RESEARCH

PGE_2 inhibits spermatogonia differentiation in zebrafish: interaction with Fsh and an androgen

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Abstract

Changes in zebrafish testicular gene expression induced by follicle-stimulating hormone (Fsh) or anti-Mullerian hormone (Amh) suggested that Amh inhibition and Fsh stimulation of spermatogenesis involved up and downregulation, respectively, of prostaglandin (PG) signaling. We found that Sertoli cells contacting type A undifferentiated (A_{und}) and differentiating (A_{diff}) spermatogonia expressed a key enzyme of PG production (Ptgs2); previous work showed that Sertoli cells contacting A_{diff} and B spermatogonia and spermatocytes showed *ptges3b* expression, an enzyme catalyzing PGE_2 production. In primary testis tissue cultures, PGE_2, but not PGD_2 or $PGF_{2\alpha}$, reduced the mitotic activity of A_{diff} and their development into B spermatogonia. *Vice versa*, inhibiting PG production increased the mitotic activity of A_{diff} and B spermatogonia. Studies with pharmacological PG receptor antagonists suggest that an Ep4 receptor mediates the inhibitory effects on the development of spermatogonia, and cell-sorting experiments indicated this receptor is expressed mainly by testicular somatic cells. Combined inhibition of PG and steroid production moreover reduced the mitotic activity of A_{und} spermatogonia and led to their partial depletion, suggesting that androgens (and/or other testicular steroids), supported by PGE_2, otherwise prevent depletion of A_{und}. Androgens also decreased testicular PGE_2 production, increased the transcript levels of the enzyme-catabolizing PGs and decreased PGE_2 receptor *ptger4b* transcript levels. Also Fsh potentially reduced, independent of androgens, PGE_2 production by decreasing *ptges3b* transcript levels. Taken together, our results indicate that PGE_2, via Ep4 receptors, favors self-renewal in conjunction with androgens and, independent of Fsh and androgens, inhibits differentiating divisions of spermatogonia.

Key Words

- Fsh
- androgen
- prostaglandin E_2
- spermatogonia
- proliferation
- differentiation
- zebrafish

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Introduction

Prostaglandins (PGs) are low-molecular-weight (~350 Da), polyunsaturated lipophilic signaling molecules. They are derived from arachidonic acid, which can be liberated from membrane phospholipids by phospholipase A2 (PLA2) enzymes. For PG biosynthesis, arachidonic acid is metabolized by cyclooxygenase enzymes 1 and 2 (COX 1 and 2, a.k.a. PTGS (prostaglandin synthase) 1 and 2) to PGH_2, which is converted further by specific synthases, for example, PGD synthase (PTGDS) or PGE synthase (PTGES), to produce the main bioactive PGs (i.e. PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (Simmons *et al.* 2004, Ricciotti & FitzGerald 2011)). PGs develop biological activity by interacting with one of the prostanoid receptors, a group of G-protein-coupled membrane receptors showing five subtypes with in total nine receptors (D1 and 2, E1-4, FP, IP and TP (Breyer *et al.* 2001, Bos *et al.* 2004)).

PLA2, COX1/2 and PG-specific synthases as well as prostanoid receptors are expressed by different tissues and cell types. It is therefore not surprising that PGs influence in a paracrine and/or autocrine manner several physiological systems including the CNS, cardiovascular, gastrointestinal, excretory, respiratory, immune, reproductive and endocrine systems (Hata & Breyer 2004). The half-life time of PGs measures in minutes in mammals (Shrestha *et al.* 2012). Despite this short half-life time, there is a specific dehydrogenase inactivating PGs (HPGD). Its physiological relevance is exemplified by the several phenotypes that have been associated with mutations in this gene in humans (https://www.genecards.org/cgi-bin/carddisp.pl?gene=HPGD), highlighting the wide range of processes modulated by PGs.

Inhibitors of COX1/2 activities block the biosynthesis of all PGs and are known as non-steroidal anti-inflammatory drugs (NSAIDs) (Hata & Breyer 2004). Early *in vivo* experiments indicated that treatment with NSAIDs or different PGs modulated spermatogenesis in mammals (e.g. mouse (Abbatiello *et al.* 1975, 1976) and dog (Moskovitz *et al.* 1987)). Later studies in rat suggested that part of these effects reflect PG-mediated modulation of Leydig cell steroidogenesis, considering that PDG_2-promoted while $PGF_{2\alpha}$ suppressed androgen production (Frungieri *et al.* 2015). In rat, FSH stimulated Sertoli cell PG production (Jannini *et al.* 1994). In fetal mice, Sertoli cell-derived PGD_2 keeps spermatogonia in an undifferentiated state by autocrine stimulation of CYP26B1 activity that inactivates retinoic acid (Rossitto *et al.* 2015). Moreover, PGD_2 triggered changes in gene expression in spermatogonia, reduced their cell cycling activity and upregulated *Nanos2*, in turn preventing *Stra8* expression, which contributed to keep spermatogonia in an undifferentiated state in fetal mice (Moniot *et al.* 2014). PG effects on stem cells have also been described in certain malignancies, such as bladder cancer (Kurtova *et al.* 2015), where blocking PGE_2 production prevented cancer stem cell self-renewal proliferation between chemotherapy cycles. A role for PGE_2 in promoting stem cell production was also reported from non-malignant tissue, such as the hematopoietic stem cells in zebrafish (Choudhuri *et al.* 2017).

A number of reproductive processes in adult teleost fish have been reported to be influenced by PGs, such as sex steroid production (Wade & Van der Kraak 1993) or ovulation and reproductive behavior (Sorensen & Goetz 1993, Takahashi *et al.* 2018). With respect to an early life-stage during zebrafish sex differentiation, it is interesting to note that PGD_2 resulted in male-biased, PGE_2 in female-biased sex ratios (Pradhan & Olsson 2014). However, we are not aware of studies on the role of PGs in adult spermatogenesis in fish. In our studies on the endocrine and paracrine regulation of adult spermatogenesis in zebrafish, we have focused on the mitotic phase of spermatogenesis. This included gene expression analyses of zebrafish testis tissue exposed to recombinant hormones and growth factors (Crespo *et al.* 2016, Morais *et al.* 2017). We found that Fsh downregulated testicular *ptgs2a*, encoding Cox2a (Crespo *et al.* 2016), while this transcript was upregulated by Amh (Morais *et al.* 2017); Amh also upregulated the PGE receptor 4b transcript *ptger4b*, while Fsh decreased the expression of the PGE synthase *ptges3b* (Crespo *et al.* 2016). These data set suggest that Fsh (promoting spermatogenesis) downregulated PGE_2 signaling, while Amh (inhibiting spermatogenesis) promoted PGE_2 signaling. Our first experiments to test this assumption indeed showed that in the presence of Fsh, PGE_2 decreased the proliferation activity of type A differentiating (A_{diff}) and type B spermatogonia (Morais *et al.* 2017). Here, we report our follow-up studies on the role of PGs in zebrafish spermatogenesis. Our data suggest that PGE_2 production and signaling is part of the testicular regulatory network implementing the endocrine control of spermatogenesis and functions to prevent depletion of A_{und} spermatogonia while dampening both basal and Fsh- or 11-ketotestosterone (11-KT)-stimulated differentiation of spermatogonia.

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Materials and methods

Animals

Wild-type and transgenic *Tg(vasa:EGFP)* adult male zebrafish (*Danio rerio*) between 4 and 12 months of age were used in the present study. Animal housing 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, which were carried out under permission NVWA 10800.

Testis tissue culture

A previously established testis tissue culture system (Leal *et al.* 2009*b*) was used to investigate the involvement of prostaglandins and related compounds in zebrafish spermatogenesis including PGE_2, PGD_2, $PGF_{2\alpha}$, indomethacin (INDO), GW627368X (EP4 receptor antagonist) and PF04418948 (EP2 receptor antagonist; all purchased from Cayman Chemical) and used at a final concentration of 5 µM (except for INDO and PF04418948, which were used at 3 µM). Additional testis tissue culture experiments were carried out to investigate the interaction of PGE_2 with the androgen 11-ketotestosterone (200 nM (Skaar *et al.* 2011); Sigma-Aldrich) or recombinant zebrafish Fsh (100 ng/mL (Nobrega *et al.* 2015)). To study PGE_2 and/or Fsh effects in the absence of steroid hormones, incubations were carried out in the presence of trilostane (Tril, 25 µg/mL (Garcia-Lopez *et al.* 2010); Sigma-Aldrich), which prevents the production of biologically active steroids.

