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Exogenous estradiol alters gonadal growth and timing of temperature sex determination in gonads of sea turtle

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ABSTRACT

Temperature sex determining species offer a model for investigating how environmental cues become integrated to the regulation of patterning genes and growth, among bipotential gonads. Manipulation of steroid hormones has revealed the important role of aromatase in the regulation of the estrogen levels involved in temperature-dependent sex determination. Estradiol treatment counteracts the effect of male-promoting temperature, but the resulting ovarian developmental pattern differs from that manifested with the female-promoting temperature. Hypoplastic gonads have been reported among estradiol-treated turtles; however the estradiol effect on gonadal size has not been examined. Here we focused on the sea turtle *Lepidochelys olivacea*, which develops hypoplastic gonads with estradiol treatment. We studied the effect of estradiol on cell proliferation and on candidate genes involved in ovarian pattern. We found this effect is organ specific, causing a dramatic reduction in gonadal cell proliferation during the temperature-sensitive period. Although the incipient gonads resembled tiny ovaries, remodeling of the medullary cords and down-regulation of testicular factor Sox9 were considerably delayed. Contrastingly, with ovarian promoting temperature as a cue, exogenous estradiol induced the up-regulation of the ovary factor FoxL2, prior to the expression of aromatase. The strong expression of estrogen receptor alpha at the time of treatment suggests that it mediates estradiol effects. Overall results indicate that estradiol levels required for gonadal growth and to establish the female genetic network are delicately regulated by temperature.

1. Introduction

After Charnier (Charnier, 1966) discovered that incubation temperature influences sex determination, several studies have sought to explain the mechanism(s) by which temperature affects the gene networks underlying gonadal sex determination. Pioneer studies used treatments with estrogens (E2), antiestrogens and aromatase inhibitors, which could counteract the sex-promoting effect of temperature. This led to the hypothesis that endogenous E2 levels in the bipotential gonad are directly involved in the process of ovary or testis commitment (Bull et al., 1988; Pieau, 1974). Thus, incubation temperature was assumed to regulate the expression and/or activity of aromatase, the enzyme that converts androgens to estrogens and plays a key role in sex determination of species with temperature-dependent sex determination (TSD) (Pieau and Dorizzi, 2004).

Furthermore, the temperature-dependent activity of aromatase in the sea-turtle *Dermochelys coriacea* (Desvages et al., 1993) and the dimorphic expression of the aromatase gene in *Emys orbicularis* (Desvages and Pieau, 1992) *Trachemys scripta* (Ramsey et al., 2007), *Chelydra serpentina* (Rhen et al., 2007), and *Crysemys picta* (Valenzuela et al., 2013) support the role of endogenous E2 levels in chelonian sex determination. However, although exogenous manipulation of gonadal estrogen levels can override the temperature cues, the proximate mechanisms by which temperature exerts its action remain unknown.

Important differences are detected in the gene expression profiles of bipotential gonads that either develop into ovaries under the effect of female-promoting temperature (FPT), or form ovaries when induced with exogenous E2 at the male promoting temperature (MPT) (Matsumoto et al., 2013; Ramsey and Crews, 2009; Valenzuela et al., 2013). For example, steroid hormone receptors (ER alpha, ER beta and androgen receptor) show different expression patterns in *Chelydra serpentina* (Rhen et al., 2007) and *Trachemys scripta* (Ramsey and Crews, 2007). However, since the

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MPT and FPT induce dimorphic expression of aromatase at the end of the temperature-sensitive period (Desvages et al., 1993; Desvages and Pieau, 1992; Ramsey et al., 2007) thresholds of endogenous E2 levels involved in gonadal differentiation may be tightly regulated by temperature prior to the stabilization of transcriptional networks. Steroid hormone and metabolite levels in developing gonads depend on regulatory gene networks. These mainly involve autonomous cell transcription, cell-cell signaling and transducing factors. In mammals and birds, FoxL2 regulates aromatase expression (Batista et al., 2007; Govoroun et al., 2004; Hudson et al., 2005; Pannetier et al., 2006). Similar regulation is inferred in reptiles with TSD; however, direct evidence is not yet available. In the slider turtle, higher FoxL2 and aromatase levels are expressed in gonads from embryos at FPT than at MPT (Loffler et al., 2003; Matsumoto and Crews, 2012; Ramsey et al., 2007; Shoemaker et al., 2007). Furthermore, gonads of the snapping turtle *Chelydra serpentina* shifted from MPT to FPT show up-regulation of FoxL2 and aromatase (Rhen et al., 2007).

Steroid hormone signals involve the corresponding receptors. In the slider turtle, ER alpha is expressed in bipotential gonads at both male- and female-promoting temperatures, but the expression drops during ovarian differentiation (Bergeron et al., 1998; Matsumoto and Crews, 2012; Ramsey and Crews, 2007), in contrast, the opposite occurs in the snapping turtle, *Chelydra serpentina* (Rhen et al., 2007).

Several genes present in species with TSD are conserved in mammals, where they are associated with gonadal sex determination and maintenance of the differentiated state (Trukhina et al., 2013). The expression schedule of these genes differs among species, indicating that the gonadal sex networks are diversified. In male mice, Sry up regulates *Sox9* in medullary supporting cells; while in females, *Sox9* remains downregulated in bipotential gonads prior to morphological differentiation (da Silva et al., 1996; Moreno-Mendoza et al., 2003). In contrast, in reptiles with TSD Sox9 is expressed in medullary cords during several days in bipotential gonads at both, MPT and FPT (Barske and Capel, 2010; Moreno-Mendoza et al., 1999). Thus, the dimorphic regulation of Sox9 in turtles with TSD offers a good marker to study the possible effects of experimental manipulation of endogenous steroid hormones on gonadal morphogenesis.

