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Temporal expression and steroidal regulation of piRNA pathway genes (*mael*, *piwi*, *vasa*) during *Silurana* (*Xenopus*) *tropicalis* embryogenesis and early larval development

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ABSTRACT

It has been extensively documented that exposure of amphibians and teleost fish to exogenous steroid hormones like estrogen, androgen, xenoestrogen or steroid biosynthesis inhibitors can impair their gonadal development or induce sex reversal against genotypic sex. However, the molecular pathways underlying sexual development and the effects of sex steroids or other exogenous hormones in these aquatic vertebrates remain elusive. Recently, a germ plasm-associated piRNA (piwi-interacting RNA) pathway has been shown to be a determinant in the development of animal gonadal germline cells. In the current study, we examined whether this piRNA pathway is involved in the regulation of sex steroid hormones in gonadal development. We firstly established developmental expression patterns of three key piRNA pathway genes (*mael*, *piwi* and *vasa*), during *Silurana* (*Xenopus*) *tropicalis* embryogenesis and early larval development. All three genes exhibit high expression at early developmental stages and have significantly decreased expression thereafter, indicating a very active involvement of piRNA pathway at the beginning of embryogenesis. We further examined gene expression changes of those genes in frog larvae exposed to two sex steroid biosynthesis inhibitors, fadrozole and finasteride, both of which are known to result in male-biased or female-biased phenotypes, respectively. We found that fadrozole and finasteride exposures increased the expression of piRNA pathway genes such as *mael* and *vasa* at the larval stage when the expression of piRNA pathway genes is programmed to be very low. Therefore, our results indicate that the piRNA pathway is likely a common pathway by which different sex steroid hormones regulate gonadal sex differentiation.

1. Introduction

Gonadal development involves the differentiation of both germline and somatic cells. In this process, germline cells specifically differentiate into sperm (spermatogenesis) or eggs (oogenesis), whereas somatic cells differentiate into interstitial gonadal tissue (Hayes, 1998). Sex steroid hormones including estrogen (e.g., 17-beta-estradiol, E2) and androgen (e.g., testosterone, T and 5-alpha-dihydrotestosterone, 5α-DHT) exert critical roles in regulating amphibian gonadal sex differentiation (Hayes, 1998). It has been extensively demonstrated that exposure of different amphibian species to sex steroids (Saidapur et al., 2001), estrogenic or androgenic anthropogenic chemicals (e.g., 17alpha-methyltestosterone, MT, 17alpha-ethinylestradiol, EE2 and bisphenol A) (Levy et al., 2004; Pettersson et al., 2006; Hogan et al., 2008) or aromatase inhibitors (e.g., fadrozole) (Olmstead et al., 2008) can alter gonadal differentiation and result in the presence of intersex phenotypes, female- or male-biased sex ratio, or even sex reversal (complete feminization or masculinization). However, molecular pathways underlying hormonal regulation of gonadal development remain largely elusive. Since sex reversal by exogenous chemicals necessarily requires the regeneration or reprogramming of germline cells which are the precursors of sperm and eggs, in this study we are motivated to explore certain molecular pathways that mediate the regulation of germline cells by sex steroid hormones.

One common characteristic of germline cells among different species is the presence of a morphologically unique organelle called the germ plasm which is also referred to as nuage, dense bodies, mitochondrial clouds or germinal granules (Ikenishi, 1998). This organelle is considered the determinant of germline development since only cells inheriting the germ plasm in the early stage of embryogenesis can develop into primordial germ cells (PGC) (Ikenishi, 1998). Furthermore, transplantation of the germ plasm to the presumptive somatic region results in PGC formation (Illmensee and Mahowald, 1974). Recently, a new type of small RNAs called piwi-interacting small RNAs (piRNAs) and a series of germ plasm-specific proteins including vasa, piwi, maelstrom (mael) have been identified

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to regulate germline development and silence certain endogenous genetic elements such as retrotransposons (Vagin et al., 2006; Klattenhoff and Theurkauf, 2008). Genetic mutations of these genes are commonly associated with a huge reduction in the amount of piRNAs, an increase in transcript level of transposable elements in germline cells (Lim and Kai, 2007; Nishida et al., 2007), a failure in establishing anterior/posterior polarity in early oocytes, disrupted asymmetric subcellular mRNA localization of *Oskar*, *Gurken* and *Biocoid*, ectopic expression of Oskar and Gurken, and a failure to proceed to the karyosome stage (Findley et al., 2003; Lim and Kai, 2007; Klattenhoff and Theurkauf, 2008). Given the importance of this piRNA pathway and the germ plasm in germline development, we hypothesize that the piRNA pathway and its associated proteins may be direct targets of sex steroid hormone actions.

In our recent studies, we have profiled the gene expression of many sex steroid-related genes such as estrogen receptor α, β, androgen receptor, aromatase and 5α/β-reductases in *Silurana* (*Xenopus*) *tropicalis* model (Duarte-Guterman et al., 2010; Langlois et al., 2010). The results showed that sex steroid-related genes are expressed as early as NF2 (2-cell stage). In addition we have also measured aromatase activity and 5β-reductase activity in eggs and embryos and we found that enzyme activity is already detected at NF7 (the earliest stage tested). Detection of sex steroid synthesis enzyme activity suggests that sex steroids are being produced much earlier than NF46. Therefore, in the present study we intend to clarify the potential steroidal regulation of germline piRNA pathway during embryogenesis and early larval development, by 1) profiling developmental expression patterns of three key piRNA pathway genes, *mael*, *piwi*, and *vasa*; 2) and examining expression changes for those genes in frog larvae exposed to two sex steroid biosynthesis inhibitors fadrozole and finasteride, either of which are known to result in male-biased or female-biased phenotypes in tadpoles, respectively.

