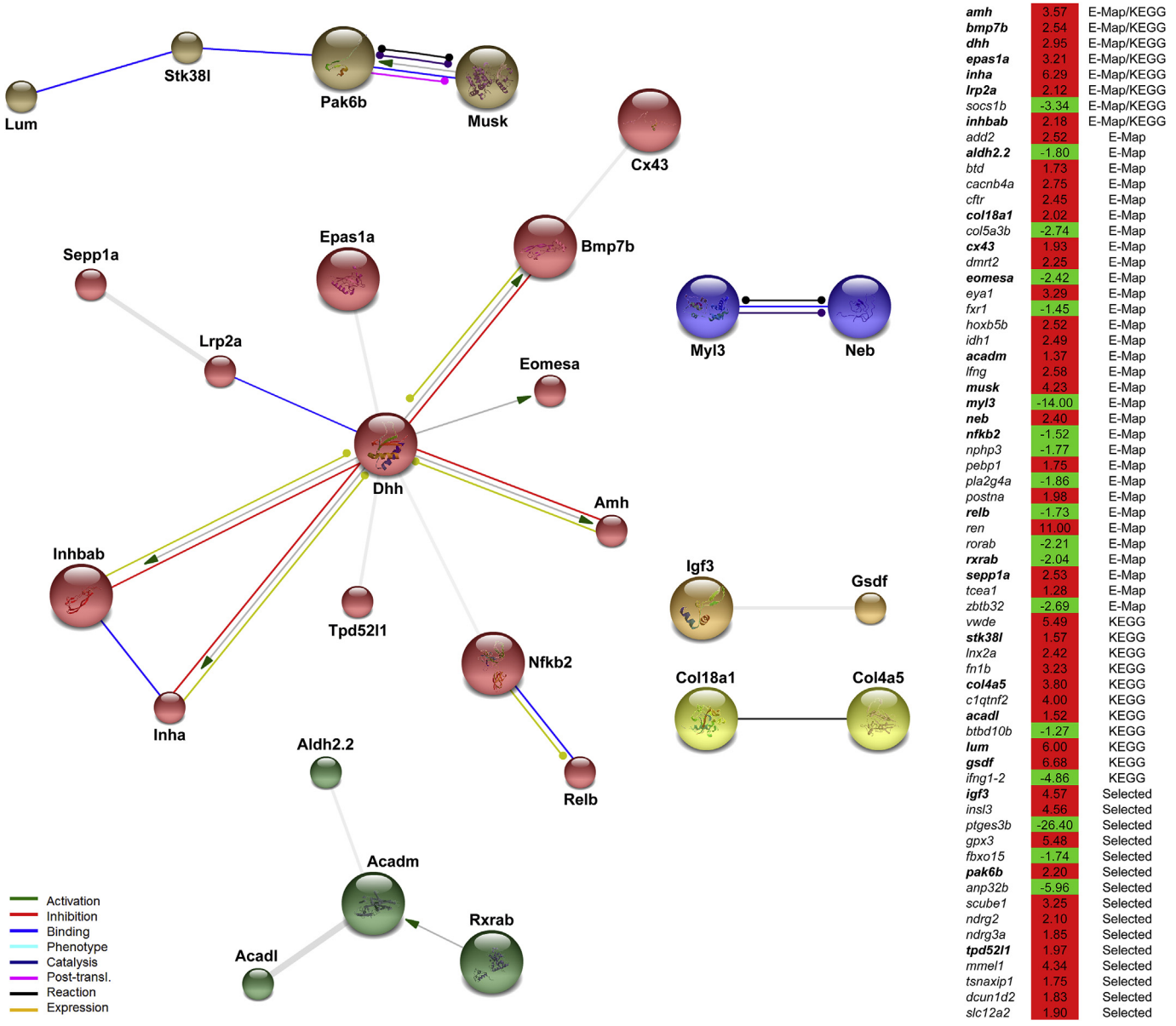

amh	3.57	E-Map/KEGG
bmp7b	2.54	E-Map/KEGG
dhh	2.95	E-Map/KEGG
epas1a	3.21	E-Map/KEGG
inha	6.29	E-Map/KEGG
lrp2a	2.12	E-Map/KEGG
socs1b	-3.34	E-Map/KEGG
inhbab	2.18	E-Map/KEGG
add2	2.52	E-Map
aldh2.2	-1.80	E-Map
btd	1.73	E-Map
cacnb4a	2.75	E-Map
cftr	2.45	E-Map
col18a1	2.02	E-Map
col5a3b	-2.74	E-Map
cx43	1.93	E-Map
dmrt2	2.25	E-Map
eomesa	-2.42	E-Map
eya1	3.29	E-Map
fxr1	-1.45	E-Map
hoxb5b	2.52	E-Map
idh1	2.49	E-Map
acadm	1.37	E-Map
lfng	2.58	E-Map
musk	4.23	E-Map
myl3	-14.00	E-Map
neb	2.40	E-Map
nfkb2	-1.52	E-Map
nphp3	-1.77	E-Map
pebp1	1.75	E-Map
pla2g4a	-1.86	E-Map
postna	1.98	E-Map
relb	-1.73	E-Map
ren	11.00	E-Map
rorab	-2.21	E-Map
rxrab	-2.04	E-Map
sepp1a	2.53	E-Map
tcea1	1.28	E-Map
zbtb32	-2.69	E-Map
vwde	5.49	KEGG
stk38l	1.57	KEGG
lnx2a	2.42	KEGG
fn1b	3.23	KEGG
col4a5	3.80	KEGG
c1qtnf2	4.00	KEGG
acadl	1.52	KEGG
btbd10b	-1.27	KEGG
lum	6.00	KEGG
gsdf	6.68	KEGG
ifng1-2	-4.86	KEGG
igf3	4.57	Selected
insl3	4.56	Selected
ptges3b	-26.40	Selected
gpx3	5.48	Selected
fbxo15	-1.74	Selected
pak6b	2.20	Selected
anp32b	-5.96	Selected
scube1	3.25	Selected
ndrg2	2.10	Selected
ndrg3a	1.85	Selected
tpd52l1	1.97	Selected
mmel1	4.34	Selected
tsnaxip1	1.75	Selected
dcun1d2	1.83	Selected
slc12a2	1.90	Selected