Morphometric analyses

To evaluate the proportion of area occupied by different germ cell types, testis tissue was fixed in 4% glutaraldehyde (4°C, overnight), dehydrated, embedded in Technovit 7100 (Kulzer), and 4 µm thick sections were stained with toluidine blue. Ten to fifteen randomly chosen, non-overlapping fields were photographed (Olympus AX70 microscope; ×400 magnification), and the images were analyzed quantitatively using a plugin of the ImageJ software, as previously described (Assis *et al.* 2016). The germ cell types analyzed (type A undifferentiated, A_{und}; A differentiating, A_{diff}; and B spermatogonia) were identified according to previously published morphological criteria (Leal *et al.* 2009*a*).

Immunohistochemistry (IHC)

After fixation of testis tissue from adult zebrafish in 4% paraformaldehyde and embedding in paraffin, 4 µm thick sections were prepared for fluorescent IHC. Briefly, slides were heated for 10 min in sodium citrate buffer pH 6.0 for antigen retrieval and subsequently cooled to room temperature for 30 min. After washing in PBS and blocking with 5% BSA (in PBS), slides were incubated with a primary goat anti-(human)COX2 (a.k.a. PTGS2) polyclonal antibody (Cayman Chemical; previously used in zebrafish (Feng *et al.* 2012)), diluted 1:100 in 1% BSA/PBS. Then, slides were incubated with the secondary antibody (chicken anti-goat IgG Alexa Fluor 488; Life Technologies) for 90 min. Propidium iodide (Sigma-Aldrich) was used as nuclear counterstain and slides were mounted with Vectashield H-1000 (Vector Laboratories). Testis sections were analyzed using a confocal laser scanning microscope (Zeiss LSM 700).

The proliferation activity of type A and B spermatogonia was investigated by quantifying the cell fraction incorporating the S-phase marker bromodeoxyuridine (BrdU; 50 µg/mL, Sigma-Aldrich), which was added to the medium for the last 6 h of tissue culture. 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. Testis tissue was dehydrated, embedded in Technovit 7100 (Heraeus Kulzer), sectioned at a thickness of 4 µm. BrdU immunodetection was carried out as previously described (Leal *et al.* 2009*a*). To quantify spermatogonial proliferation, the mitotic index was determined by examining at least 100 germ cells/cysts, differentiating between BrdU-labeled and unlabeled cells.

PGE_2 and 11-KT measurements by ELISA

The levels of PGE_2 produced by testis explants were analyzed directly in the incubation media using a commercial immunoassay (Prostaglandin E_2 ELISA Kit-Monoclonal; Cayman Chemical). The levels of 11-KT in the incubation media were analyzed by ELISA, as previously described (Cuisset *et al.* 1994). Acetylcholine esterase-labeled tracers and microplates pre-coated with monoclonal mouse anti-rabbit IgG were supplied by Cayman Chemicals. Anti-11-KT was a kind gift from David E Kime (Sheffield University, UK). All samples were analyzed in duplicate.

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Testicular gene expression

After tissue culture, testis tissue was frozen in liquid N and stored at −80°C until RNA isolation. Total RNA extraction, cDNA synthesis and real-time quantitative PCR (qPCR) were carried out as described previously (Nobrega *et al.* 2015). Data were normalized to *eef1a1l1* (eukaryotic translation elongation factor 1 alpha 1, like 1) due to its stable expression in all sample groups analyzed. All qPCRs were performed in 20 µL reactions and quantification cycle (Cq) values were obtained in a Step One Plus Real-Time PCR system (Applied Biosystems) using default settings. Relative mRNA levels were calculated as reported previously (Bogerd *et al.* 2001). Primers used in the qPCR analyses are listed in Table 1.

To investigate the cellular expression of Ep2a and Ep4b receptors in the testis, the transcript levels of *ptger2a* and *ptger4b*, respectively, were retrieved from an RNA sequencing (RNAseq) dataset available in our group and submitted to the NCBI GEO database (GSE116611).

Table 1 Primers used for gene expression studies by qPCR analysis.

Target gene	Gene description	Primer sequence (5′→3′)
amh	anti-Müllerian hormone	CTCTGACCTTGATGAGCCTCATTT
		GGATGTCCCTTAAGAACTTTTGCA
ar	androgen receptor	ACGTGCCTGGCGTGAAAA
		CAAACCTGCCATCCGTGAAC
cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1	GGGAGGCCACGGACTGTTA
		CCATGTGGAACTGTAGTCAGCAA
dazl	deleted in azoospermia-like	AGTGCAGACTTTGCTAACCCTTATGTA
		GTCCACTGCTCCAAGTTGCTCT
eef1a1l1	eukaryotic translation elongation factor 1 alpha 1, like 1	GCCGTCCCACCGACAAG
		CCACACGACCCACAGGTACAG
foxa2	forkhead box A2	GTCAAAATGGAGGGACACGAAC
		CATGTTGCTGACCGAGGTGTAA
gsdf	gonadal somatic cell derived factor	CATCTGCGGGAGTCATTGAAA
		CAGAGTCCTCCGGCAAGCT
hpgd	15-hydroxyprostaglandin dehydrogenase	GAGTAAAGAGTACGGAAAGCAAGGA
		GGTGAGGAGAATGGAGAAAAGCT
igf3	insulin-like growth factor 3	TGTGCGGAGACAGAGGCTTT
		CGCCGCACTTTCTTGGATT
insl3	insulin-like 3 (Leydig cell)	TCGCATCGTGTGGGAGTTT
		TGCACAACGAGGTCTCTATCCA
piwil1	piwi-like protein 1	GATACCGCTGCTGGAAAAAGG
		TGGTTCTCCAAGTGTGTCTTGC
ptger2a	prostaglandin E receptor 2a (subtype EP2)	CTGTGGTTCAAACGGCGTATTT
		ACACACCGCATGAGTCTTGCT
ptger4a	prostaglandin E receptor 4 (subtype EP4) a	GCGGAGATCCAGATGGTCAT
		TGGGTTTTTATCCAGACGCTTCT
ptger4b	prostaglandin E receptor 4 (subtype EP4) b	GTGCTCATCTGCTCCACTCCTT
		GCAGAGTTAAACAGCTGGTTCACA
ptges	prostaglandin E synthase	GCCAAGTGAGACTTCGGAAAAA
		AACTGCACACCTCCGTGTCTCT
ptgesl	prostaglandin E synthase 2-like	GCAATTCATGGGAGGTGATGA
		TCCATAACCCTCAGAACTCCAAAC
ptges3b	prostaglandin E synthase 3b (cytosolic)	GACAGCAAAGACGTGAAAGTAAATTTT
		CGGCTCCACTGAGACAGCTAA
ptgs1	prostaglandin-endoperoxide synthase 1	ATTCAATCTGAAACCCTACACATCCT
		CGTATAGTTCCTCCAGCTCTTTAGACA
ptgs2a	prostaglandin-endoperoxide synthase 2a	ACGCTGGAGGTTCAACACAAA
		CACCTGGACGTCCTTCACAAG
rpl13a	ribosomal protein L13a	GAGCCCCCAGCAGAATCTTC
		AGCCTGACCCCTCTTGGTTTT
star	steroidogenic acute regulatory protein	CCTGGAATGCCTGAGCAGAA
		ATCTGCACTTGGTCGCATGAC
ubc	ubiquitin C	CCATACACCGCACTCTTACAGAAA
		CCAGTCAGCGTCTTCACAAAGAT
wnt5a	wingless-type MMTV integration site family, member 5a	TGGAGATCGTGGACGCAAA
		CACTTCAGGAATCAGCAGAGGATT

Fw, forward; Rv, reverse.

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This set contains expression data from control testes, from germ cell-depleted testes following treatment with the cytotoxic agent busulfan, and from testes with spermatogenesis recovering from the busulfan treatment. A complete description of this data set, involving the three experimental groups, will be given elsewhere. The data – also used to examine testicular PG-related gene expression in the untreated control group shown in Table 2 – were generated, assembled and analyzed as previously described (Morais *et al.* 2017). Briefly, RNAseq sequencing libraries (five biological replicates per treatment) were prepared from 2 µg total RNA using the Illumina TruSeq RNA Sample Prep Kit v2 and sequenced on an Illumina HiSeq2500 sequencer (Illumina, Inc.) as 1 × 50 nucleotide reads. Data analysis was performed using the R/Bioconductor package DESeq.

