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Expression of prostaglandin synthases (*pgds* and *pges*) during zebrafish gonadal differentiation

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ABSTRACT

The present study aimed at elucidating whether the expression pattern of the membrane bound form of prostaglandin E_2 synthase (*pges*) and especially the lipocalin-type prostaglandin D_2 synthase (*pgds*) indicates involvement in gonadal sex differentiation in zebrafish as has previously been found in other species. In mice and chicken, the lipocalin-type *Pgds* is specifically expressed in pre-Sertoli cells just after *Sry* and *Sox9* and is involved in masculinisation of the developing testis. Furthermore, *Pges* are implicated in female reproduction including follicular development and ovulation. In this study, a sexually dimorphic expression of *pgds* was found in gonads of adult zebrafish with expression in testis but not in ovaries. To determine whether the sex-specific expression pattern of *pgds* was present in gonads of juvenile zebrafish and therefore could be an early marker of sex in zebrafish, we microdissected gonads from four randomly selected individual zebrafish for every second day in the period 2–20 days post hatch (dph) and 0–1 dph. The temporal expression of *pgds* and *pges* was investigated in the microdissected gonads, however, no differential expression that could indicate sex-specific difference between individual juvenile zebrafish was observed.

1. Introduction

The mechanism of sex determination is currently not elucidated in zebrafish. No sex chromosomes have been identified and homologues of mammalian and Japanese rice fish (*Oryzias latipes*) testis-determining genes, *Sry* and *dmy*, are not present in the zebrafish genome. Sex determination is therefore most likely mediated by genetic signals from autosomal genes. However, environmental factors such as temperature and hypoxia also affect the sex ratio in zebrafish populations (Uchida et al., 2004; Shang et al., 2006). Although the mechanism of sex determination is diverse among vertebrate species, genes involved in gonadal sex differentiation appear to be relatively conserved (Ijiri et al., 2008; Moniot et al., 2008). The expression pattern of several genes (*dmrt1*, *sox9a*, *fig α*, *ar* and *cyp19a1a*) implicated in gonadal sex differentiation has previously been investigated in zebrafish during the expected time of sex determination and gonadal sex differentiation (2–40 dph), however, this was conducted on whole fish (Jørgensen et al., 2008). Some genes that are implicated in sex differentiation in other vertebrates including *dmrt1*, *sox9a*, *foxl2* and *fig α* show a differential expression pattern between sexes in adult zebrafish (Onichtchouk et al., 2003; Guo et al., 2005; Rodriguez-Mari et al., 2005). This could indicate that genes expressed in a sexually dimorphic pattern in adult zebrafish might also play a role in early gonadal sex differentiation.

In mammals, the testis-determining gene *Sry* initiates the male developmental pathway by inducing *Sox9* expression in pre-Sertoli cells. It appears that the Sry/Sox9 system is rather inefficient and needs a booster to fully masculinise the testis. Thus, *Sox9* upregulates the lipocalin-type prostaglandin D_2 synthase (*Pgds*) leading to prostaglandin D_2 (PGD_2) synthesis and secretion. PGD_2 then mediates an upregulation of *Sox9* expression in an autocrine and paracrine manner, resulting in full masculinisation of the testis (Adams and McLaren, 2002; Wilhelm et al., 2005; Wilhelm et al., 2007a; Wilhelm et al., 2007b). The importance of *Pgds* in early sex differentiation was recently confirmed in chicken where *Pgds* showed a male specific gene expression during testicular development and temporal co-expression with *Sox9* in Sertoli cells (Moniot et al., 2008). As a proof of concept it was shown that addition of exogenous PGD_2 can partially masculinise female embryonic gonads of mice and chicken in culture, probably by inducing female supporting cells (granulosa cells) to differentiate into Sertoli cells (Adams and McLaren, 2002; Moniot et al., 2008). As the genetic cue(s)

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that direct the development of the bipotential gonad towards male or female differentiation in zebrafish are not known, *pgds* could be a candidate for an early marker of sex during gonadal sex differentiation. Involvement of prostaglandins in early zebrafish development including several mesodermal structures and tissues has previously been shown (Cha et al., 2006; Yeh and Wang, 2006). Furthermore, a lipocalin-type prostaglandin D_2 synthase homologue has previously been identified in zebrafish and appears to have evolved from the same ancestral gene as the mammalian *Pgds* (Fujimori et al., 2006).

Prostaglandin E_2 synthase (*pges*) and prostaglandin E_2 (PGE_2) are involved in female reproduction, especially in follicular development and ovulation (Sun et al., 2006). In zebrafish, a recent study has shown that PGE_2 is involved in regulation of oocyte maturation and ovulation (Lister and Van Der Kraak, 2008, 2009). In contrast, studies in goldfish (*Carassius auratus*) have shown that PGE_2 stimulates testosterone production in testis *in vitro* suggesting that PGE_2 may be involved in the control of steroidogenesis in the goldfish testis (Wade and Van der Kraak, 1993; Wade et al., 1994).

Prostaglandins play important roles in many processes including vascular, pulmonary, renal and reproductive physiology. In this study, we investigate a potential role in reproductive biology by determining whether *pgds* and *pges* are expressed in a sex dependent manner in zebrafish during gonadal differentiation. We found a sex-specific difference in expression of *pgds* in gonads of adult zebrafish, which was confirmed by *in situ* hybridisation (ISH). However, we did not observe differential expression of *pgds* and *pges* in microdissected gonads of individual zebrafish during the period from 0 to 20 dph.