Fig. 4. Protein-protein interaction map for Fsh-targeted candidates. The interaction network was generated using STRING database v10 (default settings; medium confidence of 0.4). A total number of 65 candidate genes (50 previously identified by Enrichment Map and KEGG pathway analyses plus 15 additional genes selected; right panel) was considered for the analysis. Genes in bold letter type denote an interaction association for their corresponding proteins found by STRING analysis. Proteins are represented as nodes, highlighted by a different color after hierarchical clustering, and lines indicate associations based on known functional interactions (see left bottom corner). Light grey lines represent predicted interactions (line thickness indicates the level of confidence of the association, reported for putative homologs). The network is significantly enriched in interactions ($P < 0.01$).

functionally associated. Hierarchical clustering displayed interacting proteins in particular groups (indicated by different colors), demonstrating that Hedgehog (Dhh and Lrp2a) and Tgf-β signaling (Bmp7b, Inha, Inhbab, Amh) pathways formed a clearly delineated network targeted by Fsh (in red; Fig. 4). This analysis suggests an important role for Dhh, potentially connecting different signaling pathways in Fsh-regulated zebrafish spermatogenesis. Other protein groups identified within the network contained factors with known functions in the fish testis (Igf3, Nóbrega et al., 2015; and Gsdf, Sawatari et al., 2007) and kinases (Stk38l, Pak6b and Musk), as well as proteins related to fatty acid metabolism (Acadl, Acadm, Rxrab, Aldh2.2) and structural proteins (Col18a1, Col4a5, Neb, Myl3) (Fig. 4). Using the human orthologues of the 65 DEGs as input provided a more extended version of the protein-protein interaction network (Supplemental Fig. 4), with an extended main group (in red) that includes next to Hedgehog/TGF-β members also several proteins of interest in the context of testicular function such as INSL3, LFNG, CFTR, DMRT2, REN and SLC12A2.

3.3. Characterization of candidate gene expression behavior in the zebrafish testis: steroid influence on Fsh-mediated effects

Since RNAseq data were obtained by examining the Fsh-mediated re-start of spermatogenesis in testis tissue obtained from an androgen insufficiency model, we asked if the expression of some of the Fsh-target genes also would be androgen responsive. Using tissue from males not exposed to E_2 *in vivo*, we observed a clear increase in the expression of *insl3* and *igf3* in response to Fsh, irrespective of blocking steroid production by trilostane or not (Fig. 5A and B). A second group of genes, *lfng*, *dhh*, *amh*, and *ptges3b* showed Fsh-modulated expression only in the absence of steroids (Fig. 5A and B). Conversely, Fsh regulated a third gene group (*inha*,

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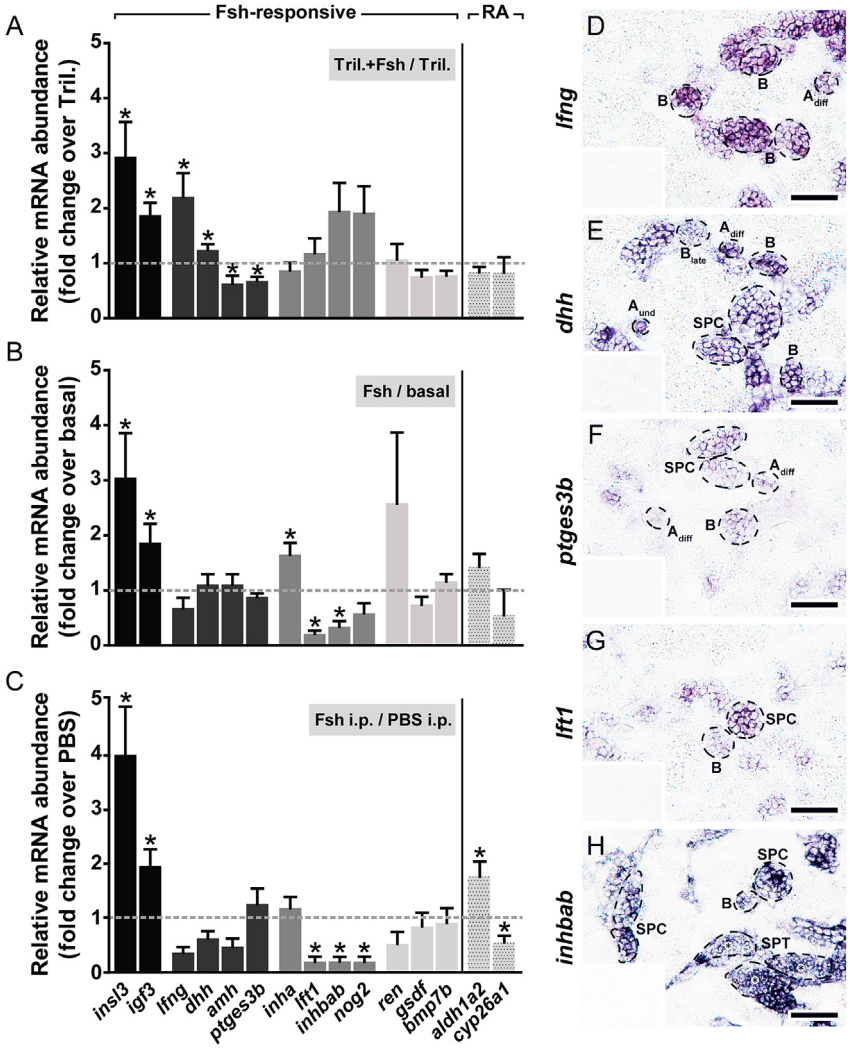

