Endocrine and local signaling interact to regulate spermatogenesis in zebrafish: follicle-stimulating hormone, retinoic acid and androgens

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ABSTRACT

Retinoic acid (RA) is crucial for mammalian spermatogonia differentiation, and stimulates Stra8 expression, a gene required for meiosis. Certain fish species, including zebrafish, have lost the stra8 gene. While RA still seems important for spermatogenesis in fish, it is not known which stage(s) respond to RA or whether its effects are integrated into the endocrine regulation of spermatogenesis. In zebrafish, RA promoted spermatogonia differentiation, supported androgen-stimulated meiosis, and reduced spermatocyte and spermatid apoptosis. Follicle-stimulating hormone (Fsh) stimulated RA production. Expressing a dominant-negative RA receptor variant in germ cells clearly disturbed spermatogenesis but meiosis and spermiogenesis still took place, although sperm quality was low in 6-month-old adults. This condition also activated Leydig cells. Three months later, spermatogenesis apparently had recovered, but doubling of testis weight demonstrated hypertrophy, apoptosis/DNA damage among spermatids was high and sperm quality remained low. We conclude that RA signaling is important for zebrafish spermatogenesis but is not of crucial relevance. As Fsh stimulates androgen and RA production, germ cell-mediated, RA-dependent reduction of Leydig cell activity may form a hitherto unknown intratesticular negative-feedback loop.

KEY WORDS: Retinoic acid, 11-ketotestosterone, Spermatogenesis, RNA sequencing, Zebrafish

INTRODUCTION

All-trans retinoic acid (RA) is crucial for mammalian spermatogenesis (Griswold, 2016). In mice, RA action, usually mediated by RA receptor (RAR) γ (Gely-Pernot et al., 2012; Ikami et al., 2015; Lord et al., 2018), is required for spermatogonia to differentiate (Busada and Geyer, 2016). A vitamin A (RA precursor)-deficient diet arrests spermatogonial differentiation (McLean et al., 1995), which is reversed by retinoid supplementation (Morales and Griswold, 1987; van Pelt and de Rooij, 1990). RA also stimulates Stra8 (stimulated by retinoic acid 8) gene expression in the testis (Endo et al., 2017, 2015). However, although Stra8 is required for meiosis, recent genetic studies suggest that its expression does not depend on RA (Teletin et al., 2019). Moreover, STRA8-independent pathways fine-tune RA action in the testis, as shown for SALL4 (spalt-like transcription factor 4; Gely-Pernot et al., 2015) and REC8 (REC8 meiotic recombination protein; Koubova et al., 2014). Dominant-negative mutant models of RARα have allowed further characterization of its essential molecular functions for germ cell development (Chen et al., 2016; Hasegawa and Saga, 2012).

It is not clear whether the RA dependency of mammalian spermatogenesis represents an evolutionary conserved feature, as little is known in this regard in non-mammalian vertebrates. stra8-like genes were lost during the evolution of the Acanthomorpha and Cypriniformes fish lineages, including a number of model species [e.g. zebrafish (Danio rerio) and medaka (Oryzias latipes)]. Although stra8 was identified in other fishes (Pasquier et al., 2016; Rodriguez-Mari et al., 2013), functional studies in the Southern catfish (Silurus meridionalis) showed that entry into meiosis is a stra8-sensitive but not stra8-dependent process (Dong et al., 2013; Li et al., 2016). On the other hand, in species lacking stra8, RA nevertheless promoted meiotic initiation (Adolfi et al., 2016; Feng et al., 2015). In zebrafish, chemical inhibition of RA production reduced sperm production/fecundity (Pradhan and Olsson, 2015), but the males remained fertile.

In addition to RA signaling, other signaling systems contribute to the regulation of spermatogenesis. Androgens, which are produced by Leydig cells, are crucial for mammalian spermatogenesis (McLachlan et al., 2002; Sharpe et al., 1992), and promote germ cell development in GnRH (gonadotropin-releasing hormone)-deficient mice (Singh et al., 1995) and hypophysectomized rats (El Shennawy et al., 1998). Sertoli cell-specific androgen receptor (Ar) knockout led to meiotic arrest and the absence of spermatids (De Gendt and Verhoeven, 2012). Testosterone also promoted spermatogonial differentiation by suppressing the Sertoli cell production of WNT5A that would otherwise stimulate spermatogonial stem cell (SSC) self-renewal (Tanaka et al., 2016). On the other hand, testosterone stimulated myoid cells to produce glial cell-derived neurotrophic factor (GDNF), in turn promoting SSC self-renewal (Chen et al., 2014). Hence, androgen signaling is important for the mitotic, meiotic as well as spermiogenic phases of mammalian spermatogenesis.

In teleost fish, 11-ketotestosterone (11-KT) is the main androgen (Borg, 1994) and its stimulatory effect on testicular gene expression (Martinovic-Weigelt et al., 2011; Rolland et al., 2013; Sambroni et al., 2013) or spermatogenesis has been described in different species (de Castro Assis et al., 2018; Leal et al., 2009b; Miura et al.,
RESULTS
Gene expression profiling during spermatogenic recovery from busulfan treatment

To investigate which signaling systems are modulated during the initial stages of spermatogenesis, we interrupted the process experimentally, using the cystostatic agent busulfan in combination with elevated water temperature, and then allowed spermatogenesis to recover spontaneously from surviving stem cells (Nóbrega et al., 2010) (Fig. S1A-B). Subsequently, we analyzed testis tissue morphologically and by RNA-sequencing (RNAseq) in control (before treatment), depleted (maximum germ cell loss) and recovering (enriched in spermatogonia) testes. Maximum germ cell depletion occurred 10 days post-injection (Fig. S1Bii). Most spermatogenic tubuli contained only Sertoli cells and very few type A und spermatogonia (Fig. S1, inset in Biii). Most spermatogenic tubuli contained only Sertoli cells and very few type A und spermatogonia (Fig. S1, inset in Biii).