To examine if *ptger2a* and *ptger4b* are expressed in somatic or in germ cells, we also used testes from transgenic *Tg(vasa:EGFP)* zebrafish expressing enhanced green fluorescent protein (EGFP) under the control of the germ cell-specific *vasa* promoter (Krovel & Olsen 2002). Testicular cell suspensions were prepared as described previously (Hinfray *et al.* 2013) and immediately submitted to fluorescence-activated cell sorting (FACS) using an in Flux cell sorter (BD Bioscience). EGFP-positive and -negative cells were collected, centrifuged in PBS at 100 ***g*** for 10 min and the pellet stored at −80°C until use for gene expression analysis by qPCR.

Statistical analysis

GraphPad Prism 5.0 package (GraphPad Software, Inc.) 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 (*$P<0.05$; **$P<0.01$; ***$P<0.001$). Data are represented as mean ± S.E.M.

Results

Prostaglandin-related gene and protein expression in the zebrafish testis

Analysis of RNAseq data obtained using testis samples of untreated adult zebrafish (data set GSE116611) revealed expression of numerous PG-related genes, such as key enzymes involved in the production of all PG series (*ptgs1*, *ptgs2a*, *ptgs2b*; Table 2). The majority of transcripts retrieved were associated to PGE_2 signaling (including several receptors and synthases), but their expression levels often were low (<15 normalized reads for 6 out of 12 transcripts identified; Table 2), compared to the 31,855, 7223 and 14,203 reads sequenced for three selected housekeeping genes *eef1a1l1*, *rpl13a* and *ubc*, respectively (data set GSE116611). Additional receptors and synthases for other PG types (i.e. PGD_2, $PGF_{2\alpha}$ and PGI_2; Table 2) were also detected.

Table 2 Prostaglandin-related gene expression in the adult zebrafish testis.

Gene ID	Gene description	Gene symbol	Read number
ENSDARG00000052148	prostaglandin-endoperoxide synthase 1	*ptgs1*[a]	87.4
ENSDARG00000004539	prostaglandin-endoperoxide synthase 2a	*ptgs2a*[a]	62.3
ENSDARG00000010276	prostaglandin-endoperoxide synthase 2b	*ptgs2b*	32.5
ENSDARG00000069439	prostaglandin D2 synthase a	*ptgdsa*	9.9
ENSDARG00000027088	prostaglandin D2 synthase b, tandem duplicate 1	*ptgdsb.1*	231.2
ENSDARG00000071626	prostaglandin D2 synthase b, tandem duplicate 2	*ptgdsb.2*	88.5
ENSDARG00000078605	prostaglandin E receptor 1a (subtype EP1)	*ptger1a*	12.9
ENSDARG00000078602	prostaglandin E receptor 1c (subtype EP1)	*ptger1c*	2.4
ENSDARG00000011434	prostaglandin E receptor 2a (subtype EP2)	*ptger2a*[a]	28.9
ENSDARG00000037033	prostaglandin E receptor 2b (subtype EP2)	*ptger2b*	8.9
ENSDARG00000055781	prostaglandin E receptor 3 (subtype EP3)	*ptger3*	12.8
ENSDARG00000059236	prostaglandin E receptor 4 (subtype EP4) a	*ptger4a*[a]	84.9
ENSDARG00000035415	prostaglandin E receptor 4 (subtype EP4) b	*ptger4b*[a]	7.6
ENSDARG00000079907	prostaglandin E receptor 4 (subtype EP4) c	*ptger4c*	12.1
ENSDARG00000020136	prostaglandin E synthase	*ptges*[a]	24.0
ENSDARG00000068415	prostaglandin E synthase 2-like	*ptgesl*[a]	756.8
ENSDARG00000037284	prostaglandin E synthase 3a (cytosolic)	*ptges3a*	1872.1
ENSDARG00000089626	prostaglandin E synthase 3b (cytosolic)	*ptges3b*[a]	16.9
ENSDARG00000074016	prostaglandin F receptor (FP)	*ptgfr*	2.6
ENSDARG00000078172	prostaglandin F2 receptor inhibitor	CU984600.1	48.1
ENSDARG00000060094	prostaglandin I2 (prostacyclin) synthase	*ptgis*	51.1

[a]Indicates genes for which a qPCR system was developed (as shown in Table 1).

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In order to investigate which cell type(s) are involved in testicular PG production, an fluorescent immunodetection approach for Ptgs2 was used. Ptgs2 labeling was observed in Sertoli cells, particularly in the nuclei of those Sertoli cells supporting type A (both A_{und} and A_{diff}) spermatogonia (Fig. 1A, B, C, D, E and F). Pre-adsorbing the antibody with the peptide sequence used for its generation eliminated the staining (Fig. 1G, H and I), showing that unspecific staining did not contribute to the labeling pattern observed.

Blocking testicular PG production increased spermatogonial proliferation and differentiation

To investigate the possible involvement of PGs in regulating zebrafish spermatogenesis, we incubated testicular explants in the absence or presence of a potent PG production inhibitor (indomethacin; INDO). First, we examined if INDO treatment efficiently inhibited testicular PGE_2 production. After both 1 and 4 days of incubation, PGE_2 levels in the culture medium were significantly lower in the presence of INDO (Fig. 2A). Blocking testicular PG production elevated the proliferation activity of differentiating (type A_{diff} and B) spermatogonia (Fig. 2B), associated with an increase in the proportion of area occupied by type B spermatogonia (Fig. 2C). The expression of steroidogenesis-related genes (*star* and *cyp17a1*) was significantly reduced by INDO, while the other transcripts analyzed (germ cell markers and growth factors) were not affected by the treatment (Fig. 2D). Moreover, transcript levels of several PG-related genes (*ptgs1*, *ptgs2a*, *ptges*, *ptgesl*, *ptges3b*, *ptger2a*, *ptger4a*, *ptger4b*) did not change in response to INDO (data not shown). Hence, blocking PG production facilitated the proliferation of differentiating spermatogonia.

In view of the reduced levels of steroidogenesis-related transcripts following INDO treatment, we investigated possible interactions between PG and steroid signaling. First, we examined if the response to INDO is modulated when blocking the production of biologically active steroids with trilostane (Tril). Similar to the previous experiments (Fig. 2B and C), the differentiating spermatogonia showed higher proliferation rates and occupied increased proportions of the testicular area (Fig. 3A and B, respectively). In addition, A_{und} spermatogonia showed a lower proliferation activity and occupied a smaller proportion of the section surface. Also, lower transcripts levels of *foxa2* (a potential marker for A_{und}

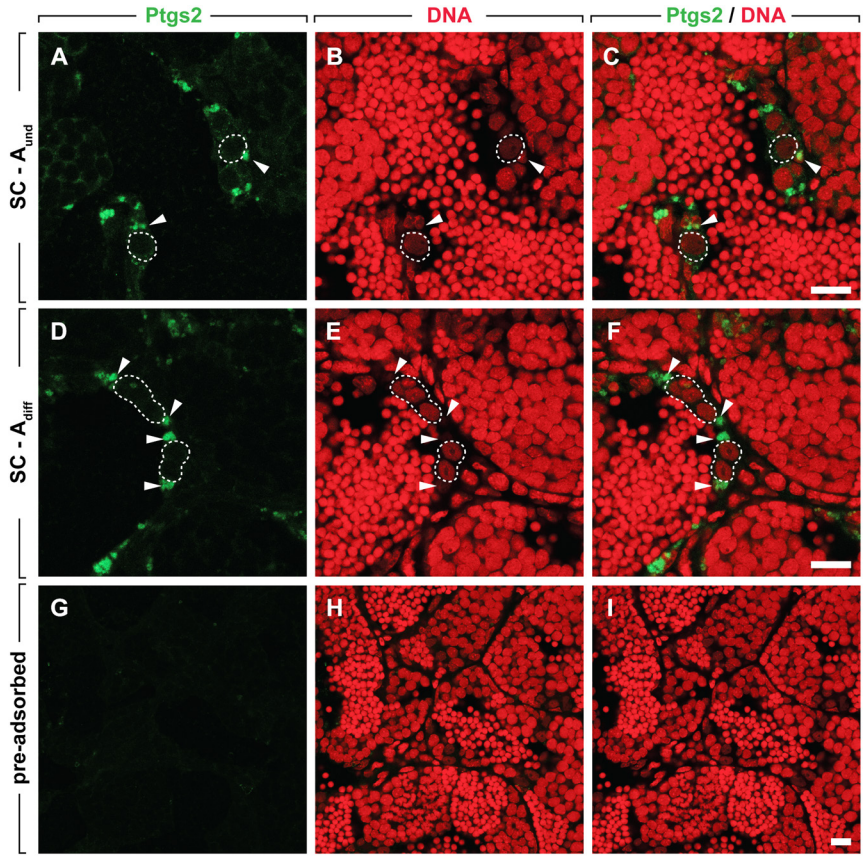

Figure 1
PGs are produced by Sertoli cells in adult zebrafish testes. (A, B, C, D, E and F) Detection of Ptgs2 protein by fluorescent immunohistochemistry. Green staining indicates Ptgs2-positive Sertoli cells and red staining indicates DNA (propidium iodide counterstain). (G, H and I) Ptgs2 antibody pre-adsorbed with blocking peptide as negative control. Arrowheads indicate nuclear Sertoli cell (SC) staining, and representative type A undifferentiated (A_{und}) and differentiating (A_{diff}) spermatogonia are encircled with a white dashed line. Scale bars in C, F and I represent 10 µm. Ptgs2-positive nuclei were restricted to Sertoli cells contacting type A_{und} and A_{diff} spermatogonia. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0309.