2. Materials and methods

2.1. Preparation of tissue from adult zebrafish

Adult male and female zebrafish were anaesthetised in a buffered solution of MS-222 (0.1 g/L) and quickly dissected into brain, gonads, liver, eyes, spleen, heart, gut, kidney, gall bladder, muscle and gills. The dissected tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was purified from approximately 5 mg zebrafish tissue that was homogenised for 20 s using an Ultra-Turrax homogenizer (IKA-Werke GmbH, Germany). RNA was purified using the Total RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA concentration was measured spectrophotometrically (Gene-Quant, Pharmacia Biotech) and the quality verified by gel electrophoresis (1% agarose gel). cDNA was obtained from 0.3 μg RNA using SuperScript II reverse transcriptase kit with Oligo dT primer (Invitrogen, Calsbad, CA, USA) according to manufacturer's instructions. Adult zebrafish used for *in situ* hybridisation were fixed in Stieves fixative as described below.

2.2. Preparation of tissue from juvenile zebrafish

Juvenile zebrafish originated from a brood population of fish. In the evening breeding boxes were placed in an aquarium with parent fish and eggs were collected the following morning. Non-fertilised eggs were removed while the fertilised eggs were placed in 900 mL glass beakers and kept at 26 ± 1 °C and a light–dark period of 14:10 h. In the interval 3–22 dph the larvae were fed two times daily with powdered dry food (Sera Micron, Sera, Heinsburg, Germany) and one time daily with newly hatched *artemia* sp. nauplii (Intér Ryba GmbH, Germany). At 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 dph zebrafish were frozen individually in liquid nitrogen and stored at −80 °C until cryosectioning and staining of gonads (Jørgensen et al. 2009). The 0 dph zebrafish were defined as the day of hatch. Zebrafish used for ISH (2, 5, 10, 15 and 20 dph) were fixed in Stieves fixative (solution I: 90 g $HgCl_2$ in 1.5 L H_2O; solution II: 400 g 40% formaldehyde and 80 g glacial acetic acid; just before use mix 38 mL solution I and 12 mL solution II) at room temperature.

2.3. Identification and isolation of gonads from juvenile zebrafish

Laser Capture Microdissection of gonads from juvenile zebrafish was conducted as previously described (Jørgensen et al. 2009). Briefly, zebrafish were placed in tissue-tek (Sakura Fintek Europe, Zoeterwonde, NL), cooled down on dryice and 20 μm sections were cut on a Cryostat. Sections were mounted on RNase free membrane slides (Molecular Machines & Industries, Glatbrugg, Switzerland) and fixed in 75% ethanol for 10 min at room temperature, followed by 99% ethanol in −80 °C. The sections were stained with NBT BCIP (as described in Jørgensen et al. 2009) and the germ cells/gonocytes were dissected using Olympus SmartCut microdissection system according to manufacturer's instructions. Lysisbuffer from RNAqueous-Micro Kit (Ambion, TX, USA) was added to the microdissected tissue that was stored at −80 °C. RNA was purified using RNAqueous-Micro Kit (Ambion) according to the manufacturer's instructions for microdissected tissue. The quality of the RNA was determined using Agilent Bioanalyser 2100 and Aglient RNA 6000 Pico Kit. The RNA was amplified twice using MessageAmpII™ II aRNA Amplification Kit. After the first round, the amplification was analysed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In order to determine the quality of the RNA, both amplifications were analysed using Agilent Bioanalyser 2100 and Agilent RNA 6000 Pico Kit.

2.4. RT-PCR

Primers for RT-PCR analysis are shown in Table 1. Primers were designed for the lipocalin-type *pgds*, the membrane form of *pges*, the germ cell marker *vasa* and the reference gene *β-actin*. Furthermore, primers were placed in the 3′ end of the gene to compensate for the 5′ lost of microdissected samples and spanning exon–intron. cDNA synthesis of RNA was performed using 50 ng/μL random hexamer primers, cDNA control was performed without RNA. Reverse transcriptase polymerase chain reaction (RT-PCR) using gene specific primers placed just upstream of the polyA site was performed (in final concentrations): 10 mM Tris–HCL, pH 8.3; 50 mM KCl; 1.8 mM $MgCl_2$; 0.1% Triton X-100; 0.0005% gelantine; 250 μM dNTP and 1 pmol/μL primer. The cycle conditions were: one cycle of 2 min at 95 °C; 40 cycles of 30 s at 95 °C, 1 min at 62 °C, 1 min at 72 °C and finally one cycle of 5 min at 72 °C. The amplicon size was checked electrophoretically on a 2% agarose gel for each primer set and bands were excised from the gel and sequenced for verification (Eurofins MWG, GmbH, Germany). EC numbers of *pgds* and *pges* are EC 5.3.99.2 and EC 5.3.99.3, respectively.

2.5. Preparation of biotin labelled probe for in situ hybridisation

Probes for ISH were prepared by RT-PCR amplification of *pgds* and *pges* transcripts and reamplification of PCR fragments using nested primers specific to the fragments with an added T3-promotor sequence in combination with the T7-extended downstream primer (Table 2). PCR conditions were: 5 min 95 °C; five cycles of 30 s 95 °C, 1 min 45 °C, 1 min 72 °C; 20 cycles of 30 s 95°C, 1 min 65°C, 1 min 72 °C and finally 5 min 72 °C. The resulting PCR product was purified on a 1% agarose gel and sequenced (Eurofins MWG, GmbH, Germany).

Table 1
Oligonucleotide primers used for RT-PCR analysis.

Primer	Amplicon	Sense primer 5′–3′	Antisense primer 5′–3′
pgds	221 nt	cgcgtggttgacgccaagt	cggcaactgagcattctcca
pges	273 nt	gagctgctgccatgtggatc	catgttgtgtggccttctgca
vasa	295 nt	tggcgtctcctggtatgcctt	accacaccaacagcaaggaa
β-actin	272 nt	cctgaccgagagaggctaca	cgcaagattccatacccaag