In depleted testes, containing only surviving A und spermatogonia as germ cells, marker gene expression for SSCs and self-renewal factors (Hammoud et al., 2014) was weak, while expression of these genes decreased in recovering testes containing more and also other germ cell types (Fig. S1C). In contrast, markers for differentiating spermatogonia were reduced in germ cell-depleted and elevated in recovering testes (Fig. S1C). Comparison of the mRNA profiles of untreated and germ cell-depleted testes revealed that two-thirds of the differentially expressed genes (DEGs) were downregulated in depleted testes (1940 of 2934, respectively; Fig. S2A). Similarly, the majority of KEGG terms significantly enriched in germ cell-depleted testes were downregulated (Fig. S2A), including pathways related to cell cycle and meiosis, as well as others involved in developmental processes (i.e. Jak-STAT, Hedgehog, ErbB or mTOR signaling). Interestingly, among the minority of upregulated KEGG terms, retinol metabolism (e.g. aldh1a2 and cyp26b1) and steroid hormone biosynthesis were identified. Most of the significantly modulated signaling pathways in germ cell-depleted testes were also observed in recovering testes but followed the opposite expression pattern (Fig. S2A-B), and the number of upregulated DEGs exceeded the downregulated ones (2344 and 1613 or 59.2% and 40.8%, respectively; Fig. S2B).

Comparing recovering and control testes provided the much lower number of 442 DEGs (Fig. 1C), most of which (371 or 83.9%) were upregulated. Similarly, all KEGG pathways significantly affected were upregulated (Fig. 1E), including TGFβ, steroid hormone, GnRH, PPAR, MAPK signaling systems, as well as extracellular matrix (ECM) structure and remodeling-related pathways (such as focal adhesion, cell-adhesion molecules and ECM-receptor interaction). When searching for common and specific DEGs, 123 genes were found in both datasets and 3834 or 319 specifically regulated during recovery with respect to the depleted or control conditions, respectively (Fig. 1D). Examining the biological functions enriched among 442 recovery-regulated genes by GO analysis showed numerous overlapping gene sets (sharing a similar function/GO identifier) that were grouped in eight different clusters (encircled in Fig. 1F). One-hundred and thirty-one DEGs form the main cluster (Developmental process; Fig. 1F), which includes genes associated with organ development (e.g. clu, dcn, cx43, mmp2, krt4, cxcl12b and fabp7a), cell differentiation [e.g. tnfa, tfc23, ecsscr, sox18, ppdpla, Wnt-related (sfrp2, fzd9a and ndp)], growth factor activity [e.g. insulin-like (ins, igfbp7 and igf5), TGFβ (stilb, tghb and ltf2), and FGF (fgf1a, fgf10a and fgf12a) family members]. However, Gdnf or Gdnf receptor genes were not among the DEGs in this (recovery to control) or other comparisons, and expression levels in general were rather low (below 20 reads in most cases; data not shown but deposited in GEO under accession number GSE116611).

After demonstrating retinol metabolism and steroid hormone biosynthesis as pathways upregulated in depleted testes, it was remarkable to find retinoic acid signaling [e.g. crabp2b, rdh10a, rbp1a (rhop5) and rbp4] also among the enriched biological functions when comparing recovering and control testes (see developmental process cluster). Furthermore, the hormone metabolic process cluster contained several genes encoding steroidogenic enzymes with increased transcript levels (e.g. hsd3b1, hsd11b2 and cyp17a1). Taken together, gene expression profiling suggests that, among the signaling systems modulated in the zebrafish testis in response to busulfan treatment and recovery from it, retinoid metabolism/retinoic acid signaling is clearly represented, as is steroidogenesis.

Retinoid-mediated stimulation of spermatogonial development

To study the potential role of RA signaling system in zebrafish spermatogenesis, we examined first whether the gene expression data can be supported experimentally, using a previously established primary testis tissue culture system. Upon ex vivo exposure to the RA precursor retinol (RE), the area in testis sections occupied by type A und and B spermatogonia increased (Fig. 2A,B), and so did the proliferation activity of these germ cell types (Fig. 2D-E). Along with the morphological data, the mRNA levels of germ cell-markers increased in response to retinoid treatment (piwll1 and nanos2; Fig. S3A). Regarding retinoid metabolism, we found that aldh1a2 expression was exclusively stimulated by RE, while cyp26a1 mRNA levels increased in response to both, RE and RA, although more clearly to RA (Fig. S3A). Transcript levels of...
Fig. 1. Morphometric and transcriptomic analyses of zebrafish testis during spermatogenic recovery from a cytotoxic (busulfan) insult. (A,B) Qualitative (A) and quantitative (B) evaluation of the recovery of different germ and somatic cell types when compared with untreated control testes. Aund, type A undifferentiated spermatogonia; Adiff, type A differentiating spermatogonia (arrowheads); SPB, type B spermatogonia (arrows); SPC, spermatocytes; SPT, spermatids; SPZ, spermatozoa (dashed line); Leydig, Leydig cells (yellow background, including other interstitial cells). Data are mean±s.e.m. (n=5; *P<0.05, ***P<0.001). Scale bar: 25 µm. (C) Up (red)- and down (green)-regulated genes identified by RNAseq at the beginning of the recovery period (n=5, P<0.005, fold change [FC]≥3.0). (D) Common and specific recovery-associated genes retrieved when tested against the depleted (gray set) and control (white set) testis transcriptomes. (E) KEGG pathways modulated in recovering testes. Each pathway is highlighted in red (up-) or green (downregulated). (F) Functional enrichment of recovery-induced gene expression. Groups of closely related GO terms are encircled and labeled (numbers of regulated genes are shown). The groups labeled as ‘developmental process’ and ‘hormone metabolic process’ are outlined with dashed lines and examples of identified DEGs in those sets are shown.
selected growth factors were unaffected (amh, igf3 and insulin; data not shown). Consistent with the RE-induced increase in type Adiff and B spermatogonia (Fig. 2B), chemical inhibition of RA production by DEAB decreased the frequency of these spermatogonia in tissue culture (Fig. 2C). DEAB treatment increased germ cell apoptosis (Fig. 2C), while exposure to RE reduced the volume fraction of apoptotic cells (Fig. 2B). These data indicate that RA signaling promotes differentiating spermatogonia (Adiff and B) numbers by stimulating their proliferation. RA signaling also reduced germ cell apoptosis, but the changes in cell morphology associated with cell death (cell shrinkage and nuclear condensation/fragmentation) did not allow identification of the affected germ cell type(s).