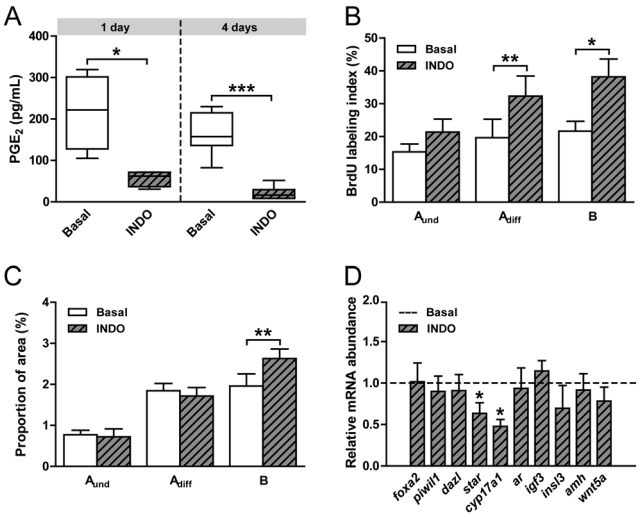

Figure 2
Inhibition of PG production by INDO promotes the differentiation of zebrafish spermatogonia. (A) Quantification of PGE_2 production by testis tissue cultured for 1 or 4 days in basal medium or in medium containing 3 µM INDO (i.e. indomethacin, a nonsteroidal anti-inflammatory drug). Data are expressed as mean ± S.E.M. (n = 4–6) and asterisks indicate significant differences between groups (*P < 0.05, ***P < 0.001, paired Student's t test). Additional testicular explants were cultured for 4 days in the absence or presence of 3 µM INDO and then used to quantify BrdU labeling indices (B), the areas occupied by type A and B spermatogonia (C), or candidate gene expression (D). Data are expressed as mean ± S.E.M. (n = 6–8) and asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01, paired Student's t test). In D, results are shown relative to the basal control condition, which is set at 1 (dashed line). A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; B, type B spermatogonia.

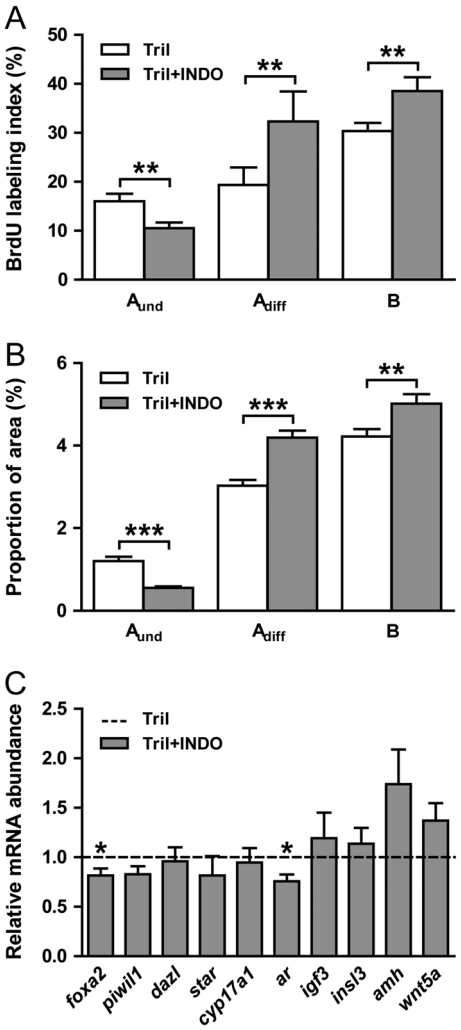

Figure 3
INDO-induced differentiation, but not self-renewal, of spermatogonia is unaffected when blocking steroid production. Determination of BrdU labeling indices (A) and frequencies (B) of type A and B spermatogonia, and (C) quantification of candidate gene expression. Testicular explants were cultured for 4 days in the absence or presence of 3 µM INDO and collected for further analysis. The medium contained trilostane (Tril, 25 µg/mL) to block the production of biologically active steroids. Data are expressed as mean ± S.E.M. (n = 6–8). Asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01, ***P < 0.001, paired Student's t test). In C, results are shown relative to the basal control condition, which is set at 1 (dashed line). A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; B, type B spermatogonia.

spermatogonia (Safian *et al.* 2017)) and *ar* were observed under these conditions (i.e. INDO plus Tril; Fig. 3C), while the level of growth factor transcripts remained unaltered. Interestingly, the expression level of genes with products related to steroidogenesis remained unchanged, so that the PGE_2 effect on *star* and *cyp17a1* transcript levels observed earlier (Fig. 2C) may depend on the presence of steroids. In summary, the proliferation of differentiating spermatogonia was still favored when blocking PG and steroid production, while type A_{und} spermatogonia became partially depleted. However, type A_{und} spermatogonia remained unaffected when blocking only PG production.

PGE_2 inhibits androgen- or Fsh-stimulated spermatogonial differentiation but promotes their self-renewal when combined with androgen

In order to study the effects of PGs on the proliferation of spermatogonia in a more direct manner, we added PGE_2 to the culture medium, asking if PGE_2 inhibits androgen-driven spermatogonia development. We found that PGE_2 reduced the proliferation activity and area occupied by type B spermatogonia (Fig. 4A and B). Type A_{und} spermatogonia, on the other hand, showed an increased proliferation activity and accumulation, suggesting that these cells preferentially underwent self-renewal divisions in the presence of 11-KT and PGE_2. Under these conditions, transcript levels of markers for differentiating spermatogonia (*piwil1* and – not significantly – *dazl*) were

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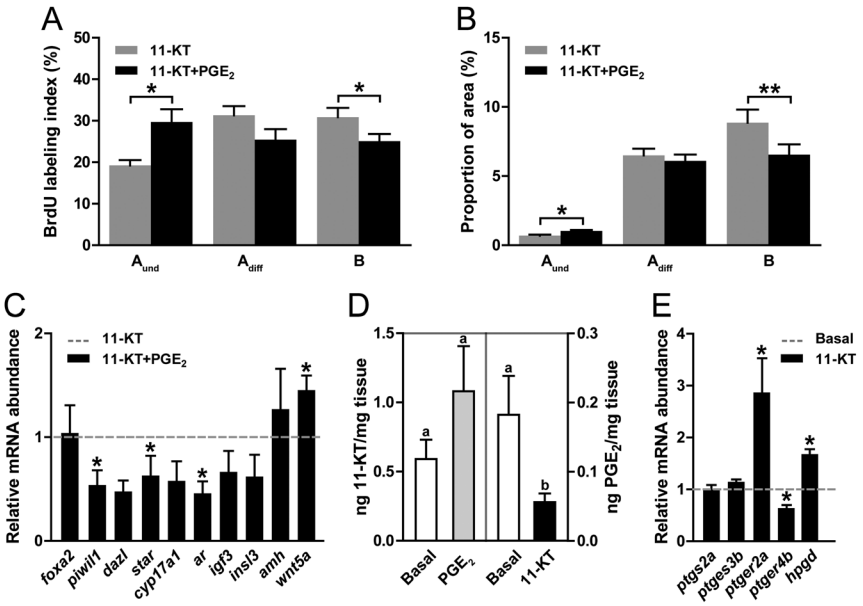

Figure 4
In the presence of the androgen 11-KT, PGE_2 increases the proliferation of type A_{und} spermatogonia while reducing their further differentiation. Testis tissue was cultured for 4 days in the absence or presence of 5 µM PGE_2, in medium containing 200 nM 11-KT, and then used for quantification of BrdU labeling indices (A), areas occupied by type A and B spermatogonia (B), or candidate gene expression (C). A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; B, type B spermatogonia. (D) Quantification of 11-KT and PGE_2 in testis tissue medium after culture for 1 day under different conditions. Different letters indicate significant differences between groups. (E) Modulation of PG-related gene expression by 11-KT. Results are expressed as mean ± S.E.M. (n = 6–17) and asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01, paired Student's t test). In C and E, results are shown relative to the basal control condition, which is set at 1 (dashed line).

downregulated, and considering growth factor expression, *wnt5a* levels increased (Fig. 4C). PGE_2 treatment reduced two genes with products related to steroidogenesis (*star* and *ar*; Fig. 4C), but testicular androgen (11-KT) release did not change significantly (left panel in Fig. 4D). Conversely, 11-KT reduced PGE_2 release (right panel in Fig. 4D), increased transcripts levels of the PG metabolizing enzyme *hpgd* (Fig. 4E), and modulated PG receptor (but not PG synthesis) gene expression (Fig. 4E).

