DEVELOPMENTAL DYNAMICS 239:1061–1075, 2010

RESEARCH ARTICLE

Genetic Network Underlying Temperature-Dependent Sex Determination Is Endogenously Regulated by Temperature in Isolated Cultured *Trachemys scripta* Gonads

Christina M. Shoemaker-Daly,[1] Kyle Jackson,[2] Ryohei Yatsu,[2] Yuiko Matsumoto,[2] and David Crews[2]*

In reptiles with temperature-dependent sex determination, gonadogenesis is initially directed by the incubation temperature of the egg during the middle third of embryonic development. The mechanism by which temperature is transduced into a sex-determining molecular signal remains a mystery, and here we examine the molecular network underlying sex determination in gonads in vitro. We use a whole organ culture system to show that expression of putative members of the sex-determining network (*Dmrt1*, *Sox9*, *Mis*, and *FoxL2*) are regulated by temperature endogenously within cells in the bipotential gonad and do not require other embryonic tissues to be expressed in a normal pattern in the red-eared slider turtle, *Trachemys scripta*. Furthermore, following a change in temperature, these factors exhibit temperature-responsive expression patterns that last for the duration of gonadogenesis. Finally, mosaic misexpression of a fusion *Sox9* construct demonstrates the ability to functionally manipulate the gonad at the molecular level. *Developmental Dynamics 239:1061–1075, 2010.* © 2010 Wiley-Liss, Inc.

Key words: gonadogenesis; sex determination; temperature; *Sox9*; *Dmrt1*; *Mis*; *FoxL2*; ovary; testis

Accepted 19 January 2010

INTRODUCTION

Sex determination in vertebrates can be broadly categorized into two modes. In genotypic sex determination (GSD), a genetic factor directs the sexual fate of the initially bipotential gonad (e.g., in mammals by means of *Sry*). Conversely, in organisms exhibiting environmental sex determination, an environmental factor determines the sex of offspring. In some species with temperature-dependent sex determination (TSD), the incubation temperature of the egg during the middle third of development establishes sex, as occurs in all crocodilians, many turtles and some lizards. In all modes of SD,

ABBREVIATIONS: AKG adrenal-kidney-gonad **AR** androgen receptor **DMRT1** doublesex mab3 related transcription factor 1 **ERα** estrogen receptor subtype α **ERβ** estrogen receptor subtype β **ESD** environmental sex determination **FOXL2** forkhead box protein L2 **FPT** female-producing temperature **GAM** gonad-adrenal-mesonephros **GSD** genotypic sex determination **ISH** in situ hybridization **MIS** Müllerian-inhibiting substance **MPT** male-producing temperature **PP1** protein phosphatase type I **qPCR** quantitative real-time polymerase chain reaction **SF1** Steroidogenic factor 1 **SOX9** SRY-like HMG-box containing transcription factor 9 **SRY** sex-determining region of the Y chromosome **TSD** temperature-dependent sex determination **TSP** temperature-sensitive period **WNT4** wingless integration site family member 4.

Additional Supporting Information may be found in the online version of this article.

[1]Molecular, Cell and Developmental Biology, University of Texas at Austin, Austin, Texas
[2]Department of Integrative Biology, University of Texas at Austin, Austin, Texas

Grant sponsor: National Science Foundation; Grant number: 200001269.

***Correspondence to: David Crews, W 24th and Speedway, Patterson Labs, Austin, TX 78705.**
E-mail: crews@mail.utexas.edu

DOI 10.1002/dvdy.22266
Published online 12 March 2010 in Wiley InterScience (www.interscience.wiley.com).

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1062

the process of forming a testis or an ovary from its bipotential gonad precursor involves complex molecular interactions and cellular behaviors. This has been well-studied in vertebrates with GSD (e.g., Brennan and Capel, 2004; Fleming and Vilain, 2004; Wilhelm et al., 2007; Blecher and Erickson, 2007; Cederroth et al., 2007; DiNapoli and Capel, 2008), and is an emerging field in reptiles and fish with TSD (e.g., Morrish and Sinclair, 2002; Yao and Capel, 2005; Fernandino et al., 2008a; Shoemaker and Crews, 2009; Ramsey and Crews, 2009; Baroiller et al., 2009). While the initial upstream factor determining sex differs between these modes, many of the downstream genes used in the formation of the gonad are thought to be conserved.