**sall4 and rec8 mediate the effects of RA in the zebrafish testis**

To determine the mechanisms potentially mediating the effects of RA in the zebrafish, we tested the expression of sall4 and rec8 (a and b paralogs), components of Stra8-independent pathways that mediate RA signaling in mammals (Gely-Pernot et al., 2015; Koubova et al., 2014). RA and RE increased the transcript levels of sall4 and rec8 (Fig. 3A). Conversely, treatment with DEAB slightly decreased sall4 and rec8a transcript levels, an effect that was clearer when using testicular cell suspensions (Fig. 3A). We then examined whether knocking down sall4, rec8a or rec8b transcripts modulated germ cell marker transcript levels. Specific GapmeRs for sall4, rec8a and rec8b reduced target transcript levels, reduced the expression of piwi1l and dazl, marker genes in differentiating spermatogonia, and reduced expression of meiotic (sycp3) and post-meiotic markers (odf3b; Fig. 3B-D). Using testicular cell suspensions from Tg(vasa:EGFP) testes (Fig. 3E) sorted for EGFP-negative and -positive cells for qPCR analysis, we found (Fig. 3F) that sall4 transcripts were enriched in EGFP-positive cells, indicating its preferential expression in germ cells in general (see Fig. S3B for further details), probably starting in type A spermatogonia that preferentially express EGFP (Fig. 3E; whole testis).

A dominant-negative raraa transgene targeted to germ cells disturbs spermatogenesis

To obtain further evidence for the germ cell-specific role(s) of RA signaling, we generated transgenic zebrafish (named dn-raraa)
expressing a truncated form of RARα α (Raraa) in germ cells, in a similar way to that described for mice (Damm et al., 1993) and zebrafish (Kikuchi et al., 2011). Transgenesis efficiency was first analyzed in larvae at 3-4 days post-fertilization, based on EGFP-positive hearts and mCherry-positive germ cells in the genital ridge (Fig. 4A). Transgene expression was confirmed in adult males in both heart and testis tissue, where mCherry staining was found in spermatogonia and spermatocytes but not in haploid germ cells (Fig. S4A). Morphological evaluation of 6-month-old adult dn-raraa testes revealed clear defects in spermatogenesis, including...
Androgen and RA signaling interact to drive spermatogenesis

We found that hindering RA signaling in germ cells increased androgen production, implying that, via mechanisms awaiting clarification, consequences of RA signaling in germ cells dampen androgen production. This suggests that androgen and RA signaling interact to promote spermatogenesis. To study this, we first examined androgen effects alone. Testis tissue responded to 11-ketotestosterone (11-KT), the main teleost androgen, by an upregulation of several germ cell-marker genes (Fig. 5A,B). Morphometric evaluation showed that 11-KT slightly increased the proportion of $A_{\text{diff}}$ spermatogonia and more clearly of spermatids, while reducing the incidence of apoptosis (Fig. 5C). Inhibiting RA production reduced the stimulatory effect of 11-KT on four out of seven germ cell markers (Fig. 5D-E), and was reflected in reduced proportions of spermatocytes and spermatids, the latter just not reaching significance (Fig. 5F). Remarkably, the presence of 11-KT could not prevent the DEAB-induced increase in apoptosis (Fig. 5F). TUNEL analysis confirmed that inhibiting RA synthesis increased the incidence of DNA fragmentation, mainly in spermatids but also in spermatocytes that were identified by the shape and size of their DAPI-stained nuclei (Fig. 5G,H and Fig. 5S), despite the presence of androgens. This suggests that in the case where RA and 11-KT interact to reduce apoptosis, 11-KT would be upstream of RA signaling. Hence, blocking steroid synthesis should not modulate apoptosis in the presence of RE, which indeed was not the case (Fig. 6A). Testing whether androgens use RA signaling to modulate spermatogenesis, we found that short-term (4 days) ex vivo and long-term (5 weeks) in vivo exposure to androgen increased aldha1a2 transcript levels; cup26a1 transcript levels also increased, although not significantly in the in vivo experiment (Fig. 6C). One explanation for these observations is that androgens increase RA production. If true, then androgen effects on RA-sensitive gene expression should be modulated by blocking RA production. Accordingly, we found that DEAB reduced rec8 transcript levels in the presence of androgen (Fig. 6D). Blocking steroid production in the presence of RE, on the other hand, did not modulate RE-stimulated sal14 or rec6a transcript levels, confirming the notion that androgens are upstream of RA in this context.

Inhibiting steroid production in the presence of RE also provided evidence for a so far undetected steroid effect: $A_{\text{und}}$ spermatogonia accumulated in the absence of steroids (Fig. 6A) without affecting their proliferative activity (Fig. 6B). This suggests that, in zebrafish, steroid hormones stimulate the transition of $A_{\text{und}}$ to $A_{\text{diff}}$ spermatogonia. Considering that the RE-induced changes of sal14 and rec6a transcript levels remained unaffected when inhibiting steroid production by trilostane (TRIL; Fig. 6D), it seems unlikely that steroids modulate retinoid effects but may support RA production as suggested above (see Fig. 6C). Similarly, the observed accumulation of spermatocytes (Fig. 6A) suggests that steroid hormones stimulate spermatocyte development, which is in line with the androgen-induced increase in spermatid numbers (Fig. 5C).