Fig. 5. Expression behavior and mRNA localization of candidate genes modulated by Fsh in zebrafish testis. The mRNA abundance of selected genes identified using RNAseq approach was investigated by qPCR in testes cultured for 48 h in the absence or presence of Fsh (100 ng/mL) and co-incubated **(A)** or not **(B)** with 25 μg/mL trilostane (Tril.), as well as in testes collected 4 h after intraperitoneal (i.p.) injection of Fsh (100 ng/g body weight) **(C)**. In addition, the expression of 2 retinoic acid (RA)-related enzymes was analyzed (separated by a vertical line). Adult males considered for this set of experiments were not pre-exposed to E_2 *in vivo*. Data are shown as mean of fold change ± SEM (n = 7–12) and expressed relative to the control condition, which is set at 1 (dashed line). Asterisks indicate significant differences between treated and control groups ($P < 0.05$). **(D–H)** *In situ* localization of candidate mRNA expression in zebrafish testis. Representative types of germ cell cysts are encircled and labeled. No specific staining was observed with sense cRNA probes (insets). A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; B, type B spermatogonia; SPC, spermatocytes; SPT, spermatids (indicated by an inner asterisk in panel H). Scale bar represents 25 μm.

lft1, *inhbab*, *nog2*) only in the presence of steroids (not significant for *nog2*; Fig. 5A and B), while some of the genes analyzed (*ren*, *gsdf*, *bmp7b*) did not respond to Fsh under these experimental conditions (Fig. 5A and B). Enrichment of aldehyde dehydrogenase activity (involved in retinoic acid [RA] metabolism) was retrieved by functional analysis (Fig. 2) but there was no clear Fsh-mediated modulation of RA signaling genes in our RNAseq data. Here, we determined mRNA levels of RA producing and degrading enzymes (*aldh1a2* and *cyp26a1*, respectively). However, no clear effects of Fsh in modulating the mRNA abundance of those genes were found after 2 days of tissue culture (Fig. 5A and B).

Very similar results compared to the *ex vivo* conditions were obtained *in vivo* regarding Fsh effects on *insl3* and *igf3* transcript levels but also on effects mediated by the steroidogenic activity of Fsh on *lft1*, *inhbab* and *nog2* (compare Fig. 5B and C). This is in line with the observation that intraperitoneal (i.p.) injection of Fsh increased androgen plasma levels and steroidogenesis-related gene expression (García-López et al., 2010) (Fig. 5C). In the *in vivo* experiment, RA-related enzymes were significantly regulated by Fsh, with *aldh1a2* up- and *cyp26a1* being down-regulated, suggesting elevated RA signaling in response to Fsh (Fig. 5C). The difference in time (sample collection 4 h after i.p. Fsh injection versus two days after adding Fsh to *ex vivo* cultures) may have contributed to different results in the case of RA signaling but potentially also regarding *inha*.

In the light of trilostane-dependent (i.e. absence versus presence of 3-keto,delta-4 steroids) differences in Fsh-mediated testicular gene expression, we tested directly the effects of 11-KT, the main androgen in fish, on the levels of selected transcripts following 2 days of tissue culture. The results confirmed direct inhibitory effects of androgens on *lfng*, *lft1*, and *inhbab* (Supplemental Fig. 5). Androgen effects on *amh* (Skaar et al., 2011; no effect) *insl3* (Assis et al., 2016; no effect), and *igf3* (Nóbrega et al., 2015; weak stimulation) transcript levels have been published previously and were not analyzed here.

Next, we localized selected mRNAs by ISH (Fig. 5D–H) on

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sections from adult zebrafish testes. We selected *dhh* and *lfng* as members of the Hedgehog and Notch signaling pathways, *inhbab* and *lft1* as members of the Tgf-β pathway, and *ptges3b* as the most down-regulated gene. Other genes of interest had been studied already in adult zebrafish testis, such as *insl3* (Assis et al., 2016), *igf3* (Nóbrega et al., 2015), *amh* (Rodriguez-Mari et al., 2005), *inha* (Morais et al., unpublished results), *gsdf* (Chen et al., 2013; Gautier et al., 2011), or *aldh1a2* and *cyp26a1* (Rodriguez-Mari et al., 2013). In all cases (except *insl3* and possibly *cyp26a1*), the mRNAs were localized to the cytoplasm of Sertoli cells contacting germ cell cysts at different stages of development (Fig. 5D–H). However, with respect to *lfng* and *inhbab*, expression in germ cells (Fig. 5D and H) cannot be excluded and future work will have to clarify this question. Sense riboprobes did not show specific staining (insets of Fig. 5D–H).