Numerous genes involved in mammalian gonadogenesis have now been cloned in a variety of reptilian TSD species and their expression patterns have been examined to varying degrees (for review, see Morrish and Sinclair, 2002; Place and Lance, 2004; Shoemaker and Crews, 2009; Ramsey and Crews, 2009). However, understanding the specific functions of members of the molecular network that underlie TSD remains a challenge. Candidate gene approach studies have informed our knowledge of the regulatory network putatively involved in temperature-dependent gonadogenesis, while embryonic temperature shifts and hormone treatments have begun to demonstrate sensitivities of various genes to both factors (Murdock and Wibbels, 2006; Shoemaker et al., 2007a,b; Ramsey et al., 2007; Ramsey and Crews, 2007). Nevertheless, progress in understanding the function of gene products has been hampered by technical challenges, particularly, a lack of techniques to manipulate the gonad at a molecular level. Furthermore, whether the action of temperature in determining sexual fate is mediated directly at the site of the gonad or indirectly through another embryonic tissue was initially unclear. It was originally suggested that the brain may contain the primary temperature-sensing locus in organisms with TSD and that innervation of the gonad might mediate this signal during development (Merchant-Larios et al., 1989; Crews, 1993; Gutierrez-Ospina et al., 1999). Initial evidence that the gonad itself may be the site of the temperature-sensing mechanism came from a study of *Sox9* in isolated Olive Ridley sea turtle gonads grown in culture (*Lepidochelys olivacae*, Moreno-Mendoza et al., 2001).

In the red-eared slider turtle, *Trachemys scripta*, we extend these findings more broadly and examine multiple members of the putative sex-determining network governing gonad development in both sexes. We optimize and use an in vitro whole organ culture system to show that the putative sex-determining network is regulated by temperature endogenously within the cells of the gonad in this species. Our data indicate that the bipotential gonad is capable of regulating expression of *Dmrt1*, *Sox9*, *Mis*, and *FoxL2* in response to temperature changes in isolation from surrounding embryonic tissues. Furthermore, the explant culture system facilitates manipulation of the gonad at the molecular level, opening the door to functional studies classically used in traditional model organisms.

The slider turtle exhibits a form of TSD in which cooler incubation temperatures (26.0°C) produce all male hatchlings and warmer temperatures (31.0°C) result in all female hatchlings, with varying sex ratios produced by temperatures in between (Wibbels et al., 1991). The embryo is sensitive to the effect of temperature beginning at approximately Greenbaum's stage 14 and lasting through stage 19 at female-producing temperature (FPT) and through stage 20 at male-producing temperature (MPT; Wibbels et al., 1991; Greenbaum, 2002). Furthermore, embryos shifted from one end of the temperature spectrum to the other during the temperature-sensitive period (TSP) respond by complete sex-reversal (Crews et al., 1994). Thus, the sex of a bipotential gonad is thought to become fated or "determined" near the beginning of the TSP, but this decision remains reversible until the close of the TSP, when sex becomes "committed" to an ovarian or testicular fate (Bull et al., 1990; Wibbels et al., 1991; Shoemaker and Crews, 2009).

In mammals, gonad development begins with the formation of the urogenital ridge and subsequent bipotential gonad from underlying mesonephric and coelomic epithelial tissues. In XY individuals, *Sry* expression in medullary somatic cells leads to accumulation of a threshold number of Sertoli cells thought to "tip the balance" and direct the gonad toward a testicular fate (Ross and Capel, 2005). Sertoli cells organize into medullary sex cords, precursors to seminiferous tubules, containing germ cells (for review, see Brennan and Capel, 2004). In general, the process of forming an ovary is less well understood (for review, see Yao, 2005). Ovarian development is characterized by a proliferation of cells in the gonad cortex where granulosa cells organize to surround germ cells, concurrent with medullary regression. Recently, experiments examining ovotestes have furthered understanding of the genetic network underlying development of these two compartments, the ovarian cortex and the testicular medulla (Wilhelm et al., 2009). Because of their critical role in gonadogenesis in organisms with GSD and their clear sexually dimorphic patterns of expression during gonad development in the slider turtle, we examine *Sox9*, *Mis*, *Dmrt1*, and *FoxL2* in the following study.