Retinoil signaling contributes to Fsh-stimulated spermatogonial differentiation

Androgen action was supported by RA signaling (Fig. 5), and it is possible that androgens increased RA production (Fig. 6). As Fsh is a major regulator of androgen production in fish, we asked whether Fsh modulates testicular RA production and whether the effects of Fsh on spermatogenesis are modulated by RA signaling.

Blocking RA production reduced the Fsh-stimulated proliferation of A and B spermatogonia, decreased the frequency of type $A_{\text{diff}}$ germ cell-depleted areas, abnormal cystic organization and increased germ cell apoptosis (Fig. 4Bii-vi, Fig. 5B4). Quantitative evaluation of spermatogenesis showed relative smaller areas occupied by type $A_{\text{diff}}$ spermatogonia and spermatocytes. The category ‘Others’ (including empty areas and germ cell-depleted tissue), was also higher in 6-month-old adult $dn$-raraa testes (Fig. 4C). Unexpectedly, the germinal epithelium had largely recovered in 9-month-old adult $dn$-raraa males (Fig. 4Bii-viii, C). This took place despite the even higher level of transgene expression (Fig. 4F), which may reflect in part the recovery-associated increase in germ cell numbers expressing the transgene. Moreover, $dn$-raraa testes hypertextrophied at 9 months of age with a more than 100% increase in weight compared with wild-type siblings or 6-month-old transgenic compared with wild-type siblings or 6-month-old transgenic fish (Fig. 4D). We furthermore observed a high incidence of DNA damage/apoptosis among post-meiotic germ cells in 9-month-old transgenic males (Fig. 4Bix-xi). In addition, problems with respect to fertilization and even more so regarding embryo survival were associated with the overexpression of the raraa$_{DN391}$ transgene compared with wild-type males (Fig. 4E). These experiments started with 6-month-old males and were repeated two to four times per month until 9 months of age, with similar results throughout the 3-month period. Analysis of RA-related gene expression showed a consistent downregulation of rec8a in adult $dn$-raraa males and raraa$_{DN391}$ transgene mRNA levels were strongly enhanced when compared with wild type (Fig. 4F).

Transcript levels of steroid production/signaling genes (star, hsd3b1 and ar) were significantly higher, but only in the 6-month-old $dn$-raraa males (Fig. 4G). Similarly, plasma 11-KT levels were higher in 6- but not in 9-month-old transgenic compared with wild-type males (Fig. 4H). In addition, we found that the relative area occupied by Leydig cells and the expression levels of the Leydig cell-derived growth factor insl3 increased above wild-type levels (but not the myoid cell-specific tagln; Fig. 4G). Taken together, our observations suggest that the increased production of Leydig cell products, such as 11-KT and Insl3, as well as increased sensitivity to androgens, may promote the spermatogenic recovery in the absence of germ cell RA signaling. The recovery process had reached a new equilibrium at 9 months of age, presenting full spermatogenesis but still not normal considering the duplicated testis weight and high level of DNA damage/apoptosis; in addition, sperm quality remained poor.

**Fig. 3. Involvement of sal14, rec8a and rec8b in mediating RA effects in the zebrafish testis.** (A) Modulation of sal14, rec8a and rec8b mRNA levels in response to retinoids. Testicular explants were cultured for 4 days with RA, RE or DEAB (each at 10 µM). Data are mean fold changes ± s.e.m. (n=6-15; *P<0.05, **P<0.01) and are shown relative to the control condition, which is set at 1 (dashed line). DEAB effects were also tested using testicular cell suspensions cultured for 3 days in the absence or presence of 10 µM DEAB (n=3, technical replicates; *P<0.05). (B-D) Effects of Gapm-R (1 µM)-mediated knockdown of sal14, rec8b or rec8b after 4 days of tissue culture, on the targeted and selected germ cell-marker genes. Data are mean fold change ± s.e.m. (n=3, technical replicates; *P<0.05, **P<0.01) and are normalized to the Gapm-R control (Ctrl), which is set at 1. (E) A pooled testicular cell suspension was obtained from 20 Tg(vasa:EGFP) testes and subjected to FACS. Black and white arrowheads indicate EGFP+ and EGFP- cells, respectively. Localization of the vasa:EGFP signal by CLSM in a whole-mount preparation of adult testis shows preferential expression of EGFP in single germ cells and in small germ cell groups, demonstrating expression in type A$_{\text{und}}$ and A$_{\text{we}}$ spermatogonia. (F) Relative mRNA expression of sal14, rec8a and rec8b in FACS-sorted fractions. Data are shown as mean fold change ± s.e.m. (n=3, technical replicates; *P<0.05) and are expressed relative to the EGFP- condition, which is set at 1. Scale bars: 25 µm.
**Fig. 4.** See next page for legend.
Fig. 4. Inactivation of retinoid signaling in germ cells results in severe testicular defects. (A) Confirmation of transgenesis 3 days post-fertilization in zebrafish larvae microinjected as 1-cell stage embryos. EGFP and mCherry expression was detected in the heart (arrows) and genital ridge (broken lines) of dn-rara larvae, respectively. Scale bars: 200 µm in iii; 50 µm in iii. (B-D) Qualitative (B) and quantitative (C,D) analysis of spermatogenesis (C) and gonadosomatic (D) indices of wild-type and dn-rara males 6 and 9 months post-fertilization (data are means ± s.e.m.; n = 3-5; *P < 0.05, **P < 0.01). Scale bars: 25 µm. Red asterisks indicate germ cell-depleted tissue; black asterisk indicates an empty area. Myoid, myoid cells; others, germ cell-depleted tissue plus empty areas. In Bix-xi, detection of germ cell apoptosis/DNA damage by TUNEL is presented. TUNEL+ cells/cysts are shown in green and PI (propidium iodide) counterstain is in red. Arrowheads indicate isolated TUNEL+ cells among spermatoozoa in the tubular lumen; representative spermatid cysts containing several TUNEL+ cells are encircled with a dashed line. Scale bars: 25 µm. (E) Fertilization rate (gray bars) and embryo survival of fertilized eggs (black bars) from adult wild-type and dn-rara transgenic males. Mating was repeated every 7-14 days (data are means ± s.e.m.; n = 4-6; **P < 0.01, ***P < 0.001), and fertilization and survival recorded 2 and 24 h post-fertilization (hpf), respectively. (F) qPCR quantification of RA-related gene and raraα expression was detected in the heart (arrows) and genital ridge (broken lines) of dn-rara transgenic larvae. Data are mean fold-change±s.e.m. (n = 3 or 4; *P < 0.05, **P < 0.01) and are expressed relative to the wild-type group, which is set at 1. (G) Transcript levels of steroidogenesis-related (star, hsd3b1, cyp11a1 and ar), Leydig cell (insl3 and myoid cell (lag10)) genes in wild-type and dn-rara transgenic adult testes. Data are shown as mean fold-change±s.e.m. (n = 3 or 4; *P < 0.05, **P < 0.01) and are expressed relative to the wild-type group, which is set at 1 (dashed line). (H) 11-KT plasma mean fold-change±s.e.m. (n = 3 or 4; *P < 0.05).