3.4. Studies on zebrafish Insl3 as an Fsh-responsive factor regulating zebrafish spermatogenesis

Previous work identified *insl3* as Fsh-responsive gene (García-López et al., 2010) expressed by Leydig cells in adult zebrafish testis (Good-Avila et al., 2009). We also showed that human INSL3 promoted spermatogonial differentiation in zebrafish (Assis et al., 2016). The present results pinpointed *insl3* as one of the genes most robustly up-regulated by Fsh, so that we decided to produce and test recombinant zebrafish Insl3. Incubating adult zebrafish testes with recombinant zebrafish Insl3 (100 ng/mL) clearly increased the mitotic index of type A_{und} ($P < 0.001$) but not of type A_{diff} spermatogonia (Fig. 6A). Morphometric analysis of the areas occupied by these spermatogonia revealed, on the other hand, that Insl3 decreased the area occupied by A_{und} but increased the area occupied by type A_{diff} cells (Fig. 6B). In addition, changes in transcript levels in response to Insl3 were investigated, analyzing a set of genes including growth factors, RA-related and germ cell markers (Fig. 6C). From the four growth factors analyzed, only *gsdf* responded to Insl3 *ex vivo* for 7 days. Moreover, mRNA levels of the RA-degrading enzyme *cyp26a1* were suppressed in the presence of Insl3, as was *nanos2*, a marker for type A_{und} spermatogonia (Bellaiche et al., 2014). No effect was observed for a marker gene expressed by all type A spermatogonia (i.e. *piwil1*; Houwing et al., 2007).

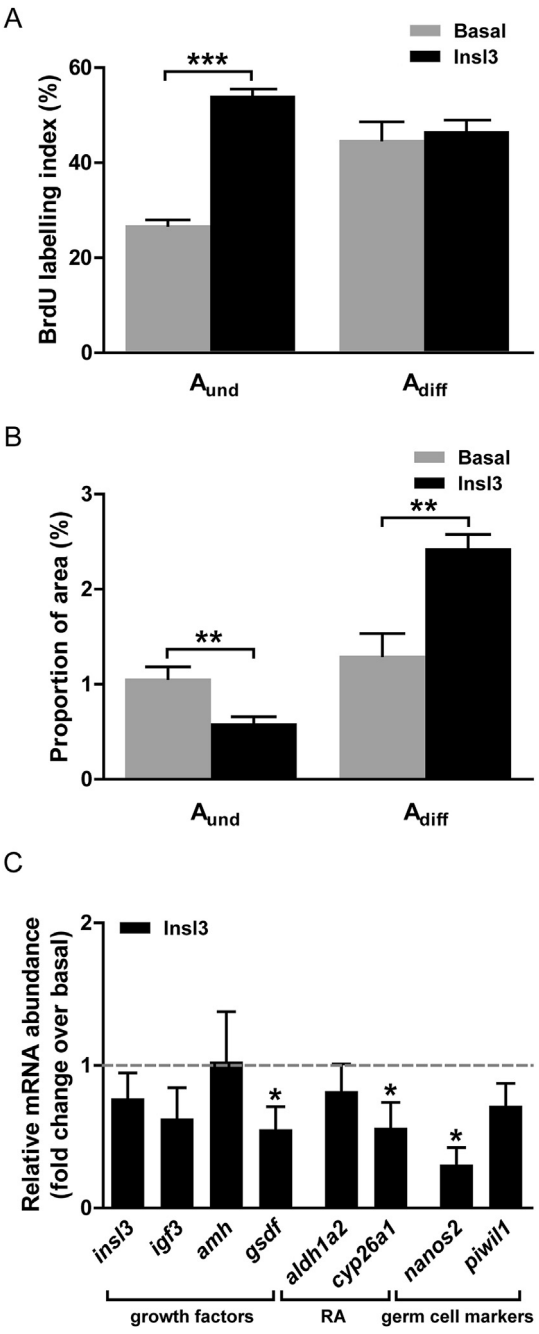

Fig. 6. Effects of recombinant zebrafish Insl3 on germ cell proliferation and testicular gene expression. (A) Quantitative determination of BrdU labeling index of type A_{und} and type A_{diff} spermatogonia in zebrafish testes cultured for 7 days in the absence or presence of 100 ng/mL Insl3. Data are shown as mean ± SEM (n = 6). ***, $P < 0.001$. **(B)** Volumetric proportion of cysts of type A (A_{und} and A_{diff}) spermatogonia upon incubation in the absence or presence of Insl3 (100 ng/mL). Data are expressed as mean ± SEM (n = 4). **, $P < 0.01$. **(C)** Expression of selected genes quantified by qPCR in testes cultured in the absence or presence of Insl3 (100 ng/mL). Data are shown as mean of fold change ± SEM (n = 12) and expressed relative to the control condition, which is set at 1 (dashed line). RA, retinoic acid-related. *, ($P < 0.05$).

