General and Comparative Endocrinology 236 (2016) 35–41

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Dimorphic DNA methylation during temperature-dependent sex determination in the sea turtle *Lepidochelys olivacea*

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ARTICLE INFO

Article history:
Received 10 February 2016
Revised 13 June 2016
Accepted 19 June 2016
Available online 21 June 2016

Keywords:
Temperature sex determination
DNA methylation/demethylation
Spatiotemporal 5hmC
Gonadal development
Sea turtle

ABSTRACT

Sex determination in vertebrates depends on the expression of a conserved network of genes. Sea turtles such as *Lepidochelys olivacea* have temperature-dependent sex determination. The present work analyses some of the epigenetic processes involved in this. We describe sexual dimorphism in global DNA methylation patterns between ovaries and testes of *L. olivacea* and show that the differences may arise from a combination of DNA methylation and demethylation events that occur during sex determination. Irrespective of incubation temperature, 5-hydroxymethylcytosine was abundant in the bipotential gonad; however, following sex determination, this modification was no longer found in pre-Sertoli cells in the testes. These changes correlate with the establishment of the sexually dimorphic DNA methylation patterns, down regulation of *Sox9* gene expression in ovaries and irreversible gonadal commitment towards a male or female differentiation pathway. Thus, DNA methylation changes may be necessary for the stabilization of the gene expression networks that drive the differentiation of the bipotential gonad to form either an ovary or a testis in *L. olivacea* and probably among other species that manifest temperature-dependent sex determination.

1. Introduction

Development of the bipotential gonad to form either an ovary or a testis depends on the dimorphic expression of a conserved network of genes (Davidson and Erwin, 2006; Shoemaker and Crews, 2009). However, the mechanisms that trigger the establishment of male or female gene expression patterns differ among taxa. Most organisms have genetic sex determination, where sex is determined by the genetic background after fertilization (Ezaz et al., 2006). Therefore, differences in sex-specific alleles are responsible for activation of male or female differentiation pathways (Matsuda et al., 2007). Among mammals, genetic sex determination relies on the XX and XY sex chromosomes, in which the *Sry* gene encoded in the Y chromosome acts as the testis-determining factor (Koopman et al., 1991). Among birds, the *Dmrt1* gene is encoded in sexual chromosome Z and dosage of this gene in paired ZZ and ZW chromosomes is important for sex determination (Smith et al., 2009).

Among vertebrates with environmental sex determination, sex depends on external cues, mostly in the absence of sex chromosomes (Rhen and Schroeder, 2010). Several species of turtles, lizards and all crocodiles are subject to temperature-dependent sex determination (TSD) (reviewed in Merchant-Larios and Díaz-Hernández (2013)). In these organisms, the molecular mechanism(s) involved in translation of temperature cues to dimorphic regulation of sex-determining genes in the gonad remain poorly understood. Epigenetic mechanisms play an important role in translating environmental and cellular stimuli into gene expression patterns and may also be involved in TSD (Navarro-Martin et al., 2011; Matsumoto et al., 2013; reviewed in Piferrer (2013)). Among epigenetic mechanisms, some directly affect DNA molecules, such as methylation of carbon 5 of cytosine in the CpG context (5mC), associated with transcriptional repression and stabilization of transposable elements. DNA methylation is important in inactivation of the X-chromosome among mammals and Z chromosome in birds (Simon et al., 2013; Teranishi et al., 2001; Yang et al., 2011). Furthermore, active DNA demethylation has recently been described in many embryonic cell types, proving

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http://dx.doi.org/10.1016/j.ygcen.2016.06.026
0016-6480/

DNA methylation and demethylation to be highly dynamic during development (Kinney et al., 2011; Szwagierczak et al., 2010). In particular, DNA demethylation involves hydroxylation of 5mC (5hmC) and its subsequent excision and replacement by a non-methylated cytosine (Sun et al., 2014; Ito et al., 2010; Koh et al., 2011). Another versatile epigenetic mechanism is that associated with a sophisticated set of histone post-translational modifications. Different histone residues can be targets for changes in acetylation, methylation, ubiquitination, or phosphorylation, among others that have direct effect on gene expression with biological significance (Gardner et al., 2011; Zentner and Henikoff, 2013).

Recent reports correlate epigenetic processes with sex differentiation: DNA methylation is necessary for Sertoli cell differentiation and formation of testicular cords in murine gonads (Mizukami et al., 2008). In birds, the DNA methylation over the promoter sequence of the aromatase gene *CYP19A1* is more abundant in male than in female gonads (Ellis et al., 2012). In species with TSD, such as the European sea bass, it has also been found that the aromatase promoter shows higher levels of DNA methylation in males than in females (Navarro-Martin et al., 2011). This type of regulation has also been described in two reptile species: the red-eared turtle *Trachemys scripta* (Matsumoto et al., 2013) and the alligator *Alligator mississippiensis* (Parrott et al., 2014). Furthermore, in the alligator, a putative promoter sequence of the *Sox9* gene showed higher DNA methylation levels in gonads from embryos incubated at female-promoting temperature (FPT) than when they were incubated at the male-promoting temperature (MPT) (Parrott et al., 2014). These results strongly corroborate the idea that epigenetic modifications are involved in the regulation of certain genes from the sex-determining networks in species with TSD. However, much has still to be clarified regarding global epigenetic changes to the DNA and epigenetic signals for specific gonadal cells involved in the establishment of TSD networks.