and B (but not Aund) spermatogonia, and increased the incidence of apoptosis among germ cells (Fig. 7A,B). Interestingly, Fsh increased testicular RA production (Fig. 7C) and also the apoptosis among germ cells (Fig. 7A,B). Interestingly, Fsh increased testicular RA production (Fig. 7C) and also the increase of the area occupied by type Aund spermatogonia, reducing the apoptotic loss of spermatocytes and spermatids. Moreover, we present the first evidence in vertebrates for a regulatory link between the reproductive hormones Fsh and androgens, and RA production and action, potentially forming an intratesticular feedback loop to prevent testis hypertrophy (summarized schematically in Fig. 8).

Transcriptomic profiling identifies retinoid and steroid signaling pathway activation during germ cell recovery

Using an established experimental model, depletion of most germ cells resulted in spermatogenic tubules containing Sertoli cells and a small number of Aund spermatogonia, probably surviving SSCs, which fueled the spermatogenic recovery observed some days later. Depleted testis tissue was characterized by the transcriptional enrichment of the “Retinol metabolism” and of the “Steroid hormone biosynthesis” pathways, among others, the latter being consistent with the enhanced androgen production found previously in busulfan-depleted zebrafish testis (Nóbrega et al., 2010). Increased androgen signaling may have supported the spermatogenic recovery (Crowder et al., 2018; de Castro Assis et al., 2018; Tang et al., 2018) (Fig. 5).

Genes encoding SSC self-renewal factors expressed in Thy+ undifferentiated mammalian spermatogonia (Hammoud et al., 2014), were upregulated in depleted zebrafish testes (Fig. S1C). This gene set included colony stimulating factor 1 receptors (csf1r and csf1rb). Enhanced expression of Csf1 ligand (csf1a) followed during recovery (Table S1). In mice, macrophages, peritubular myoid and Leydig cells located adjacent to the seminiferous tubules secrete Csf1 (Potter and DeFalco, 2017), which promotes SSC self-renewal (Oatley et al., 2009) and – together with RA signaling – spermatogonia differentiation (DeFalco et al., 2015). We found a remarkable increase of differentiating spermatogonia during recovery, suggesting that after SSC regeneration, rapid and widespread differentiation of spermatogonia is the response of the zebrafish testis to the cytostatic insult, involving retinoid and steroid signaling. These coincident changes prompted us to examine experimentally retinoid/androgen signaling interactions.

Retinooids directly control spermatogonia differentiation in zebrafish

As in mammals (Chen et al., 2016; Endo et al., 2017; McLean et al., 2002), RA promotes spermatogonia differentiation in zebrafish. However, besides RA, androgens (de Castro Assis et al., 2018) and Fsh (Crespo et al., 2016) also stimulate spermatogonia differentiation in zebrafish. Yet the inhibitory effect of blocking RA production and the stimulatory effect of adding RE, were evident in the presence of Fsh (Fig. 7A-B,G-H). Moreover, the effect of RE in stimulating differentiating spermatogonia proliferation was evident in the absence of sex steroids (Fig. 7G, H). These observations demonstrate that RA signaling also has effects that are independent of Fsh or androgens. In addition, Fsh can create a growth factor environment with reduced Amh (Skar et al., 2011), and increased Wnt5a (Safian et al., 2018), Ins13 (Crespo et al., 2016) and Igfβ levels (Nóbrega et al., 2015) in which retinoids can further stimulate the differentiating proliferation of type A spermatogonia.

RA-stimulated spermatogonia differentiation in mice involves KIT (reviewed by Busada and Geyer, 2016). In zebrafish, we did not observe a modulation of Kit receptor transcript levels in response to RE or DEAB, in particular transcript levels of kitb (Fig. S6A,B), the receptor probably expressed by germ cells, as suggested by the changes in its transcript levels after busulfan treatment (Fig. S6E).

DISCUSSION

The initial stages of spermatogenic recovery after pharmaceutical germ cell depletion included activation of a transcriptional network with growth and differentiation factors, retinoid and androgen signaling. For the first time in fish, we have identified specific germ cell stages responding to RA signaling (stimulating the proliferation of differentiating spermatogonia, reducing the apoptotic loss of spermatocytes and spermatids). Moreover, we present the first evidence in vertebrates for a regulatory link between the reproductive hormones Fsh and androgens, and RA production and action, potentially forming an intratesticular feedback loop to prevent testis hypertrophy (summarized schematically in Fig. 8).
However, the previously described mouse RA-regulated germ cell factors SALL4 (Gely-Pernot et al., 2015; Hobbs et al., 2012) and REC8 (Koubova et al., 2014), may induce germ cell differentiation in zebrafish. Our gene expression studies on FACS-enriched germ cells suggest direct retinoid effects on these transcripts. In mice, the germ cell-specific expression of Sall4 is controlled by RARγ (Gely-Pernot et al., 2015) and genetic ablation of Sall4 in spermatogonia results in the loss of differentiating germ cells (Hobbs et al., 2012).