Next, we examined the effect of PGE_2 on Fsh-stimulated spermatogonia development. To focus on the steroid-independent effects of Fsh, trilostane (Tril) was included in the incubation medium. Similar to the findings in the presence of androgen, addition of PGE_2 reduced Fsh-stimulated proliferation activity and relative numbers of differentiating spermatogonia (Fig. 5A and B). The effect of other PG types (PGD_2 and $PGF_{2\alpha}$) on the proliferation activity were also evaluated in the presence of Fsh, but no significant changes were found for any of the three types of spermatogonia analyzed (data not shown).

The *star* transcript levels were upregulated by PGE_2 in the presence of Fsh (Fig. 5C). However, Fsh-stimulated androgen release, being about 10-fold higher than basal (Fig. 4D, left panel), was not changed by PGE_2 (Fig. 5D, left panel), suggesting that PGE_2 is not acutely modulating basal or gonadotropin-stimulated testicular androgen production. Fsh tended to reduce PGE_2 production (Fig. 5D, right panel), but different from the androgen effect (Fig. 4D, right panel), statistical significance was not reached. However, blocking steroid production in the presence of Fsh increased testicular PGE_2 release compared to Fsh alone, but not compared to basal conditions, suggesting that this increase reflects the (trilostane-based) removal of steroid-mediated inhibition of PGE_2 production. Taken together, the stimulatory effects of both, Fsh and androgens on spermatogonial differentiation were reduced by PGE_2, androgens diminished PGE_2 production, and PGE_2 in combination with androgen induced an accumulation of A_{und} spermatogonia.

Ep4 receptor mediates PGE_2-induced effects in the zebrafish testis

PGE_2, but not PGD_2 or $PGF_{2\alpha}$, modulated the activity of spermatogonia. To gain additional knowledge on the mechanism mediating PGE_2 effects, we aimed at investigating which receptor(s) mediate these effects. We have found previously (Crespo *et al.* 2016, Morais *et al.* 2017) that two PG synthases (*ptgs2a* and *ptges3b*) and two receptors (*ptger2a* and *ptger4b*) were modulated by different hormones and growth factors (summarized in Fig. 6A). *In situ* hybridization studies failed to localize the cellular expression of *ptger2a* and *ptger4b*, possibly due to their low expression levels in the testis (Table 2). Using alternative approaches, we first, analyzed *ptger2a* and *ptger4b* transcript levels available from an RNAseq dataset that compares control, germ cell-depleted, and recovering testes (data set GSE116611). This suggested an enrichment of receptor expression in somatic cells, since *ptger2a* and *ptger4b* transcript levels were upregulated in germ cell-depleted testes and returned to control levels during the

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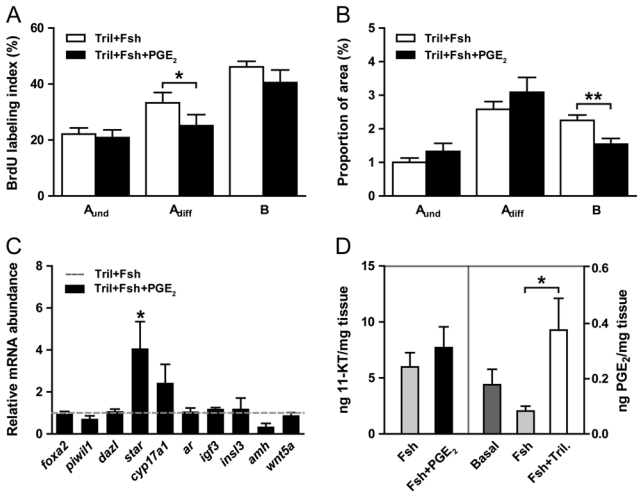

Figure 5

PGE$_2$ inhibits Fsh-stimulated differentiation of spermatogonia. Testis explants were cultured for 4 days with Fsh (100 ng/mL) in the absence or presence of 5 µM PGE$_2$, while inhibiting steroid production with trilostane (Tril, 25 µg/mL). After 4 days, testis tissue was used for quantification of BrdU labeling indices (A), the areas occupied by type A and B spermatogonia (B), or of candidate gene expression (C). Quantification of 11-KT and PGE$_2$ (D) in testis tissue medium after culture for 1 day under different conditions. Results are expressed as mean ± S.E.M. (n = 4–9) and asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01, paired Student's t test (A, B, C, and left panel in D) or one-way ANOVA followed by Tukey's multiple comparison test (right panel in D). In (C), results are shown relative to the basal control condition, which is set at 1 (dashed line). A$_{und}$, type A undifferentiated spermatogonia; A$_{diff}$, type A differentiating spermatogonia; B, type B spermatogonia.

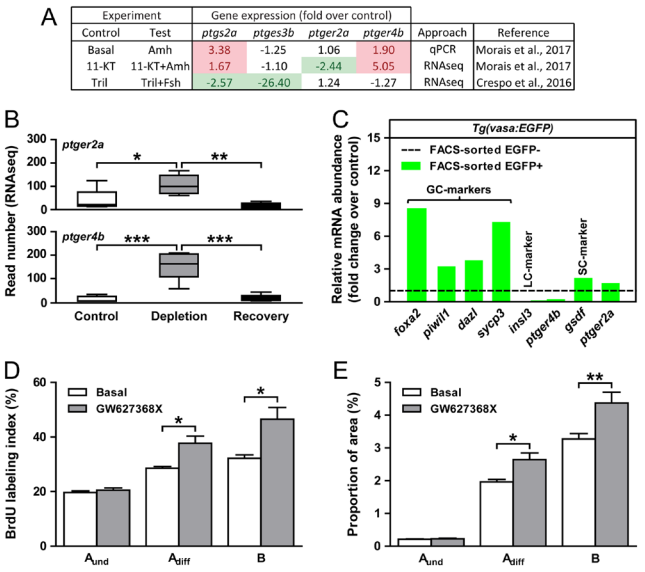

Figure 6

Ep4 receptor (*ptger4b*) mediates PGE$_2$ signaling in the zebrafish testis. (A) Modulation of PG-related gene expression in testis tissue cultures exposed to different treatments, and analyzed by qPCR or RNAseq approaches. (B) Expression levels of PGE$_2$ receptors in control, germ cell-depleted (by exposure to the cytostatic agent busulfan, as previously described (Nobrega *et al.* 2010)), and testes with recovering (from busulfan) spermatogenesis, as described in the NCBI GEO database (data set GSE116611). (C) qPCR analysis of EGFP- and EGFP+ cell fractions. A testicular cell suspension was obtained from testes of transgenic *Tg(vasa:EGFP)* fish and subjected to FACS. EGFP$^-$ and EGFP$^+$ cell fractions were collected and RNA was isolated for analysis of germ (GC), Leydig (LC) and Sertoli cell (SC) markers, as well as PGE$_2$ receptors. Data are expressed relative to the EGFP$^-$ condition, which is set at 1. (D and E) Effects of an Ep4 receptor antagonist on germ cell development. Testicular explants were cultured for 4 days in the absence or presence of GW627368X (5 µM). Testis tissue was used to quantify BrdU labeling indices (D), and the areas occupied by the type A and B spermatogonia (E). Results are expressed as mean ± S.E.M. (n = 5–8) and asterisks indicate significant differences between groups (*P < 0.05, **P < 0.01, ***P < 0.001). A$_{und}$, type A undifferentiated spermatogonia; A$_{diff}$, type A differentiating spermatogonia; B, type B spermatogonia. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0309.

recovery of spermatogenesis (Fig. 6B). Enrichment of the *ptger4b* transcript in somatic cells was confirmed using testes from transgenic *Tg(vasa:EGFP)* zebrafish. Analyzing FACS-sorted testicular cell suspensions by qPCR showed that *ptger4b* followed an expression pattern similar to the one obtained for Leydig cell marker *insl3* (Fig. 6C). This did not apply to the same stringency to *ptger2a* that followed the Sertoli cell marker *gsdf* (Fig. 6C) that probably reflects re-association of some EGFP-positive germ cells with Sertoli cells. Therefore, it cannot be excluded that some *ptger2a* expression was associated with germ cells.