The transcription factor *Sry-like HMG-box 9* (*Sox9*) is the direct target of *Sry* in mammals and is both necessary and sufficient for normal testicular development (Vidal et al., 2001; Sekido and Lovell-Badge, 2008). The ability of *Sox9* to "replace" the action of *Sry* in mammals makes it an obvious candidate to examine for an early, critical role in testis development in TSD organisms. Its expression pattern becomes sexually dimorphic in the developing gonad of five reptile species with TSD at varying points of development, ranging from the end of the TSP to later during testicular differentiation (for review, see Shoemaker and Crews, 2009). In both humans and mice, *Sox9* directly upregulates the expression of *Müllerian-inhibiting substance* (*Mis* or *Anti-Müllerian hormone*; De Santa Barbara et al., 1998; Arango et al., 1999). MIS, a member of the transforming growth factor-β (TGF-β) superfamily, is secreted by differentiated Sertoli cells in the testis and causes the

ID: kumarpr | Black Lining: [ON] | Time: 17:23 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: **Z7H** Customer A_ID: **DVDY22266** Cadmus Art: **DVDY22266** Ed. Ref. No.: **09-0462** Date: **8-March-10** Stage: Page: **1063**

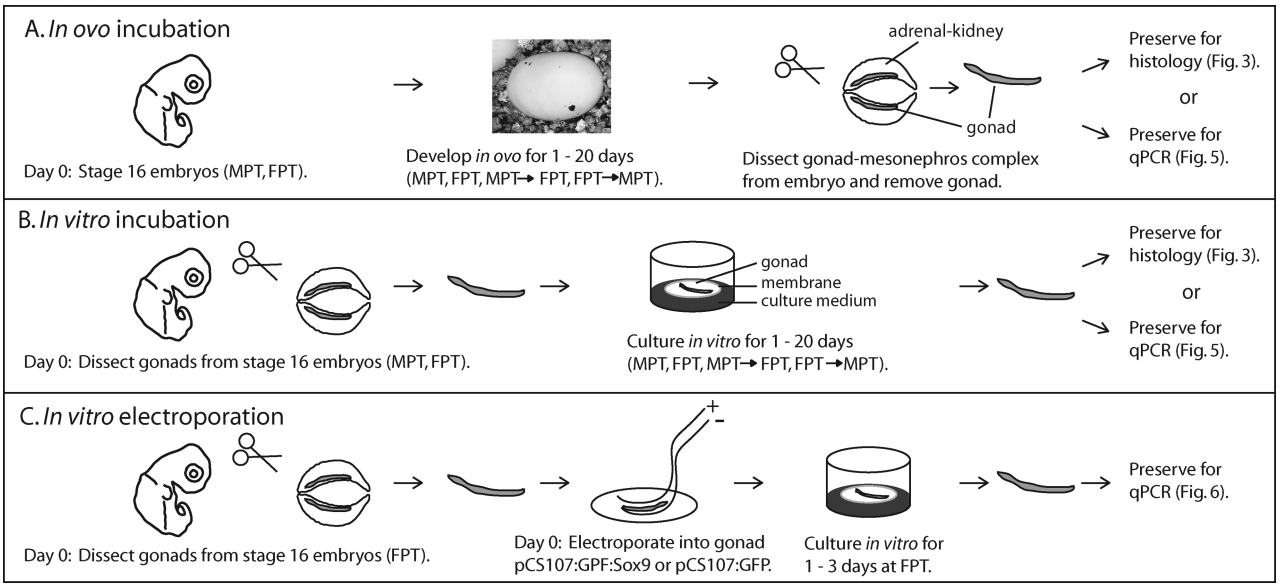


Fig. 1. Experimental design. **A**: Developmental progression of red-eared slider turtle (*Trachemys scripta*) embryos incubating in ovo at male-producing temperature (MPT) or female-producing temperature (FPT) was monitored. At Greenbaum's embryonic stage 16 (= Day 0), some eggs were shifted to the opposite temperature while others remained at constant temperature. After up to 20 days further in ovo incubation, embryos were killed and gonads dissected for individual quantitative real-time polymerase chain reaction (qPCR) and histological analysis. **B**: Developmental progression of embryos incubating in ovo at MPT or FPT was monitored and at Greenbaum's embryonic stage 16 (= Day 0), embryos were killed, adrenal-kidney-gonads were dissected, and gonads were separated. Gonads from each original incubation temperature were cultured in vitro at either constant and shifted temperature for up to 20 days and preserved for individual qPCR and histological analysis. **C**: Developmental progression of embryos incubating in ovo at FPT was monitored and at Greenbaum's embryonic stage 16 (= Day 0), embryos were killed and gonads dissected. Gonads were electroporated with pCS107:GFP:Sox9 or pCS107:GFP, cultured in vitro for up to 3 days, and preserved for individual qPCR analysis.