Sea turtles manifest TSD, where low and high temperatures produce male and female hatchlings, respectively. In the olive ridley turtle, *L. olivacea,* differentiation of the bipotential gonad into a testis or ovary takes several days, enabling the study of structural and molecular changes during sex determination and differentiation (Merchant-Larios and Díaz-Hernández, 2013). In the current study, we extended the investigation of this species using unbiased, global approaches and we provide evidence of dimorphism of DNA methylation patterns between ovaries and testes. We found a correlation between levels of 5hmC DNA modification in medullary cords and sexual commitment. Together, our results suggest that changes in DNA methylation are necessary for the stabilization of gene expression networks of ovaries and testes in *L. olivacea* and probably in other species with TSD.

2. Methods

2.1. Embryos

Freshly laid eggs of the olive ridley turtle (*L. olivacea*) were collected at La Escobilla beach on the Pacific Coast of Oaxaca, Mexico (96_2701600W, 15_4003600N). The eggs were transported to the laboratory in Mexico City and incubated at MPT (26 °C ± 0.5) or FPT (33 °C ± 0.5). The total number of embryos used in the present study is shown in Supplementary Table 1. All protocols were approved by local animal rights committees from the Secretary for Environment and Natural Resources (SEMARNAT) and the National Autonomous University of Mexico. Embryos were sampled before and after sex determination: at Stages 24 and 27 for MPT and 23 and 27 for FPT; samples for immunofluorescence were taken also at stages 25 and 26 (Supplementary Table 1). Stages are according to Miller's criteria (Miller, 1985).

2.2. Genomic DNA isolation

Urogenital complexes were removed from embryos incubated at MPT or FPT. Gonads were separated from the adjacent mesonephros and grouped into pools containing tissue from 10 embryos each. Gonads from embryos of at least 2 different nests were included in each pool. Samples were sliced into 5–8 pieces smaller than 2 mm^3 and incubated in Lysis Buffer (10 mM NaCl, 10 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 2% SDS, 1 mg/ml Proteinase K and 150 μg/ml RNase) for 16 h at 65 °C. DNA was purified with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Biological replicates for other experiments were prepared from another 10 embryos originating from 2 different nests each time. Mouse DNA was obtained from a pool of 5 brains from new born male CD-1 strain.

2.3. Amplification of Inter-methylated Sites (AIMS)

The AIMS method was adapted from the original (Frigola et al., 2002). Embryos came from the same 2 nests, sampled at different developmental stages. DNA samples from different incubation temperatures and developmental stages came from pools of gonads of 10 embryos, these being 5 embryos from each nest. A biological replicate used embryos from 2 other nests. (Supplementary Table 1). Each sample of 5 μg DNA was treated with 100 U methylation-sensitive endonuclease HpaII for 16 h at 37 °C and then purified with phenol-chloroform-isoamyl alcohol, as previously described. From each sample, 2 μg were treated with 10 U DNA polymerase I, Large (Klenow) fragment (New England BioLabs) following the manufacturer's instructions, for 4 h at 30 °C until blunt ends remained. The reaction was arrested at 75 °C for 30 min and DNA was precipitated with 100% ethanol, 0.1 volumes of sodium acetate 3 M pH5 and 20 μg glycogen (Roche). This DNA was treated with 100 U methylation-insensitive MspI endonuclease for 16 h at 37 °C, and 20 U enzyme MspI was then added to the reaction and incubated for 4 h at 37 °C, prior to heat inactivation of the enzyme. DNA was once again precipitated, as previously described. To prepare the linkers, 2 nmoles of each primer Blue (5′-ATTCGCAAAGCTCTGA-3′) and Blue-GC (Phos-5′-CGTCA GAGCTTTGCGAAT-3′) were incubated together at 65 °C for 2 min and then at room temperature for 30 min. Linkers were ligated to treated DNA with 800 U T4 Ligase (New England BioLabs) for 16 h at 14 °C. Unincorporated linkers were removed with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and DNA was re-suspended in 250 μl DNase-free H_2O. PCRs were prepared with 3 μl sample DNA and 1 μCi [α-^{32}P]dCTP with Hot Start Taq (Quiagen), using 5 pmol of primer Blue1 (5′-ATTCGCAAAGCTCT GACGGATTAG-3′), Blue2 (5′-ATTCGCAAAGCTCTGACGGATATC-3′), Blue3 (5′-ATTCGCAAAGCTCTGACGGATGC-3′). Reactions were performed in quadruplicate, using 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a last extended stage of 10 min at 72 °C. PCR products were precipitated as previously described and re-suspended in 2 μl loading buffer. Products were run on 7.5% polyacrylamide denaturing gels, 0.4 mm thick with 7 M urea on TBE buffer. Gels were dried and auto-radiographs were produced from X-ray films that had been exposed for 48 h. AIMS was performed twice for stage 24 and 3 times for embryos at stage 27, plus one extra time for a biological replicate to corroborate the results. Quantity One Analysis Software was used to generate histograms and evaluate relative intensity of the banding patterns. Only bands showing dimorphic intensity in every PCR and biological replicate were considered as indicating differences in DNA