In zebrafish testis tissue, RA signaling increased sall4 transcript levels, and knockdown of sall4 decreased expression of germ cell-marker genes, in line with a reduction of differentiating spermatoocytes in rodents (Eijpe et al., 2003; Lee et al., 2003). Loss of Rec8 in mice results in high mortality rates and sterility in both sexes; in males surviving until adulthood, haploid germ cells...
are absent (Xu et al., 2005). As both isoforms of the rec8 gene are upregulated by retinoids, it is possible that Rec8 also mediates RA-stimulated germ cell development in zebrafish. Indeed, knockdown of rec8a and rec8b causes a significant decrease in germ cell-marker gene expression, suggesting that rec8 may mediate part of the effects of RA on zebrafish germ cells.

Although the relevance of RARγ has been established with respect to the differentiation of spermatogonia in adult mice (Gely-Pernot et al., 2012; Ikami et al., 2015), it has also been discussed whether the level of RARγ expression, or rather RA availability, is relevant for the RA-mediated differentiation of spermatogonia (Lord et al., 2018; Velte et al., 2019). The four Rar and six Rxr paralogs present in zebrafish are all expressed in testis tissue (see Lord et al., 2018; Velte et al., 2019). The four Rar and six Rxr paralogs present in zebrafish are all expressed in testis tissue (see GEO deposit with accession number GSE116611), but there is no information on the exact cellular location of these proteins.

**Germ cell-specific inhibition of RA signaling**

Our dominant-negative transgenic zebrafish model with a germ cell-specific inactivation of RA signaling showed partial germ cell depletion and increased apoptosis at 6 months of age. Spermatogonia differentiation, meiosis and the production of mature sperm still took place, resulting in a quite heterogeneous testis histology. This differs from the generalized block of spermatogonia differentiation found in mice using a similar transgenic approach (Chen et al., 2016). However, sperm quality was clearly compromised in transgenic zebrafish, with reduced fertilization rates and a high mortality of the (few) resulting embryos within the first 24 h of development. Interestingly, we also found higher numbers of interstitial cells, duplicated plasma androgen levels and elevated levels of steroidogenesis-related and androgen receptor transcripts. Hindering RA signaling in germ cells apparently leads, via mechanisms yet unknown and involving a spermatogenesis defect associated with partial germ cell loss, to an activation of Leydig cells. This, in turn, suggests that the consequences of RA signaling in germ cells normally dampen Leydig cell activity. However, the elevated Leydig cell activity in dn-raraa males may support the spermatogenic recovery that is achieved at the cost of testicular hypertrophy and elevated levels of DNA damage/apoptosis, while not improving the low sperm quality. Considering direct RA effects in germ cells, we assume that, in particular, rec8a plays an important role, as indicated by its consistent downregulation in the testes of dn-raraa transgenic fish. Taken together, our functional evidence shows that the germ cell-specific lack of RA signaling results in a clear spermatogenesis phenotype in young adults that partially recovers within 3 months, is associated with a transient Leydig cell over-activation and results in a hypertrophied testis still showing some abnormalities. In this regard, it is possible that the duplicated germ cell mass re-establishes germ cell-mediated dampening of Leydig cell activity in 9-month-old males.

**Androgens and retinoids jointly promote spermatogenesis**

Next to retinoids, androgens also stimulate vertebrate spermatogenesis (O’Shaughnessy et al., 2010; Verhoeven et al., 2010). In mice, Sertoli cell-selective knockout of the androgen receptor (SCARKO) blocked meiosis (De Gendt and Verhoeven, 2012), while its ubiquitous ablation (ARKO) also reduced spermatogonia numbers (Tan et al., 2005). 11-KT (the main androgen in fish) initiated spermatogonial differentiation (Melo et al., 2015; Miura et al., 1991). In zebrafish deficient for the androgen receptor, mutants showed clearly smaller testes than wild-type fish, although some spermatozoa still were produced (Crowder et al., 2018; Tang et al., 2018). In mice, meiosis and spermiogenesis depend on androgen signaling (De Gendt and Verhoeven, 2012; O’Shaughnessy et al., 2010; Verhoeven et al., 2010), unless Sertoli cells express a constitutively active FSH receptor (Oduwole et al., 2018). In zebrafish, androgen-induced increases in transcript levels of mitotic and meiotic germ cell markers were reduced by blocking RA production, which also elevated apoptosis among spermatocytes and, in particular, spermatids (Fig. 5). Blocking steroid production in the presence of RE, on the other hand, led to an accumulation of type A spermatogonia and spermatocytes (Fig. 6). This suggests that retinoid-stimulated production of type A spermatogonia and meiotic cells is complemented by androgen signaling that promotes spermatogonial differentiation and completion of meiosis. Therefore, we propose that androgens and retinoids regulate spermatogenesis in a complementary manner to guarantee efficient sperm production in zebrafish. Although an androgen/retinoid
interplay promoting spermatogonia differentiation is a new proposal, interactions between androgen and RA signaling have been demonstrated in other tissues/cell types (Li et al., 2002; Munetsuna et al., 2009; Ubels et al., 2003). In contrast to mammalian models, where preventing either androgen or retinoid signaling completely blocked spermatogenesis, removing one of these pathways in zebrafish still allows (some) sperm production to occur, albeit in a compromised manner.