regression of the Müllerian ducts, anlagen which otherwise develop into the uterus, cervix, and fallopian tubes in females (Behringer et al., 1990). However, the regulatory relationship between *Sox9* and *Mis* in mammals may not be strictly conserved across phyla or mechanisms of sex determination. Similar to the case in chicken, two reptiles with TSD exhibit strongly sexually dimorphic *Mis* expression early in the sex-determining period, before sexually dimorphic *Sox9* expression (Shoemaker et al., 2007b; Takada et al., 2004; Western et al., 1999; Oreal et al., 1998; Smith et al., 1999a). These data support a role for both *Sox9* and *Mis* in testicular development in organisms with TSD, and suggests a divergence from the relationship seen in mammals in which *Sox9* initially up-regulates *Mis* expression.

Doublesex-, mab3-related transcription factor 1 (*Dmrt1*) is one of the few genes identified with homologs involved in gonad development across widely diverse taxa, including *Drosophila*, *C. elegans*, mammals, fish, and reptiles (Burtis and Baker, 1989; Shen and Hodgkin, 1998; Raymond et al., 1998; Smith et al., 1999b; Kettlewell et al., 2000; Nanda et al., 2002; Matsuda et al., 2002; Sreenivasulu et al., 2002; Shoemaker et al., 2007a,b). In mammals, *Dmrt1* function is critical to testis differentiation after sex has been determined (Veitia et al., 1997; Raymond et al., 2000). However, in both chicken and medaka (*Oryzias latipes*) it has been proposed to be an early-acting sex-determining gene, i.e., a nonmammalian equivalent of SRY (Nanda et al., 2002; Matsuda et al., 2002; Kobayashi et al., 2004; Smith and Sinclair, 2004), although further studies are needed to confirm this role (e.g., Zhao et al., 2007). In three reptiles with TSD, *Dmrt1* expression is enhanced at MPT at varying times during gonad development, implicating an involvement in testis formation although its functional role remains unknown (Smith et al., 1999b; Kettlewell et al., 2000; Torres Maldonado et al., 2002; Murdock and Wibbels, 2003; Shoemaker et al., 2007a,b).

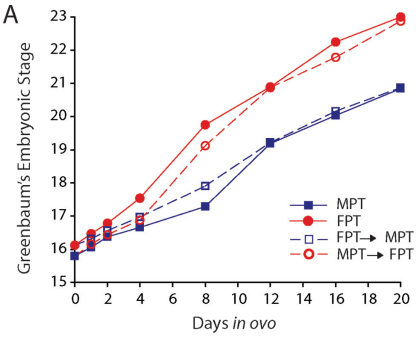
Forkhead box protein L2 (*FoxL2*) is a single-exon transcription factor required for ovarian development in mammals (Crisponi et al., 2001; Udar et al., 2003; Schmidt et al., 2004). A *FoxL2* binding "response element" has been identified that when mutated, may be responsible for the female-to-male sex-reversal observed in *FoxL2*/*Wnt4* double knockout XX mice (Ottolenghi et al., 2007; Benayoun et al., 2008). In two turtle species with TSD, *FoxL2* expression becomes restricted to the developing ovary during the end of the TSP (Loffler et al., 2003; Shoemaker et al., 2007b; Rhen et al., 2007), suggesting a role in ovarian commitment or differentiation.

The present study examines the ability of temperature to regulate expression of multiple components of the putative sex-determining network throughout gonadogenesis of the red-eared slider turtle and demonstrates the following. (1) Expression of *Sox9*, *Dmrt1*, *Mis*, and *FoxL2* is regulated in ovo in response to sex-reversing temperature shifts in a manner that is sustained and lasts from the sex-determining period through differentiation; this reveals that temperature

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1064

COLOR

A



B

Days in ovo	Corresponding Developmental Stage			
	FPT	FPT→ MPT	MPT	MPT→ FPT
0	16		early 16	
1	16.5	late 16	16	late 16
2	early 17	16.5	late 16	16.5
4	17.5	17	early 17	17
8	early 20	18	late 17	19
12	21	late 19	late 19	21
16	late 22	late 20	20	early 22
20	23	21	early 21	23

Fig. 2. Developmental rate variation at different incubation temperatures. Red-eared slider turtle embryos developing in ovo were dissected and staged by a variety of morphological characteristics, including digit, limb, eye, head, caruncle, carapace, plastron, and body size phenotype (Greenbaum, 2002). The number of days development post-stage 16 (= Day 0) is plotted against corresponding average developmental stage to show overall rate of embryonic morphological development (n = 5–8 per data point). **A**: Comparison of variation in rates of development between male-producing temperature (MPT), female-producing temperature (FPT), MPT→FPT, and FPT→MPT. **B**: Summary of average stages graphed in A is given for reference; data from individuals and numerical averages are given in Supp. Table S1.