2. Materials and methods

2.1. Tissue collection for developmental profiles

Eggs and larvae of *S.* (*X.*) *tropicalis* were raised in Petri dishes containing modified Ringer's solution (0.1 M NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 300 mg/L $NaHCO_3$; 1:9 v/v) and 0.04 mg/L of the antibiotic gentamycin (Sandoz Canada Inc.). Whole embryos and larvae were sampled at Nieuwkoop and Faber (NF) (Nieuwkoop and Faber, 1994) 2, 7, 16, 21, 26–27, 34, 41 and 46, which corresponds to 1, 4.5, 13, 15, 20, 36, 44 and 72 h post-fertilization (hpf) under our husbandry conditions. Pools of 20–25 embryos and larvae were used to ensure a sufficient amount of RNA for gene expression ($n=6$–8). Embryos were frozen immediately in dry ice and stored at −80 °C. Larvae at NF 41 and NF 46 were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222; 0.01%; Sigma) before freezing.

2.2. Fadrozole and finasteride exposure

Eggs were allowed to develop to NF 6, at which point they were collected and dejellied with 2% (w/v) L-cysteine (pH 8.0; Sigma). The eggs were washed 3 times with modified Ringer's solution (1:9 v/v) following cysteine treatment and placed in Petri dishes (containing modified Ringer's solution and antibiotic) at a density of 50 eggs per dish. Embryos were exposed from NF 12 to NF 46 (8 to 72 hpf) to nominal concentrations of fadrozole (0.5, 1.0, 2.0 μM; Novartis Pharma AG, Basel, Switzerland) dissolved in water and finasteride (25, 50, 100 μM; Sigma Canada Ltd, Oakville, ON) delivered in ethanol (0.05%). Embryos were also exposed to water and ethanol (0.05%) controls. Throughout the exposure, the medium and antibiotic (Gentamycin, Sandoz Canada Inc., Boucherville, QC, Canada) were refreshed daily. Whole NF 46 larvae were sampled from each treatment for gene expression (10 per sample; $n=5$–8).

2.3. RNA extraction, quality assessment and cDNA synthesis

Total RNA was isolated using RNase Micro Kit (Qiagen, Mississauga, ON, Canada) as described in the manufacturer's protocol. Total cDNA was prepared from 2 μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H-reverse transcriptase (SSII) as described by the manufacturer (Invitrogen). A no reverse transcriptase control (NRT) was made where RNase-free water was added to the cDNA synthesis reaction instead of the SSII enzyme.

2.4. Real-time RT-PCR assay

SYBR green real-time RT-PCR assays were used to measure the relative mRNA expression of *mael*, *piwi*, and *vasa*. Primers (Table 1) were designed using Primer3 (You et al., 2008) and synthesized by Invitrogen. The Mx3000 Quantitative PCR System (Stratagene, La Jolla, CA, USA) was used to amplify and detect the transcripts of interest. Each real-time RT-PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3.5 mM $MgCl_2$, 150–300 nM gene-specific primer (Invitrogen), 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100 nM ROX reference dye, in a 25 μL reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 59–60 °C for 5 s, 72 °C for 30 s, and a detection step at 80 °C for 8 s. Dilutions of a cDNA mix from all samples were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55 °C (+1 °C/30 s) to 95 °C. Samples were run in duplicate along with a no-template control (RNase-free water was added to the reaction instead of the cDNA template) and a NRT control (previously described). The SYBR green assay for every target gene was optimized for primer concentration and annealing temperature to obtain for the standard curve an $R^2>0.985$, amplification efficiency between 90 and 110% and a single sequence-specific peak in the dissociation curve.

2.5. Statistical analysis

Data were analyzed using the MxPro QPCR software package (Stratagene). The relative mRNA level of each gene was normalized based on total RNA content. All data were first tested for normality and those data with non-normal distribution were subjected to log transformations prior to statistical analyses. A one-way analysis of variance (ANOVA) followed by a Holm–Sidak post-hoc test in SigmaStat 3.5 (SPSS Inc.) was used to evaluate significant changes in gene expression between samples ($p<0.05$).

3. Results and discussion

3.1. Developmental profiles of piRNA genes

For the first experiment, we established developmental profiles of *mael*, *piwi* and *vasa* from NF 2 until NF 46 in *S. tropicalis* (Fig. 1). Both *mael* and *piwi* exhibit the highest expression at the first two sampled stages (NF 2 and 7) and very low expression after NF 24. In contrast,

Table 1
Oligonucleotide primers used for real-time RT-PCR assays.

Target gene	GenBank ID	Sequence (5′–3′)	Amplicon size
mael	NM_001079177	F: ATCCCCATCTCTGGCTTTG R: GGTTTTCCATTCCTGCTTTG	178 bp
piwi	NM_001016823	F: GGCAGGGGGTTTCTTTCTAC R: TGTGGCTTTTCCATACTCACC	179 bp
vasa	NM_001016823	F: GCAAAAGAGGAGCGACAAAC R: TAATCCGACCACGACAAACA	111 bp