The Ep4 receptor antagonist GW627368X increased the proliferation activity and relative numbers of differentiating spermatogonia (Fig. 6D and E), a response similar to the one seen when blocking PG production by INDO (Fig. 2A and B). Analyzing the transcript levels also quantified when blocking PG production by INDO (Fig. 2D), we did not find changes in these transcript levels in response to GW627368X (data not shown). Since *ptger2a* transcript levels were modulated in response to Amh (Fig. 6A) and may be associated with germ cells (Fig. 6C), we also tested the Ep2 receptor antagonist PF04418948. However, this compound did not affect the proliferation activity or relative numbers of spermatogonia (data not shown). Taken together, our results indicate that the *b* paralogue of the Ep4 receptor is relevant for mediating PGE$_2$ action in the zebrafish testis, and that expression of this receptor is associated preferentially with testicular somatic cells.

Discussion

Our main findings are that locally produced PGE$_2$, but not PGD$_2$ or PGF$_{2\alpha}$, reduced the proliferation activity of type A$_{diff}$ and/or type B spermatogonia, both under basal

and Fsh- or androgen-stimulated conditions. Moreover, PGE_2 increased the self-renewal proliferation of type A_{und} spermatogonia resulting in their accumulation, which depended on a permissive effect of Fsh-stimulated androgen/steroid productions. Hence, local PGE_2 signaling in the testis lowered the production of differentiating spermatogonia but expanded the pool of type A_{und} spermatogonia, suggesting overall a reduced spermatogenic activity. An inhibitory effect of PGE_2 on spermatogenesis was reported earlier based on *in vivo* studies in mammals (mice (Abbatiello *et al.* 1975) and dogs (Moskovitz *et al.* 1987)), describing reduced numbers of spermatocytes and spermatids.

Considering the site of PG production, we found Cox2 protein in Sertoli cells contacting type A spermatogonia. In rat (Winnall *et al.* 2007) and dog (Korber & Goericke-Pesch 2019) testis, COX2 likewise was present in Sertoli cells but also in Leydig cells, which was not the case in zebrafish. COX2 produces PGH_2, which requires PGE synthase to be converted to PGE_2. The *ptges3b* transcript has been localized to Sertoli cells contacting A_{diff} and B type spermatogonia and spermatocytes in zebrafish (Crespo *et al.* 2016). This opens the possibility that in tubuli showing a high number of these more differentiated germ cells high PGE_2 levels are present, a setting compatible with a local negative feedback loop to reduce the production of further differentiating spermatogonia while expanding the pool of A_{und} (Fig. 7).

Although zebrafish Leydig cells were negative for Cox2 or *ptges3b*, Leydig cell-derived androgens are not only stimulators of spermatogenesis (Crowder *et al.* 2018, de Castro Assis *et al.* 2018, Tang *et al.* 2018), but also seem relevant in the context of PG signaling. Part of the stimulatory action of androgens on spermatogenesis may be related to reducing testicular PGE_2 production via increasing *hpgd* mRNA, coding for an enzyme that catabolizes PGs. Also decreasing transcript levels of *ptger4b* can contribute to an androgen-mediated limitation of PGE_2 signaling. Different from androgens, another stimulator of spermatogenesis, Fsh (Crespo *et al.* 2016) had no rapid (overnight) inhibitory effect on PGE_2 production but reduced the transcript levels of two PG synthases (*ptgs2a* and *ptges3b*) after 4 days of tissue culture. Therefore, it seems possible that androgen-independent effects of Fsh also reduce PGE_2 synthesis. FSH-modulated *Cox2* expression and PG production has been reported from primary Sertoli cell cultures of immature rat (Jannini *et al.* 1994) or adult hamster (Matzkin *et al.* 2012). It remains to be clarified what the functional connotation is of the nuclear localization of Cox2 in zebrafish Sertoli cell nuclei; a nuclear localization of COX2 has been reported earlier for rat spermatogonia (Neeraja *et al.* 2003).

While stimulators of spermatogenesis (androgens and Fsh) inhibited PGE_2 signaling, we have found previously that Amh, an inhibitor of zebrafish spermatogenesis (Skaar *et al.* 2011), promoted PGE_2 signaling: Amh elevated transcript levels for *ptgs2a* and *ptger4b* (Morais *et al.* 2017), compatible with increasing PG production and PGE_2 sensitivity. A schematic working model summarizing effects of regulators of PGE_2 production is presented in Fig. 7, placing PGE_2 downstream of Fsh and intratesticular factors targeted by Fsh, such as androgens and Amh. However, we did not find evidence for PG effects on Leydig cell androgen production. This is different from the situation in mammals (see 'Introduction' section), and also from previous work in a relative of the zebrafish, the goldfish, reporting PGE_2-stimulated testicular androgen production (Wade & Van der Kraak 1993). However, later experiments involving treatment of adult zebrafish with an NSAID (ibuprofen) did not result in changes of sex steroid plasma levels (Morthorst *et al.* 2012). Similarly, in our experiments, PGE_2 did not modulate acute (overnight) basal or Fsh-stimulated androgen release but PGE_2 did change *star*, *cyp17a1* or *ar* transcript levels after 3 or 4 days of tissue culture. We may have missed such intermediate-term effects of PGE_2 on androgen production with our approach, but the type of effect is not clear. For example, we recorded reduced *star* and *cyp17a1* transcript levels when blocking PG production, but only when steroid production was not blocked at the same time (Figs 2 and 3). One possibility to understand

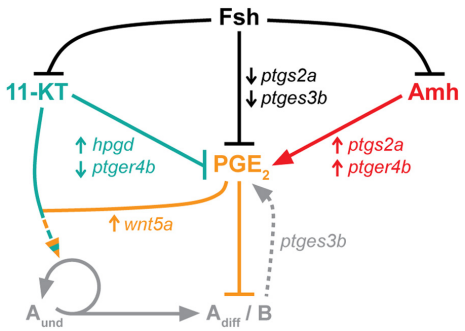

Figure 7
Schematic illustration showing the endocrine and paracrine regulation of zebrafish testicular PGE_2 production and the stages of spermatogonial development affected by PGE_2. Described effects by secreted factors are indicated by black (Fsh), green (11-KT), red (Amh) and orange (PGE_2) arrows, while germ cell development or germ cell-mediated effects are indicated in grey. Fsh, follicle-stimulating hormone; 11-KT, 11-ketotestosterone; Amh, anti-Müllerian hormone; PGE_2, prostaglandin E_2; A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiating spermatogonia; B, type B spermatogonia. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0309.

this data set is assuming that PGs may prevent steroid-induced downregulation of *star* and *cyp17a1*. However, this possibility does not fit to the PGE_2-induced decrease of *star*, *cyp17a1* and *ar* transcript levels when 11-KT was present in the incubation medium (Fig. 4C). More work is required to clarify the relation between PG and androgen/steroid production. For example, differences between observations made by blocking PG production using INDO and adding PGE_2 may reflect effects of PGs other than PGE_2 that are also no longer produced when using INDO.