4. Discussion

We describe transcriptional changes in zebrafish testis induced by Fsh. The gene expression profile includes signaling pathways mainly operating in Sertoli but also in Leydig cells (illustrated in Fig. 7). Previous studies reporting large-scale gonadotropin-induced changes of testicular gene expression in fish were carried out in rainbow trout using microarray technology and purified pituitary hormones from a related fish species (Sambroni et al., 2012, 2013). Here, we used homologous recombinant zebrafish Fsh and RNA sequencing to identify target genes. Our experimental model was designed to focus on somatic cell gene expression not related to steroid signaling, while concentrating on the mitotic phase of spermatogenesis. To our knowledge, this study is the first to present evidence in fish for the involvement of Hedgehog and Notch signaling pathways in Fsh-stimulated spermatogenesis, and provides further evidence for the involvement of Wnt and Tgf-β family members in the regulation of spermatogonial development. In addition, our *in silico* studies hint to substantial crosstalk among the signaling systems modulated by Fsh, with Hedgehog signaling potentially playing an integrative role.

Among the genes identified in this study, the expression of several Tgf-β family members (*inhbab*, *inha*, *lft1*, *bmp7b*, *nog2*, *amh*, *gsdf*) was modulated by Fsh. Activin and inhibin are dimeric proteins first described as reproductive hormones with opposite effects on FSH secretion (Ling et al., 1986). Apart from stimulating FSH release, activin A (INHBA; homodimer composed of two identical β_A subunits) is an important regulator of Sertoli cell function in the mouse testis throughout development. *Inhba* knock-out mice show

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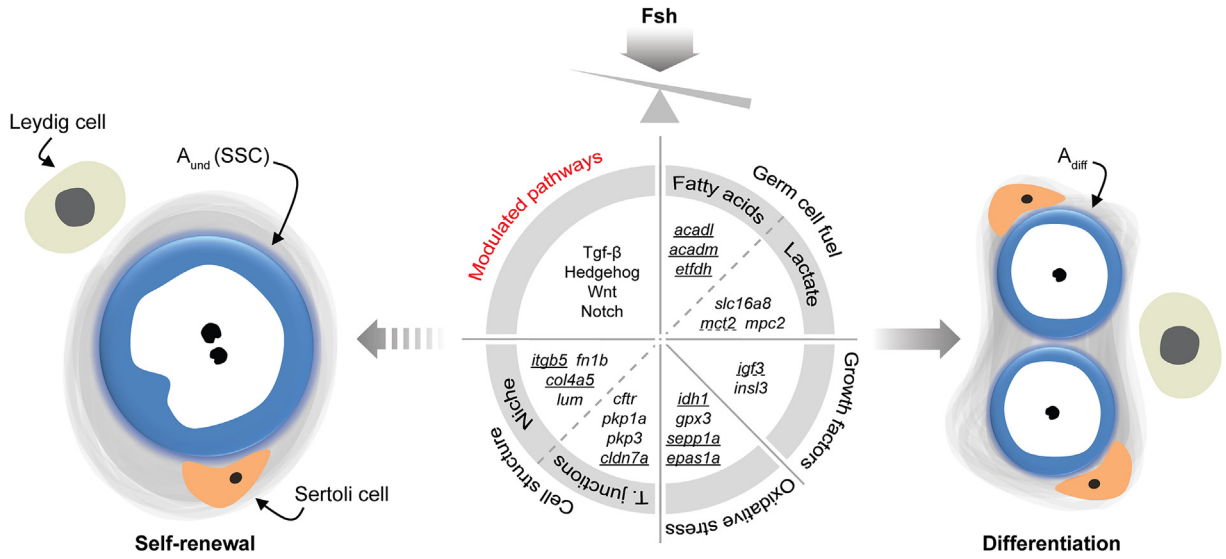

Fig. 7. Schematic illustration showing main genes/pathways involved in Fsh-stimulated restoration of spermatogenesis. SSC, spermatogonial stem cell; T. junctions, tight junctions. Solid and dashed underline indicate Sertoli cell specific and enriched expression, respectively.

smaller testes and a reduced Sertoli cell number at birth (Mendis et al., 2011), suggesting that activin establishes the normal Sertoli cell number until birth. Postnatal Sertoli cell proliferation ceases at puberty, which is accompanied by a dramatic decrease of activin A levels (Barakat et al., 2008). This decrease is necessary for establishing adult Sertoli cell structures and functions, including the blood-testis-barrier (BTB), as shown by activin overexpression in the adult mouse testis (Nicholls et al., 2012). Activin and inhibin bind to the same transmembrane type II receptors (ACVRIIA or ACVRII2B). Enhanced binding of inhibin to type II receptors, via the co-receptor betaglycan, antagonizes activin signaling (Lewis et al., 2000). Inhibin heterodimers are formed by an α subunit and a β_A or β_B subunit, constituting INHA or INHB, respectively. Our *ex vivo* results showed that *inhbab* transcripts were down-regulated by Fsh, whereas the expression of *inha* was stimulated. Interestingly, this pattern disappeared after addition of trilostane, suggesting an androgen-mediated regulation that was confirmed in our experiments for *inhbab ex vivo* and *in vivo*, similar to what has been reported in rainbow trout previously (Rolland et al., 2013). These observations suggest that in the presence of low androgen levels (such as before puberty, in our insufficiency model, or when trilostane is present) Fsh increased *inhbab*, while its transcript levels become down-regulated via Fsh-stimulated androgen production after puberty. The situation with *inha* appears more complex. Its acute Fsh stimulation in normal adult testis seems to depend on steroids, though not on androgens. We propose that the Fsh-triggered, androgen-mediated shift to a reduced activin/elevated inhibin tone may represent a mechanism to facilitate germ cell development in the adult zebrafish testis, potentially via promoting Sertoli cell differentiation.