In zebrafish, a remarkable effect of blocking retinoid signaling is increased apoptosis among spermatocytes and in particular spermatids, while spermatogonia differentiation is reduced but not blocked. In mammals, spermatogonia differentiation is completely blocked in the absence of RA signaling. Considering apoptosis, the meiotic block in ARKO mice is associated with increased germ cell apoptosis (De Gendt et al., 2004), but an androgen-independent role for RA signaling as regards germ cell apoptosis has not been reported so far. In zebrafish, inhibition of steroid production in the presence of RE has no effect on germ cell apoptosis, while the androgen-induced decrease of apoptosis (Fig. 6C) may reflect androgen support of RA production. Overall, our data suggest that 11-KT and RA jointly promote spermatogonia differentiation, while 11-KT (but not RA) also promotes the completion of meiosis, and
spermatogenesis is mediated by RA signaling, which leads to testicular failure and spermatogonia apoptosis in mammals. In contrast, in zebrafish, blocking RA signaling results in complete spermatogenic failure. Hence, RA and androgen signaling represent critical bottlenecks that cannot be bypassed physiologically. In zebrafish, on the other hand, loss of RA signaling in germ cells reducing androgen production, and the main stages of zebrafish spermatogenesis affected by RA (orange) and/or androgens (green), both of which are regulated by Fsh (black). The shaded areas surrounding germ cells represent Sertoli cell cytoplasm containing orange nuclei with nucleoli. RA (but not 11-KT) increases the efficiency of spermiogenesis by reducing the apoptotic loss of spermatids.

**Fsh increases RA production and uses retinoid signaling to stimulate spermatogenesis**

Fsh is an important hormone that also regulates spermatogenesis in zebrafish (Crespo et al., 2016). Its action in the fish testis is mediated by Sertoli and Leydig cells, both expressing the fshr gene (Chauvigné et al., 2012; García-López et al., 2009, 2010; Ohta et al., 2007), so that Fsh is a potent steroidogenic hormone (García-López et al., 2009, 2010; Mazón et al., 2014; Planas and Swanson, 1995) and modulates growth factor production (Crespo et al., 2016). We show here that another role of Fsh is to increase testicular RA production, thereby stimulating RA target gene transcript levels. Moreover, blocking RA production in the presence of Fsh reduced the differentiating proliferation of spermatogonia and increased germ cell apoptosis (Fig. 7A,B). Collectively, these observations indicate that the biological activity of Fsh in the zebrafish testis is, in part, mediated by RA signaling.

In conclusion, RA signaling is also of significant relevance for testicular physiology in zebrafish, with regard to both classic functions of germ cell development and androgen production. Particularly interesting is the observation that blocking RA signaling in germ cells activated Leydig cell steroidogenesis, suggesting that physiologically, RA signaling in germ cells dampens Leydig cell function. We also found that Fsh stimulates testicular RA production, which, when combined with previous findings that fish Leydig cells express the Fsh receptor (rendering Fsh a potent steroidogenic hormone), led us to propose a new intratesticular feedback loop (Fig. 8). This loop may have remained undetected so far in mammals. Alternatively, it may represent a specific adaptation in teleost fish, where Fsh is also a potent steroidogenic hormone, thereby preventing testicular hypertrophy. In mammals, blocking RA signaling leads to a complete failure of spermatogonia differentiation; blocking androgen signaling also results in complete spermatogenic failure. Hence, RA and androgen signaling represent critical bottlenecks that cannot be bypassed physiologically. In zebrafish, on the other hand, loss of RA signaling (this MS) or loss of the androgen receptor (Crowder et al., 2018; Tang et al., 2018), both disturb but do not completely block spermatogenesis, and compensatory mechanisms rescue at least some fertility. The concept therefore is that of a regulatory system using parallel regulatory routes to avoid bottleneck situations upon failure of a single system.
biological replicates per condition) have been deposited in the NCBI GEO database under the accession number GSE116611. Regulated KEGG pathways were determined using the KEGG Mapper tool (Wang et al., 2014). KEGG pathways represented by at least five differentially expressed genes (DEGs) and by the ratios of regulated genes (up- or down-, and vice versa) higher than five were considered for the analysis.

Functional enrichment analyses were carried out using a plug-in available at www.baderlab.org/Software/EnrichmentMap (Merico et al., 2011) for the Cytoscape network environment (Shannon et al., 2003). The Enrichment Map plug-in 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., 2009) was used to retrieve GO terms from the list of DEGs and exported as the input for each functional enrichment analysis.

**Testis tissue culture**

Using a previously established ex vivo culture system (Leal et al., 2009b), adult zebrafish testis tissue was incubated in the presence of various test compounds to investigate retinoid involvement in spermatogenesis, including retinoid acid (RA), retinol (RE, RA precursor), 4-diethylaminovalproylgender (DEAV, RA production inhibitor; Mu et al., 2013; Perz-Edwards et al., 2001) and BMS493 (pan-RA receptor antagonist; Stafford and Prince, 2002; Wang et al., 2016). All these compounds were produced from Sigma-Aldrich and used at the same final concentration of 10 µM. Additional ex vivo experiments were performed to test the effects of recombinant zebrafish Fsh (100 ng/ml; García-López et al., 2010) and 11-ketotestosterone (11-KT, 200 nM; Sigma-Aldrich). To exclude the potential effects of steroid hormones, incubations were carried out in the presence of trilostane (TRIL, 25 µg/ml; Sigma-Aldrich), which prevents the production of biologically active steroids. Moreover, an androgen insufficiency model was employed to obtain the results presented in Fig. 8E-G. Briefly, before collecting testis tissue for ex vivo culture, the fish were exposed to 10 nM 17-β-estradiol (E2; Sigma-Aldrich) in vivo for 3 weeks with a daily change of the E2-containing water. Using this approach, endogenous spermatogenesis is interrupted such that type A spermatogonia accumulate while the testes become depleted of type B spermatogonia, spermatocytes and spermatids (de Waal et al., 2009).