Our working model also summarizes the main effects of PGE_2 on type A and B spermatogonia (Fig. 7). We recorded inhibitory effects on the development of type A_{diff} and B spermatogonia that were independent of the absence or presence of steroids/androgen or Fsh. The self-renewal proliferation of type A_{und} spermatogonia, on the other hand, was stimulated by PGE_2 but this effect depended either on the presence of steroids/androgen (Figs 2, 3 and 4), or on undisturbed steroid production in the presence of Fsh (Fig. 5 of the present MS and Fig. 6 of Morais *et al.* 2017). It therefore appears that steroids play a (yet to be characterized) permissive role for the PGE_2-triggered effects on type A_{und} spermatogonia. In addition, an unexpected, PG-independent androgen effect on type A_{und} spermatogonia became evident when comparing the results presented in Figs 2 and 3. In these two experiments, PG production was blocked but it required the additional blocking of steroid production to decrease A_{und} proliferation and to partially deplete these cells (Fig. 3). This suggested that steroid hormones otherwise support self-renewal proliferation of A_{und}. Previous work has shown already that PGE_2 promoted self-renewal proliferation and accumulation of A_{und} in the presence of Fsh and undisturbed steroid production (Fig. 6C and D in Morais *et al.* 2017). In the light of the results presented in Figs 2 and 3, we speculated that this effect seen in response to the combined presence of Fsh and PGE_2, may depend on the capacity to produce biologically active steroids. Repeating this experiment in the presence of trilostane indeed abolished the effect of PGE_2 on A_{und} but not on the more differentiated A_{diff} and B spermatogonia (Fig. 5A and B). Since androgens are likely candidates for mediating this action, we examined if the PGE_2 effect on A_{und} spermatogonia is recovered in the presence of 11-KT, which indeed was the case (Fig. 4). Hence, in the absence of PGs, sex steroids alone already stimulate self-renewal/accumulation of A_{und}, which is further stimulated when PGE_2 is present as well (Fig. 7). Also in salmon testis tissue collected from fish that have just entered pubertal development and showed an elevated level of single cell proliferation activity, PGE_2 production is upregulated (Crespo *et al.* 2019). Since in these males *fshb* transcript levels in the pituitary and plasma androgen levels were also elevated, it seems possible that in salmon testis, a combined Fsh/androgen/PGE_2 signaling accompanies the expansion of the population of type A_{und} spermatogonia observed in the early pubertal salmon testis (Crespo *et al.* 2019, Schulz *et al.* 2019).

Zebrafish testis tissue expresses eight PGE_2 receptor subtypes (Table 2). Previous work (Morais *et al.* 2017) suggested that Ptger2a and Ptger4b are relevant candidates and the present experiments involving EP2 and EP4 receptor antagonists further narrowed it down to Ptger4b. The latter is preferentially expressed by testicular somatic cells, showing in particular a pattern similar to the Leydig cell marker gene *insl3*. Interestingly, the only growth factor gene that responded to PGE_2 with elevated transcript levels was *wnt5a* (Fig. 4C). Expression of this Wnt ligand is stimulated by Fsh in Leydig cells in the zebrafish testis and stimulates the self-renewal proliferation of type A_{und} spermatogonia (Safian *et al.* 2018). Therefore, one possibility for PGE_2 to promote the self-renewal may involve elevated *wnt5a* expression. However, so far, we cannot offer a mechanistic hypothesis for the inhibitory effect of PGE_2 on type A_{diff} and B spermatogonia.

Other PGs had no effect in zebrafish in our experiments. In mice, PGD_2 inhibited the differentiation of fetal spermatogonia, using different mechanisms that relied on Sertoli and/or germ cell expression of PGD_2 receptors (Moniot *et al.* 2014, Rossitto *et al.* 2015). These receptors are not expressed by the adult zebrafish testis (Table 2). Work on bladder cancer stem cells showed that PGE_2 promoted stem cell production (Kurtova *et al.* 2015). Similarly, PGE_2 treatment increased hematopoietic stem cell numbers and cyclooxygenase activity was required for their formation (North *et al.* 2007). Recently, it has also been shown that isolated human testicular peritubular cells secrete PGE_2 and that PGE_2, as well as EP1 and EP4 receptor agonists, increased glia cell line-derived neurotrophic factor (GDNF) levels (Rey-Ares *et al.* 2018), a growth factor that maintains the stem cell pool by promoting SSC self-renewal proliferation (Chen *et al.* 2016, Potter & DeFalco 2017). Evidence for a potential role of Gndf in the rainbow trout testis to prevent differentiation of A_{und} spermatogonia has been published (Bellaiche *et al.* 2014). Although a *gdnf* gene has been annotated in the zebrafish genome, information on its functions in the testis is not available yet.

Taken together, it appears that prostaglandin signaling inhibits the production of more differentiated cells while

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stimulating stem cell self-renewal in undifferentiated cells in a range of vertebrate models and cell types. For the adult zebrafish testis, we propose that PGE_2 signaling is an integral part of the local regulatory network used by reproductive hormones to adjust spermatogenic activity to the changing requirements via endocrine signals, in particular pituitary Fsh, while safeguarding testis tissue homeostasis.

Declaration of interest

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

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References

Abbatiello ER, Kaminsky M & Weisbroth S 1975 The effect of prostaglandins and prostaglandin inhibitors on spermatogenesis. *International Journal of Fertility* **20** 177–182.

Abbatiello ER, Kaminsky M & Weisbroth S 1976 The effect of prostaglandins F1 alpha and F2 alpha on spermatogenesis. *International Journal of Fertility* 82–88.

Assis LH, Crespo D, Morais RD, Franca LR, Bogerd J & Schulz RW 2016 INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (*Danio rerio*). *Cell and Tissue Research* **363** 579–588. (https://doi.org/10.1007/s00441-015-2213-9)

Bellaiche J, Goupil AS, Sambroni E, Lareyre JJ & Le Gac F 2014 Gdnf-Gfra1 pathway is expressed in a spermatogenetic-dependent manner and is regulated by Fsh in a fish testis. *Biology of Reproduction* **91** 94. (https://doi.org/10.1095/biolreprod.114.119834)

Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ & Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **64** 1633–1643. (https://doi.org/10.1095/biolreprod64.6.1633)

Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP & Versteeg HH 2004 Prostanoids and prostanoid receptors in signal transduction. *International Journal of Biochemistry and Cell Biology* **36** 1187–1205. (https://doi.org/10.1016/j.biocel.2003.08.006)

Breyer RM, Bagdassarian CK, Myers SA & Breyer MD 2001 Prostanoid receptors: subtypes and signaling. *Annual Review of Pharmacology and Toxicology* **41** 661–690. (https://doi.org/10.1146/annurev.pharmtox.41.1.661)

Chen LY, Willis WD & Eddy EM 2016 Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *PNAS* **113** 1829–1834. (https://doi.org/10.1073/pnas.1517994113)

Choudhuri A, Fast EM & Zon LI 2017 Using zebrafish to study pathways that regulate hematopoietic stem cell self-renewal and migration. *Stem Cell Reports* **8** 1465–1471. (https://doi.org/10.1016/j.stemcr.2017.05.018)

Crespo D, Assis LHC, Furmanek T, Bogerd J & Schulz RW 2016 Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. *Molecular and Cellular Endocrinology* **437** 237–251. (https://doi.org/10.1016/j.mce.2016.08.033)

Crespo D, Bogerd J, Sambroni E, LeGac F, Andersson E, Edvardsen RB, Bergman EJ, Bjornsson BT, Taranger GL & Schulz RW 2019 The initiation of puberty in Atlantic salmon brings about large changes in testicular gene expression that are modulated by the energy status. *BMC Genomics* **20** 475. (https://doi.org/10.1186/s12864-019-5869-9)

Crowder CM, Lassiter CS & Gorelick DA 2018 Nuclear androgen receptor regulates testes organization and oocyte maturation in zebrafish. *Endocrinology* **159** 980–993. (https://doi.org/10.1210/en.2017-00617)

Cuisset B, Pradelles P, Kime DE, Kühn ER, Babin P, Davail S & Le Menn F 1994 Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as laberl: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. *Comparative Biochemistry and Physiology Part C* **108** 229–241. (https://doi.org/10.1016/1367-8280(94)90035-3)

de Castro Assis LH, de Nobrega RH, Gomez-Gonzalez NE, Bogerd J & Schulz RW 2018 Estrogen-induced inhibition of spermatogenesis in zebrafish is largely reversed by androgen. *Journal of Molecular Endocrinology* **60** 273–284. (https://doi.org/10.1530/JME-17-0177)

Feng Y, Renshaw S & Martin P 2012 Live imaging of tumor initiation in zebrafish larvae reveals a trophic role for leukocyte-derived PGE(2). *Current Biology* **22** 1253–1259. (https://doi.org/10.1016/j.cub.2012.05.010)

Frungieri MB, Calandra RS, Mayerhofer A & Matzkin ME 2015 Cyclooxygenase and prostaglandins in somatic cell populations of the testis. *Reproduction* **149** R169–R180. (https://doi.org/10.1530/REP-14-0392)

Garcia-Lopez A, de Jonge H, Nobrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J & Schulz RW 2010 Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151** 2349–2360. (https://doi.org/10.1210/en.2009-1227)

Hata AN & Breyer RM 2004 Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacology and Therapeutics* **103** 147–166. (https://doi.org/10.1016/j.pharmthera.2004.06.003)