The expression of lefty1 (*lft1*), an extracellular antagonist of nodal (another ligand of ACVRIIA/ACVRII2B), was consistently decreased by Fsh, mediated by androgens. In humans, LEFTY is a TGF-β signaling inhibitor highly expressed by different stem cell populations (Tabibzadeh and Hemmati-Brivanlou, 2006). Since nodal signaling promotes stem cell self-renewal in various tissues (Pauklin and Vallier, 2015), down-regulation of *lft1* by Fsh would facilitate nodal signaling, which might promote stem cell self-renewal.

Members of the Bmp branch of the Tgf-β superfamily, were also identified in our study, e.g. Fsh increased *bmp7b* transcript levels. However, this was only observed when re-starting previously interrupted spermatogenesis. In mice, *Bmp7b* is expressed in spermatogonia and spermatocytes in the pubertal testis, and deletion of one allele aggravates the degenerative spermatogenesis phenotype when crossed with *Bmp8a* null mice (Zhao et al., 2001). In zebrafish, an inactivating mutation in the type IB Bmp receptor *alk6b* leads to over-proliferation of undifferentiated spermatogonia and testicular tumors (Neumann et al., 2011), suggesting that Bmp signaling via this receptor limits self-renewal and promotes spermatogonial differentiation. Hence, after E_2-induced interruption of spermatogenesis in a testis enriched in type A spermatogonia, Fsh might promote germ cell differentiation via Bmp7b signaling. Though differential expression of *bmp7b* was not observed in normal adult testis, mRNA levels of the Bmp antagonist noggin2 (*nog2*) were down-regulated by Fsh when steroid production was not inhibited, potentially opening another route to facilitate Bmp signaling.

A fish-specific Tgf-β member is the Sertoli cell-derived factor *gsdf* that showed, similar to *amh*, higher transcript levels in response to Fsh after E_2-induced interruption of spermatogenesis. In rainbow trout, recombinant Gsdf promoted spermatogonial proliferation (Sawatari et al., 2007), but no clear gonadotropin modulation of its transcript levels was found (Sambroni et al., 2012). Also in our experiments with testis tissue from adult males not pre-treated with E_2, Fsh did not change *gsdf* transcript levels. Still, *gsdf* expression decreased following incubation with Insl3, although *insl3* mRNA levels always were up-regulated by Fsh. Therefore, we propose that *gsdf* is not a direct (or close indirect) downstream target of Fsh or Insl3. Since *gsdf* mRNA was localized to all Sertoli cells (Chen et al., 2013; Gautier et al., 2011), expression of this gene may be relatively stable, and changes in transcript abundance might reflect changes in Sertoli cell number. In fish and amphibians, showing the cystic type of spermatogenesis, Sertoli cell numbers increase in particular during the mitotic expansion of differentiating spermatogonial clones (Franca et al., 2015), which is induced by Fsh or Insl3 *ex vivo*. More work is required to clarify the regulation of expression as well as the bioactivity of Gsdf.

The up-regulation of *amh* transcript levels would suggest a signal favoring self-renewal over differentiating divisions (Miura et al., 2002; Morinaga et al., 2007; Skaar et al., 2011). This is in line with the observation that Fsh-induced spermatogonial

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proliferation activity not only involves pro-differentiation signals but also triggers expansion of the type A_{und} spermatogonial population and Sertoli cell proliferation (Fig. 1D and F). This is the basis for generating new spermatogenic cysts, equivalent to increasing stem cell niche space in the fish testis (Franca et al., 2015). Here, down-regulation of *lft1* (see above) and elevated *amh* may represent two signals promoting spermatogonial stem cell self-renewal and hence a balanced re-start of spermatogenesis after E_2-induced interruption that includes both, self-renewal and differentiation signals. Still, it is somewhat puzzling that previous work demonstrated Fsh-mediated down-regulation of *amh* transcript levels (Skaar et al., 2011). Interestingly, the latter study used testis tissue from males without E_2 pre-treatment *in vivo* when Fsh induced down-regulation of *amh* mRNA. Also in the present study, in experiments with testis tissue from untreated males Fsh decreased *amh* transcript levels (Fig. 5A), although the effect becomes more prominent when tissue is incubated for 5 or 7 days instead of the 2 days used in the present study (Nóbrega et al., 2015). We assume that the estrogen-induced drop of *amh* transcript to very low levels in fish (Filby et al., 2007; Schulz et al., 2007; Vizziano-Cantonnet et al., 2008), in combination with the Fsh-mediated re-start of spermatogenesis, involving also the proliferation of *amh* expressing Sertoli cells, explains the elevation of *amh* transcript levels.

