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Research paper

Expression of *sf1* and *dax-1* are regulated by thyroid hormones and androgens during *Silurana tropicalis* early development

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ABSTRACT

Thyroid hormones (THs) and androgens have been shown to be extensively involved in sexual development; however, relatively little is known with regard to TH-related and androgenic actions in sex determination. We first established expression profiles of three sex-determining genes (*sf1*, *dax-1*, and *sox9*) during the embryonic development of Western clawed frogs (*Silurana tropicalis*). Transcripts of *sf1* and *sox9* were detected in embryos before the period in which embryonic transcription commences indicating maternal transfer, whereas *dax-1* transcripts were not detected until later in development. To examine whether TH status affects sex-determining gene expression in embryonic *S. tropicalis*, embryos were exposed to co-treatments of iopanoic acid (IOP), thyroxine (T4), or triiodothyronine (T3) for 96 h. Expression profiles of TH receptors and deiodinases reflect inhibition of peripheral deiodinase activity by IOP and recovery by T3. Relevantly, elevated TH levels significantly increased the expression of *sf1* and *dax-1* in embryonic *S. tropicalis*. Further supporting TH-mediated regulation, examination of the presence and frequency of transcription factor binding sites in the putative promoter regions of sex-determining genes in *S. tropicalis* and rodent and fish models using *in silico* analysis also identified TH motifs in the putative promoter regions of *sf1* and *dax-1*. Together these findings advocate that TH actions as early as the period of embryogenesis may affect gonadal fate in frogs. Mechanisms of TH and androgenic crosstalk in relation to the regulation of steroid-related gene expression were also investigated.

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1. Introduction

Sex determination is highly diverse in vertebrates. Sex determining mechanisms have broadly been divided into either genotypic sex determination (GSD) or environmental sex determination (ESD). In GSD, inherited sex chromosomes at fertilization determine gonadal fate and the ensuing sex differentiation (Barske and Capel, 2008; reviewed in Vilain and McCabe, 1998); whereas in ESD, sexual fate is controlled by environmental factors experienced after fertilization (Sarre et al., 2004; Valenzuela and Lance, 2004; Bull, 1983). Endocrine processes provide the foundation for sex determination and subsequent gonadal formation in all vertebrate species. A growing body of literature advocates that endocrine disruption can overcome the sex-determining program irrespective of GSD or ESD (Mizoguchi and Valenzuela, 2016; Golan and Levavi-Sivan, 2014; Nakamura, 2010). Thyroid hormones (THs) have pleiotropic effects in developing vertebrates, including effects on gonadal development. Several studies have demonstrated that TH status alters sex ratios in fish (Sharma and Patiño (2013); Mukhi et al., 2007; Bernhardt et al., 2006) and amphibians (Goleman et al., 2002). THs have been shown to crosstalk with both the estrogen and androgen axes regulating sex steroid-related transcription and production (reviewed in: Duarte-Guterman et al., 2014; Flood et al., 2013; Habibi et al., 2012; Wajner et al., 2009; Wagner et al., 2008; Cooke et al., 2004; Maran, 2003). Although our knowledge of the molecular mechanisms underlying TH mediated reproductive effects is increasing, relatively little is known regarding sex determination.

We previously identified potential crosstalk via several candidate sex-determining genes imperative to bipotential gonad formation and differentiation (Flood et al., 2013). The steroidogenic factor 1 (*sf1*) is encoded by the NR5A1 gene and is important for sexual differentiation as it is expressed in primordial organ cell clusters fated to differentiate into mammalian adrenal glands,

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testes, and ovaries (reviewed in: Hoivik et al., 2010; Parker and Schimmer 2002; Vilain and McCabe, 1998). Moreover, *sf1* is considered a master regulator of steroidogenic-related genes. Numerous studies have demonstrated the ability of TH status to not only influence steroid hormone production, but also the underlying transcriptional activity (Duarte-Guterman et al., 2014; Flood et al., 2013). The widespread effects of THs on steroidogenesis could thus be mediated via *sf1*. Another sex-determining gene of interest is *dax-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1). Encoded by NR0B1, *dax-1* expression is restricted to tissues directly involved in steroid hormone production and reproductive function. This gene served primarily as a negative regulator by binding to the promoter of different genes, including but not limited to, *sf1*, androgen receptor (*ar*), and aromatase (*cyp19*) (reviewed in: Orekhova and Rubtsov, 2015; Lalli and Sassone-Corsi, 2003). The expression and activity of *Dax-1* has been shown to be essential for normal testicular development in vertebrates (reviewed in: Iyer and McCabe, 2004; Lalli and Sassone-Corsi, 2003; Parker and Schimmer, 2002). Studies have shown that Dax-1 can negatively regulate thyroid hormone receptor transcription (Valadares et al., 2008; Moore et al., 2004); however, reciprocal TH regulation has not yet been investigated. The sex-determining region Y box 9 (*sox9*) is a male-specific transcription factor that mediates testis differentiation (Kobayashi et al., 2005; Kent et al., 1996). Outside the reproductive axes, studies showed that THs can influence *sox9* transcript levels. Okubo and Reddy, (2003) observed that *sox9* expression in *Mus musculus* chondrocytes significantly decreases with thyroxine (T4) exposure. Thus, THs may regulate *sox9* expression during the period of sexual determination and gonadal formation. Sex-determining genes initiate a cascade of genetic events that play a crucial role in vertebrate gonadal differentiation as well as sexual development. Consequently, TH mediated regulation of sex-determining genes may have long-lasting effects on subsequent development.