In mice, several genes upstream Sry were found to be necessary for cell proliferation at the onset of the gonadal ridge establishment (Sekido and Lovell-Badge, 2013). For example, in *Lhx9* knockout mice, cell proliferation is inhibited in the incipient genital ridges and gonads fail to form (Birk et al., 2000). Although *Wt1* and *Sf1* knockout mice also lack gonads, the role of these genes is more related to cell survival than to cell proliferation in the presumptive genital ridges (Kreidberg et al., 1993; Luo et al., 1994). Recent studies show that Wnt4/Rspo1 control cell proliferation in the genital ridges of both sexes, cell proliferation diminishes and affects gonad development. In XX double knockout gonads, the effect of reduced cell proliferation is less pronounced than in XY gonad development where testes growth is disrupted. The low numbers of pre-Sertoli cells leads to formation of scarce seminiferous cords and, consequently, the testes remain small (Chassot et al., 2012).

Chromatin remodeling plays an important role in murine developing gonads. The chromobox homolog 2 (Cbx2 or M33), subunit of the polycomb repressive complex 1, is a member of the chromatin-associated complex (Simon and Kingston, 2009). *Cbx2* knockout mice (Katoh-Fukui et al., 1998) and mutant *CBX2* in humans (Biason-Lauber et al., 2009) develop hypoplastic gonads and male-to-female sex reversal. Furthermore, mating of a *Cbx2*$^{-/-}$ strain with mice in which expression of Sry or Sox9 was forced prevented male-to-female sex reversal but the testes remained smaller in *Cbx2*$^{-/-}$ transgenic mice (Katoh-Fukui et al., 2012). Thus, results indicate that in mice, gonadal growth and sex determination are independent processes; in these two arrangements of Cbx2 target genes are involved in two parallel pathways.

The role of *Lhx9, Wt1, Sf1, Wnt4, Rspo1* and *Cbx2* involved in gonadal growth in mice remains unknown in species with TSD. Counteracting the sex-promoting effect of temperature with exogenous estrogen frequently affects gonad size in turtles with TSD: *Emys orbicularis* (Pieau and Dorizzi, 2004), *Lepidochelys olivacea* (Merchant-Larios et al., 1997) and *Trachemys scripta* (Barske and Capel, 2010; Bieser et al., 2013). Therefore, it is likely that E2 treatments may distinguish between growth, on the one side, and sex determination and differentiation on the other, as two separate processes in the gonads of TSD species.

Organ size depends on cell number, cell size and the amount of intercellular matrix. In developing organs, cell proliferation, growth and death rely on the integration of intracellular programs and on extracellular signaling factors that regulate these programs (Conlon and Raff, 1999). Some mechanisms involved in both, maintaining proportional organ growth and integrating environmental cues with sensitive developmental cues, have been elucidated in Drosophila (Mirth and Shingleton, 2012); however, such mechanisms remain to be investigated in species with TSD.

The aim of the current study was to investigate the effect of exogenous estradiol on developing gonads of the olive ridley turtle, *Lepidochelys olivacea*. We compared cell proliferation patterns and genes involved in ovary commitment of E2-treated embryos at MPT. Our results indicate that exogenous E2 alters two key processes involved in ovarian determination and differentiation: gonadal growth rate and timing of the dimorphic regulation of Sox9, *Cyp19a1* (aromatase) and *FoxL2*.

2. Materials and methods

2.1. Animals

This study was approved by the local Ethics Committee at the Instituto de Investigaciones Biomedicas, UNAM. Eggs of *L. olivacea* (sea turtle) were collected at La Escobilla Beach, Oaxaca, Mexico, and transported to Mexico City within 12 h after collecting. The eggs were kept in boxes with moistened vermiculite and incubated at either 26 ± 0.5 °C male-promoting temperature (MPT) or 33 ± 0.5 °C female-promoting temperature (FPT) (Merchant-Larios et al., 1997).Temperature fluctuations were monitored with data loggers. Experiments were performed using eggs from seven different clutches (2010–2013); developmental stages were determined according to Miller's criteria (Miller, 1985).

2.2. Total RNA extraction, cDNA synthesis and end-point PCR

The tissue was quickly pooled and frozen in dry ice. Samples were stored at −60 °C until RNA extraction. Total RNA was extracted with Trizol following the manufacturer's protocol (Invitrogen, Life Technologies, USA), then treated with Turbo DNA-free Kit (Ambion, Life Technologies, USA), and reverse-transcribed into cDNA using transcriptor reverse transcriptase (Roche, Life Science) and 900 ng–1 μg of total RNA. Relative gene expression of aromatase, ERα and FoxL2 was assayed by end-point PCR. Negative controls were prepared without template and with 50 ng of each total RNA extraction. Oligonucleotides were designed using the published Esr1 (estrogen receptor alpha) sequence for *L. olivacea* (Chavez et al., 2009) aromatase and FoxL2 of sequences reported for *T. scripta* (AF178949.1 and AY155535.1 respectively), Actb (beta-actin) amplification was used as endogenous expression control.

The oligonucleotide sequences were: aromFw1 5′ ATGCATTCCAATATCACCAG-3′, aromRv3 5′-CACATTGATGTTTCCCAGTC-3′, Esr1Fw 5′-

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