Next to Fsh effects on the Tgf-β pathway, other signaling networks known from developmental processes were identified in the present study, such as Wnt, Notch and Hedgehog. *In vitro* studies in mammals have shown that WNT ligands directly support SSC self-renewal (Yeh et al., 2011, 2012). On the other hand, sustained activation of the WNT/β-catenin canonical signaling in Sertoli cells causes seminiferous tubule degeneration and infertility, preventing Sertoli cells from supporting meiotic and postmeiotic germ cells (Boyer et al., 2008, 2012; Tanwar et al., 2010). Similarly, knockdown of the canonical WNT inhibitor *Dkk3*, highly expressed at puberty, blocks Sertoli cell maturation (Das et al., 2013). We found an up-regulation of a paralog (i.e. *vwde*) of the mammalian WNT inhibitory factor *Wif1*. Decreased Wnt signaling would also follow Fsh-stimulated expression of *ndrg2* and *ndrg3a*, inhibitors of canonical WNT signaling during metastatic progression in mammals (Hwang et al., 2011; Liu et al., 2012). *Ndrg2* (NDRG family member 2) is also expressed in the adult rat testis and may be involved in germ cell differentiation and apoptosis (Hou et al., 2009). Sambroni et al. (2012) proposed that Wnt signaling in rainbow trout testes may be required for the quiescence and/or maintenance of SSCs. Our results in zebrafish confirm an involvement of the Wnt signaling system in the endocrine regulation of spermatogenesis in fish. Genetic evidence in mice suggests that WNT/β-catenin signaling from Sertoli cells promotes the differentiation of spermatogonial progenitor cells (Takase and Nusse, 2016), although this concept has been challenged in part recently (Chen et al., 2016). Future work will have to show the exact role of Wnt in zebrafish SSC proliferation/differentiation, including potentially distinct effects of canonical versus non-canonical signaling and the cellular localization of the signaling pathway members found to respond to endocrine stimulation.

The Notch-related factor *lfng* increased in response to Fsh when steroid production was inhibited. *Lfng* null males were subfertile, associated with cystic dilation of the rete testis (Hahn et al., 2009) and inhibition of Notch signaling *in vivo* leads to disrupted germ cell development in mice (Murta et al., 2014). The Notch modulator *lnx2a* was up-regulated in our results. While no function is known in the vertebrate gonad yet for this modulator, it is involved in the differentiation of various other cell types (Won et al., 2015; Yin et al., 2015; Zhou et al., 2015). Constitutive activation of Notch signaling in murine fetal Sertoli cells led to a premature differentiation of gonocytes (embryonic SSC precursors), associated with the precocious expression of Sertoli cell genes promoting germ cell differentiation, such as *Cyp26b1* and *Gdnf* (Garcia and Hofmann, 2013). In adult testes, NOTCH1 gain of function in germ cells results in failure of spermatogenesis due to an enhanced pro-apoptotic activity in differentiating spermatogonia (Huang et al., 2013). However, loss of Notch signaling in germ cells (Hasegawa et al., 2012) and/or Sertoli cells (Batista et al., 2012) seems dispensable for mouse spermatogenesis. Much is still to be learnt about the role of Notch signaling in spermatogenesis and the zebrafish may be a suitable experimental model in this regard.

Our data also indicate that the Hedgehog pathway is relevant for mediating the Fsh-stimulated restart of spermatogonial proliferation. Transcript abundance of *dhh* and *lrp2a* (an auxiliary Hedgehog receptor in mammals; McCarthy et al., 2002) was increased by Fsh in an androgen independent manner. In mice, Sertoli cell-derived *Dhh* is required for spermatogenesis and null mutant males lack Leydig cells, associated with a block of germ cell differentiation and male infertility (Bitgood et al., 1996; Clark et al., 2000). Components of the Hedgehog signaling are expressed in both the prepubertal and adult testis (Szczepny et al., 2006). Our *in silico* protein-protein interaction analyses, using DEGs of the present study as input suggested a central role for Dhh in the regulation of Fsh-stimulated spermatogenesis, by integrating crosstalk among signaling systems regulating the stem cell self-renewal/differentiation balance (e.g. Tgf-β, Notch, and Igf/relaxin signaling factors), as demonstrated in other organ systems (reviewed in Borggrefe et al., 2016).

Members of the Igf/relaxin family (e.g. *igf3*, *insl3*) raised our particular interest among the DEGs, as they mediate pro-differentiation effects of Fsh on germ cells in an androgen-independent manner. Recently, we have reported that Fsh-mediated Sertoli cell production of Igf3 promoted the proliferation of all types of spermatogonia but also of Sertoli cells (Nóbrega et al., 2015). Another gene showing a very robust response to Fsh is *insl3*, which occurred independently of androgens (García-López et al., 2010; present study). Zebrafish Leydig cells express the *fshr* (García-López et al., 2010) and *insl3* (Assis et al., 2016) genes, so that Fsh could directly stimulate Leydig cell Insl3 production, not excluding though the possible existence of a Sertoli cell-mediated, paracrine mechanism promoting Insl3 production. Without modulating basal or gonadotropin-stimulated androgen release, human INSL3 recruited type A_{und} spermatogonia into differentiating mitosis while decreasing the proportion of type A_{und} spermatogonia in adult zebrafish testes (Assis et al., 2016). Using recombinant zebrafish Insl3, we confirmed the findings made with the human growth factor as regards the proliferation and differentiation behavior of type A spermatogonia. Insl3 receptors also are expressed in the zebrafish testis (Good et al., 2012). Although the cellular site(s) of expression are still unknown for zebrafish Insl3 receptors, they have been localized to Leydig and to different germ cell types in mammals (Pitia et al., 2015). Therefore, direct effects of Insl3 on germ cells seem possible. Again, this does not exclude additional mechanisms. Here, we found that the pro-differentiation effect of Insl3 may be related to increased RA signaling, considering the decrease of *cyp26a1* (cytochrome P450, family 26, subfamily A, polypeptide 1) expression, the main RA degrading enzyme in zebrafish (Rodriguez-Mari et al., 2013). Also, transcript levels of the RA producing enzyme *aldh1a2* (aldehyde dehydrogenase 1 family, member A2) were increased by Insl3 after a 4 days long incubation period (Supplemental Fig. 6). In mammals, RA signaling is crucial for spermatogonial differentiation (Busada and Geyer, 2016; Griswold, 2016). Information regarding RA function(s) in fish spermatogenesis is limited but an inhibitor of Aldh1a2 enzyme activity in combination with a vitamin A (RA precursor)-depleted diet,