All three genes (*sf1*, *dax-1*, and *sox9*) are co-expressed in precursor testis and ovary cells within the gonadal ridge of embryonic vertebrates (Kobayashi et al., 2005; Hoyle et al., 2002; Kent et al., 1996; Ikeda et al., 2001, 1994). The genital ridge is formed during the period of embryogenesis (i.e., 72 h post-fertilization (hpf) in amphibians; El Jamil et al., 2008) and 10 days postcoitum (dpc) in mice (Tanaka and Nishinakamura, 2014; Kent et al., 1996). Thyroid gland organogenesis begins to form at approximately Nieuwkoop and Faber (NF) stage 40 (~72 hpf) with consequential I^- uptake at approximately NF stage 46 (~96 hpf) in the frog *Xenopus laevis* (Brown, 2005). Thyroid gland activity is not detected until post-partum in mice; however, the fetus' TH requirements are met via the placenta (reviewed in Darras et al., 2015). However, several studies have detected deiodinase (*dio*) transcription and activity during the period of embryogenesis in amphibians (*Silurana tropicalis*: Tindall et al., 2007; *X. laevis*: Morvan Dubois et al., 2006) and unlike their mammalian counterparts, *dio*s serve as the only source for *de novo* production of THs prior to thyroid gland activity. Therefore, dio-related transcription and activity may play a putative role in gonadal fate in amphibians.

To understand the putative role of THs on sex-determining gene expression prior to gonadal differentiation, we first established expression profiles of the *sf1, sox9, and dax-1* from the commencement of amphibian embryogenesis (NF stage 2) to the beginning of larvae development (NF stage 46). Embryos (NF stage 10–12) were exposed to co-treatments of iopanoic acid (10 μM; IOP), thyroxine (5 nM; T4), or triiodothyronine (50 nM; T3) for 96 h to examine whether TH status affects sex-determining gene expression in embryonic *S. tropicalis*. Embryos were also exposed to either T3 (0.5, 5, or 50 nM) or 5α-dihydrotestosterone (5α-DHT; 4, 40, or 400 nM) to further study TH and androgenic mechanisms of crosstalk in relation to the regulation of sex-determining gene expression. We also conducted novel *in silico* promoter analysis to assess the presence and frequency of putative transcription factor binding sites in *S. tropicalis* and compare it with rodent and fish models.

2. Materials and methods

2.1. Animals

Sexually mature male and female *S. tropicalis* frogs were housed in the Queen's University Animal Care Facility (Kingston, ON, Canada). Adults were reared in tanks containing dechlorinated and aerated water (25 ± 1 °C) on a 12:12 h light:dark regime (light commencing at 0700 h). Fertilized eggs were obtained from three pairs of adult frogs and were mixed together in order to remove any clutch effect. Spawning was artificially induced by injecting human chorionic gonadotropin hormone (hCG; 2500 IU/mL; Sigma Canada Ltd., Oakville, ON, Canada) into the dorsal lymph sac. Both males and females received a priming injection of 50 μL hCG (12.5 IU) followed by a boosting injection of 200 μL hCG (100 IU) after 24 h, as previously outlined by Flood and Langlois (2014). Eggs were present within 2–3 h post-injection. Developmental stages were determined following the Nieuwkoop and Faber (NF) developmental staging system (Nieuwkoop and Faber, 1994). Animal care was performed in accordance with the guidelines of the Animal Care Committee of Queen's University and the Canadian Council on Animal Care.

2.2. Developmental profile

Samples of whole embryos were taken at different NF stages of development: 2, 7, 16, 21, 27, 34, 41, and 46. At each stage, embryos were pooled (20 embryos for NF 2 to NF 34 and 10 embryos for NF 41 and NF 46) to ensure sufficient material for RNA isolation. Pools (n = 6–8 per NF stage) were flash frozen on dry ice and stored at −80 °C for further analysis.

2.3. Exposures and material

Eggs were allowed to develop to NF stage 8, at which point they were collected and de-jellied with 2% (w/v) l-cysteine (pH 8.0; Sigma Canada Ltd., Oakville, ON, Canada) for 2 min. The eggs were washed three times with 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 $NaHCO_3$). Two experiments were run in parallel using methodology previously outlined by Flood and Langlois (2014). In the first experiment, we examined whether TH status affects sex-determining gene expression in embryonic *S. tropicalis*. Embryos (NF stage 10–12) were placed in either modified Ringer's solution (1:9 v/v) or one of six test solutions in 125-mL glass jars at a density of 50 embryos per jar. Embryos were exposed to DMSO (0.001%), 10 μM of IOP (TCI America), a co-treatment of 10 μM IOP + 50 nM T3 (Sigma, Oakville, Ontario, Canada), a co-treatment of 10 μM IOP + 5 nM T4 (Sigma, Oakville, Ontario, Canada), 5 nM T4 or 50 nM of T3. Of note, IOP is a known inhibitor of the enzymatic activity of both dio2 and dio3 in frogs (Becker et al., 1997). In all exposures, a concentration of 0.04 ppm of the antibiotic gentamycin (Sandoz Canada, Inc Boucherville, QC, Canada) was administered every 24 h. Water changes occurred every 24 h until NF stage 46. At NF stage 46 embryos were pooled (10 embryos per pool) to ensure sufficient material for RNA isolation. Pools (n = 6–8 per treatment) were flash frozen on dry ice and stored at −80 °C for further analysis. In the second experiment, we examined TH and androgenic mechanisms of crosstalk in embryonic *S. tropicalis*. Embryos (NF stage 10–12) were placed in either modified Ringer's solution

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