**Testicular cell suspension and culture**

To investigate the cellular localization of testicular sal14, rec8a and rec8b expression, we used testes from transgenic Tg(vasa:EGFP) zebrafish expressing enhanced green fluorescent protein under the control of the germ cell-specific vasa promoter (Krøvel and Olsen, 2002). Testis tissue from 2-10 transgenic fish was digested with 1 mg/ml collagenase/dispose solution at 27°C for 2 h with gentle shaking (Hinfray et al., 2013). The resulting cell suspension was filtered through a 70 µm filter and subsequently through a 40 µm filter (BD Bioscience) before resuspension in PBS (phosphate-buffered saline; pH 7.4) and the centrifugation at 500 rpm (50 g) for 10 min and the pellet stored at 4°C until use. Under some of the experimental conditions described in this study (testis tissue culture section), type A and type B spermatogonia proliferation activity was investigated by studying the incorporation of the S-phase marker bromodeoxyuridine (BrdU; 50 µg/ml, Sigma-Aldrich), which was added to the medium during the last 6 h of the culture period. After incubation, testis tissue was fixed at room temperature for 1 h in freshly prepared methacarn [60% (v/v) methanol, 30% chloroform and 10% acetic acid glacial; Merck Millipore] and processed for subsequent analysis. To quantify spermatogonial proliferation, the mitotic index was determined by examining at least 100 germ cells/cysts, differentiating between BrdU-unlabeled and unlabeled cells. To evaluate the proportion of area occupied by different germ cell types in 4% glutaraldehyde fixed tissue (4°C, overnight), 10-15 randomly chosen fields were photographed at ×40 magnification and the images were analyzed quantitatively using ImageJ software. With a specific plug-in, a 540-point grid was made to quantify the proportion of the area for the various germ cell types, based on the number of points counted over those germ cell types. For both purposes (BrdU incorporation and area analyses), testis tissue was dehydrated, embedded in Technovit 7100 (Heraeus Kulzer), sectioned at 4 µm. The germ cells/cysts were identified according to previously published morphological criteria (Leal et al., 2009a).

Additional ex vivo experiments and testicular cell suspensions were carried out to investigate candidate gene expression in response to different experimental conditions. For all these experiments, total RNA was isolated using the RNAqueous Kit (Ambion) following the manufacturer’s instructions. Relative mRNA levels of candidate genes were analyzed using real-time quantitative PCR (qPCR; see Table S2 for detailed primer information) as previously described (García-López et al., 2010). The geometric mean of eef1a1l1, rpl13a and ubc was used as housekeeping endogenous control owing to their constant expression under the conditions analyzed.

Furthermore, incubation medium was collected to quantify testicular RA release by enzyme immunoassay (commercial kit, MyBioSource). In parallel experiments, a blood sample was collected for 11-KT quantification, and levels were measured by enzyme immunoassay (commercial kit, Cayman Chemicals).

**Detection of DNA damage/apoptosis by TUNEL and quantification of apoptotic cells by morphological criteria**

To determine the incidence of cells showing DNA damage/apoptosis on paraffin-embedded tissue sections from testes previously incubated in the absence or presence of the test compounds, we used the deoxyuridine dioxygen dUTP nick-end labeling (TUNEL) approach. First, testis tissue was washed with PBS and subsequently fixed in 4% PBS-buffered paraformaldehyde (4°C, overnight). After a 30 min wash with PBS, testis tissue was dehydrated and embedded in paraffin wax. Sections (4 µm) were treated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 8 min. Finally, testis tissue was incubated with TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein; Roche) in the dark at 37°C for 1 h. After washing twice in PBS, sections were counterstained with DAPI mounted in Vectashield antifade mounting medium (Vector Laboratories) and analyzed by confocal laser scanning microscopy (Zeiss LSM 700). Negative and positive controls were included in each experimental set up (Fig. S5). The TUNEL technique has been used repeatedly to detect apoptotic cells in the testis of zebrafish (e.g. Saito et al., 2011) or mice (e.g. Jauregui et al., 2018), but can also detect DNA damage in necrotic degenerating cells (e.g. Loo, 2011). We therefore refer to TUNEL-positive cells as positive for DNA damage/apoptosis.

**In vivo exposure to androgen**

Adult male fish were submitted to water containing 100 nM 11-ketoandrostenedione (OA) or control untreated conditions in vivo for 5 weeks with a daily change of water (as described by de Castro Assis et al., 2018). Stock solutions were prepared in deionized water by extensive stirring at 37°C, which were further diluted to the proper concentration in aquarium water. Upon in vivo treatment, testis tissue was collected and used for gene expression analysis.

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Apoptotic cells were also quantified and analyzed using Technotiv 7100-embedded Toluidine Blue-stained 4 µm sections from glutaraldehyde-fixed testes previously incubated in the absence or presence of the test compounds. Cells were considered as apoptotic when they showed shrinkage, associated with losing at least partially the contact with the immediate tissue environment, combined with pyknosis/nuclear fragmentation (e.g. Elmore, 2007). The incidence of apoptosis was quantified using a 540-point grid, as described above for the different germ cell stages, and expressed as proportion of the total testicular section area.

**sal4, rec8a and rec8b knockdown by GapmeR technology**

sal4, rec8a and rec8b gene knockdown was investigated by using specific antisense oligonucleotides (LNA GapmeRs, Exiqon; design IDs 639263-1, 639292-1 and 639281-1, respectively). GapmeRs were dissolved in the incubation medium at a 1 µM final concentration and applied to ex vivo tissue culture conditions for 4 days. An unassisted GapmeR uptake method, called gynomix, has been previously described (Soifer et al., 2012; Stein et al., 2010). Three technical replicates for each gene were performed and knockdown efficiency was confirmed by qPCR analysis, and normalized to the negative GapmeR control.

**Statistical analysis**

GraphPad Prism 5.0 package (GraphPad Software) was used for statistical analysis. Significant differences between groups were identified using Student’s t-test (paired or unpaired, as appropriate) or one-way ANOVA followed by Tukey’s test for multiple group comparisons (*P<0.05; **P<0.01; ***P<0.001; ns, no significant changes observed). Data are mean±s.e.m.

No statistical method was applied to predetermine sample size. The number of wild-type and transgenic zebrafish males used was based on experimental considerations. For each experiment, sample size is indicated.

**Supplementary information**

The complete raw RNAseq data have been deposited in GEO under accession number GSE116611. Analyzed and filtered data are available in the article and its additional files.

**References**