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impaired zebrafish spermatogenesis and reduced sperm counts and fecundity (Pradhan and Olsson, 2015). Thus, Fsh-triggered and Insl3-mediated differentiating proliferation of zebrafish spermatogonia may be mediated in part by RA signaling, involving elevated RA production but also decreased RA breakdown.

While *igf3* and *insl3* expression is not modulated by androgens, androgen signaling may still affect other Fsh-regulated processes. Therefore, we used an experimental model focusing on steroid-independent Fsh effects. Akin approaches have been used in mammals, for example by studying FSH effects in mice lacking a functional androgen receptor in all cells or in specific cell types in the testis (Denolet et al., 2006; O'Shaughnessy et al., 2010). Physiologically, however, Fsh also would activate steroid/androgen signaling in the adult fish testis. Therefore, we carried out in addition exploratory studies as those shown in Fig. 5A–C (expression of selected genes in the absence/presence of steroids) and Supplemental Fig. 5 (expression of selected genes in response to androgen). These studies revealed different response patterns among the selected genes, demonstrating the complexity of Fsh-targeted signaling in the piscine testis. Still, in view of this complexity, we excluded the steroid-mediated effects for our RNA sequencing approach in this study.

In addition to a range of growth factors, Sertoli cells also provide nutritional support for developing germ cells by synthesizing various metabolites such as carbohydrates and fatty acids. In the mammalian testis, glucose is converted to lactate by Sertoli cells which is then provided to neighboring germ cells via the MCT2 (monocarboxylic acid transporter 2) transporter, representing the central energy substrate during mammalian spermatogenesis (Rato et al., 2012). Moreover, certain fatty acids are required for normal spermatogenesis and fertility (Zadravec et al., 2011). Therefore, it was intriguing to find in our functional enrichment analyses genes involved in lactate transport and metabolism (*mct2* [also known as *slc16a7*], *slc16a8*, *mpc2*) as well as fatty acid metabolism (*acadm*, *acadl*, *etfdh*).

Another conserved feature of Sertoli cells is the formation of tight junctions that segregate the microenvironment for meiotic and postmeiotic germ cell differentiation (i.e. the BTB) (Cheng and Mruk, 2010). Here, we found that Fsh up-regulated tight junction components (e.g. *cldn7a*, *pkp1a*, *pkp3* and, almost significantly, *cx43*). In this regard, knock-down of FSH-induced cystic fibrosis transmembrane conductance regulator (CFTR; a cAMP-activated HCO_3^- channel) signaling in mice disrupted tight junctions between adjacent Sertoli cells, associated with an increase of the NF-kB/COX2/PGE_2 pathway (Chen et al., 2012). Interestingly, transcript abundances of *cftr* and a PGE_2 synthase *ptges3b* (as well as *nfkb2*) were up- and down-regulated by Fsh, respectively. Also, factors involved in the regulation of reactive oxygen species (ROS) homeostasis were stimulated by Fsh, including the antioxidant molecules *epas1a*, *sepp1a*, *gpx3* and *idh1*. EPAS1 (endothelial PAS domain protein 1a; or HIF2-α) may be a regulator of the Sertoli cell transcriptional program (Zimmermann et al., 2015), and loss of EPAS1 function led to male infertility in the context of a compromised BTB (Gruber et al., 2010). Indeed, regulation of oxidative stress represents a critical process during spermatogenesis in mammals in context with the high proliferation rate (Guerriero et al., 2014). Interestingly, antioxidant protective mechanisms may function in the fish testis as well since protection against ROS seems particularly high in type A_{und} spermatogonia (Celino et al., 2012).

We assume that the Fsh-triggered re-activation of spermatogenesis in our experimental system is implemented by a balanced stimulation of both self-renewal and differentiating proliferation. Although our approach was not designed to search for SSC markers, we did find genes encoding extracellular matrix components (*fn1b*, *lum*, *itgb5*, *col4a5*) considered as important for the stem cell niche (Chen et al., 2007; Ryser et al., 2011) as being up-regulated by Fsh.

5. Conclusion

The data presented here show that the Fsh-mediated restart of the spermatogonial development is accompanied by transcriptional modulation of several signaling systems, and that many of these genes operate in Sertoli cells. Moreover, Fsh supports spermatogonial proliferation by regulating metabolic processes (e.g. lactate and fatty acid metabolism) as well as extracellular matrix remodeling in the testis. Taken together, our results suggest that Fsh (in a steroid independent manner in our experimental model), orchestrates the complex network of transcriptional events leading to spermatogonial development by establishing a pro-differentiation environment (illustrated in Fig. 7) that involves modulating the activity of several major signaling pathways into regulation of early stages of spermatogenesis, apparently an evolutionary old feature of vertebrate spermatogenesis.

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Disclosure statement

The authors have nothing to disclose.

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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.2016.08.033.

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