Hinfray N, Nobrega RH, Caulier M, Baudiffier D, Maillot-Marechal E, Chadili E, Palluel O, Porcher JM, Schulz R & Brion F 2013 Cyp17a1 and Cyp19a1 in the zebrafish testis are differentially affected by oestradiol. *Journal of Endocrinology* **216** 375–388. (https://doi.org/10.1530/JOE-12-0509)

Jannini EA, Ulisse S, Cecconi S, Cironi L, Colonna R, D'Armiento M, Santoni A & Cifone MG 1994 Follicle-stimulating hormone-induced phospholipase A2 activity and eicosanoid generation in rat Sertoli cells. *Biology of Reproduction* **51** 140–145. (https://doi.org/10.1095/biolreprod51.1.140)

Korber H & Goericke-Pesch S 2019 Expression of PTGS2, PGFS and PTGFR during downregulation and restart of spermatogenesis following GnRH agonist treatment in the dog. *Cell and Tissue Research* **375** 531–541. (https://doi.org/10.1007/s00441-018-2915-x)

Krovel AV & Olsen LC 2002 Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish. *Mechanisms of Development* **116** 141–150. (https://doi.org/10.1016/s0925-4773(02)00154-5)

Kurtova AV, Xiao J, Mo Q, Pazhanisamy S, Krasnow R, Lerner SP, Chen F, Roh TT, Lay E, Ho PL, *et al.* 2015 Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature* **517** 209–213. (https://doi.org/10.1038/nature14034)

Leal MC, Cardoso ER, Nobrega RH, Batlouni SR, Bogerd J, Franca LR & Schulz RW 2009*a* Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* **81** 177–187. (https://doi.org/10.1095/biolreprod.109.076299)

Leal MC, de Waal PP, Garcia-Lopez A, Chen SX, Bogerd J & Schulz RW 2009*b* Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. *General and Comparative Endocrinology* **162** 134–138. (https://doi.org/10.1016/j.ygcen.2009.03.003)

Matzkin ME, Pellizzari EH, Rossi SP, Calandra RS, Cigorraga SB & Frungieri MB 2012 Exploring the cyclooxygenase 2 (COX2)/15d-Delta(12,14)PGJ(2) system in hamster Sertoli cells: regulation by FSH/testosterone and relevance to glucose uptake. *General and Comparative Endocrinology* **179** 254–264. (https://doi.org/10.1016/j.ygcen.2012.08.020)

Moniot B, Ujjan S, Champagne J, Hirai H, Aritake K, Nagata K, Dubois E, Nidelet S, Nakamura M, Urade Y, *et al.* 2014 Prostaglandin D2 acts through the Dp2 receptor to influence male germ cell differentiation in the foetal mouse testis. *Development* **141** 3561–3571. (https://doi.org/10.1242/dev.103408)

Morais RDVS, Crespo D, Nobrega RH, Lemos MS, van de Kant HJG, de Franca LR, Male R, Bogerd J & Schulz RW 2017 Antagonistic regulation of spermatogonial differentiation in zebrafish (*Danio rerio*) by Igf3 and Amh. *Molecular and Cellular Endocrinology* **454** 112–124. (https://doi.org/10.1016/j.mce.2017.06.017)

Morthorst JE, Lister A, Bjerregaard P & Van Der Kraak G 2012 Ibuprofen reduces zebrafish PGE(2) levels but steroid hormone levels and reproductive parameters are not affected. *Comparative Biochemistry and Physiology C* **157** 251–257. (https://doi.org/10.1016/j.cbpc.2012.12.001)

Moskovitz B, Munichor M & Levin DR 1987 Effect of diclofenac sodium (Voltaren) and prostaglandin E2 on spermatogenesis in mature dogs. *European Urology* **13** 393–396. (https://doi.org/10.1159/000472832)

Neeraja S, Sreenath AS, Reddy PR & Reddanna P 2003 Expression of cyclooxygenase-2 in rat testis. *Reproductive Biomedicine Online* **6** 302–309. (https://doi.org/10.1016/s1472-6483(10)61849-4)

Nobrega RH, Greebe CD, van de Kant H, Bogerd J, de Franca LR & Schulz RW 2010 Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS ONE* **5** e12808. (https://doi.org/10.1371/journal.pone.0012808)

Nobrega RH, Morais RD, Crespo D, de Waal PP, de Franca LR, Schulz RW & Bogerd J 2015 Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. *Endocrinology* **156** 3804–3817. (https://doi.org/10.1210/en.2015-1157)

North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, *et al.* 2007 Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* **447** 1007–1011. (https://doi.org/10.1038/nature05883)

Potter SJ & DeFalco T 2017 Role of the testis interstitial compartment in spermatogonial stem cell function. *Reproduction* **153** R151–R162. (https://doi.org/10.1530/REP-16-0588)

Pradhan A & Olsson PE 2014 Juvenile ovary to testis transition in zebrafish involves inhibition of ptges. *Biology of Reproduction* **91** 33. (https://doi.org/10.1095/biolreprod.114.119016)

Rey-Ares V, Rossi SP, Dietrich KG, Kohn FM, Schwarzer JU, Welter H, Frungieri MB & Mayerhofer A 2018 Prostaglandin E2 (PGE2) is a testicular peritubular cell-derived factor involved in human testicular homeostasis. *Molecular and Cellular Endocrinology* **473** 217–224. (https://doi.org/10.1016/j.mce.2018.01.022)

Ricciotti E & FitzGerald GA 2011 Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31** 986–1000. (https://doi.org/10.1161/ATVBAHA.110.207449)

Rossitto M, Ujjan S, Poulat F & Boizet-Bonhoure B 2015 Multiple roles of the prostaglandin D2 signaling pathway in reproduction. *Reproduction* **149** R49–R58. (https://doi.org/10.1530/REP-14-0381)

Safian D, Bogerd J & Schulz RW 2018 Igf3 activates beta-catenin signaling to stimulate spermatogonial differentiation in zebrafish. *Journal of Endocrinology* **238** 245–257. (https://doi.org/10.1530/JOE-18-0124)

Safian D, van der Kant HJG, Crespo D, Bogerd J & Schulz RW 2017 Follicle-stimulating hormone regulates igfbp gene expression directly or via downstream effectors to modulate Igf3 effects on zebrafish spermatogenesis. *Frontiers in Endocrinology* **8** 328. (https://doi.org/10.3389/fendo.2017.00328)

Schulz RW, Taranger GL, Bogerd J, Nijenhuis W, Norberg B, Male R & Andersson E 2019 Entry into puberty is reflected in changes in hormone production but not in testicular receptor expression in Atlantic salmon (*Salmo salar*). *Reproductive Biology and Endocrinology* **17** 48. (https://doi.org/10.1186/s12958-019-0493-8)

Shrestha HK, Beg MA, Burnette RR & Ginther OJ 2012 Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers. *Biology of Reproduction* **87** 18, 1–6. (https://doi.org/10.1095/biolreprod.112.100776)

Simmons DL, Botting RM & Hla T 2004 Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacological Reviews* **56** 387–437. (https://doi.org/10.1124/pr.56.3.3)

Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW & Male R 2011 Proteolytically activated, recombinant anti-Mullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* **152** 3527–3540. (https://doi.org/10.1210/en.2010-1469)

Sorensen PW & Goetz FW 1993 Pheromonal and reproductive function of F prostaglandins and their metabolites in teleost fish. *Journal of Lipid Mediators* **6** 385–393.

Takahashi T, Hagiwara A & Ogiwara K 2018 Prostaglandins in teleost ovulation: a review of the roles with a view to comparison with prostaglandins in mammalian ovulation. *Molecular and Cellular Endocrinology* **461** 236–247. (https://doi.org/10.1016/j.mce.2017.09.019)

Tang H, Chen Y, Wang L, Yin Y, Li G, Guo Y, Liu Y, Lin H, Cheng CHK & Liu X 2018 Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish lacking androgen receptor. *Biology of Reproduction* **98** 227–238. (https://doi.org/10.1093/biolre/iox165)

Wade MG & Van der Kraak G 1993 Arachidonic acid and prostaglandin E2 stimulate testosterone production by goldfish testis in vitro. *General and Comparative Endocrinology* **90** 109–118. (https://doi.org/10.1006/gcen.1993.1065)

Winnall WR, Ali U, O'Bryan MK, Hirst JJ, Whiley PA, Muir JA & Hedger MP 2007 Constitutive expression of prostaglandin-endoperoxide synthase 2 by somatic and spermatogenic cells is responsible for prostaglandin E2 production in the adult rat testis. *Biology of Reproduction* **76** 759–768. (https://doi.org/10.1095/biolreprod.106.053124)

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