both induces and represses their expression. (2) In a whole-organ in vitro culture system, the expression patterns of these genes mimics the patterns seen in ovo, revealing that the isolated gonad itself senses and responds to changes in temperature by regulation of the molecular network(s) governing sexual development in both sexes. This indicates that the members of the sex-determining network examined here are regulated by temperature endogenously in cells within the gonad and do not require other embryonic tissues to be expressed in a normal pattern. (3) In vitro misexpression of a fusion construct (green fluorescent protein [GFP]:Sox9) by electroporation into gonads demonstrates for the first time in a reptile with TSD the ability to manipulate the gonad at the molecular level.

RESULTS

Rate of Development Throughout Embryonic Incubation

Embryos incubating in ovo were dissected between 1 and 20 days postembryonic stage 16 (= Day 0) for analysis, allowing a comparison of developmental rate under various incu- F1 bation conditions (Fig. 1). Due to metabolic reasons, development at warmer FPT occurs at a faster rate than at cooler MPT (McCue, 2004). After 20 days of development in the egg post-Day 0, an FPT embryo has reached stage 23, while an MPT embryo has reached early stage 21 (Fig. 2). Embryos that are shifted at Day 0 from one temperature to the other respond to the new environment with altered morphological rates of development. Developmental rate decreased in embryos shifted from FPT to MPT (FPT→MPT), and accelerated in embryos shifted from MPT to FPT (MPT→FPT). It took approximately 12 days for this adjustment period to occur, regardless of the direction of shift (Fig. 2A). By Day 12, MPT→FPT embryos reached the same overall morphological phenotype as their FPT counterparts, and FPT→MPT embryos arrived at the same morphology as MPT embryos. From Day 12 to Day 20, shifted embryos developed at temperature-typical rates. It is noted that the correlation between days of development and developmental stage of an embryo in ovo is not applicable to in vitro samples.

Morphological Development of Gonads In Ovo and In Vitro

The histology of gonads developing either within the embryo (in ovo) or isolated in culture (in vitro) under both constant and shifted temperature conditions was examined (Fig. 3).

In ovaries developing in ovo, somatic cells reorganized into two regions by Day 20, the cortex and the medulla, separated by the basal lamina. While the medullary region lacked obvious structure, primordial germ cells and granulosa cells migrated into the cortex where they will develop into follicles in the adult (Fig. 3A). This occurred in gonads developing under either constant FPT or MPT→FPT conditions (Fig. 3A, i and iv), revealing the ability of an embryo to sense and respond to its temperature environment at the level of gonadal morphological structure. In gonads shifted MPT→FPT, cells began to reorganize and move away from male-typical morphology as observed by degrading medullary sex cords by 12 days post-shift (Fig. 3A, iv). Remnants of sex cords were observable at Day 16, as well as the initial formation of a thickening cortex. By Day 20 post-shift, sex cords were no longer visible and the basal lamina boundary between cortex and medulla was distinct. F2

In testes developing in ovo, somatic pre-Sertoli cells organize into medullary sex cords surrounded by interstitial tissue composed of Leydig cells and peritubular myoid cells, and eventually lack any substantial cortical region (Fig. 3A). This occurred in embryonic gonads developing at either constant MPT or shifted FPT→MPT conditions (Fig. 3A, iii and ii). In response to a FPT→MPT shift, male-typical sex cords formed as early as 12 days post-shift (Fig. 3A, ii). The sex cords continued to condense through development, and by 20 days post-shift, the cortex was fully dissolved and sex cords were distinct.

The morphological development of gonads removed from embryos at developmental stage 16 (= Day 0) and cultured in vitro for up to 20 days was also examined (Fig. 3B). Transverse sections of in ovo gonads are characteristically round, and this morphology changes during in vitro culture. As the gonad becomes flattened from resting on the culture membrane, transverse sections taken along the same axis appear more oval-shaped. Following culture at FPT, a cortical region began to emerge and the medullary region showed signs of reorganization by Day 12 (Fig. 3B, v). Primor- F3 dial germ cells continued to migrate to the cortex, visible at Day 16, and by 20 days of culture, a more distinct

ID: kumarpr | Black Lining: [ON] | Time: 17:23 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

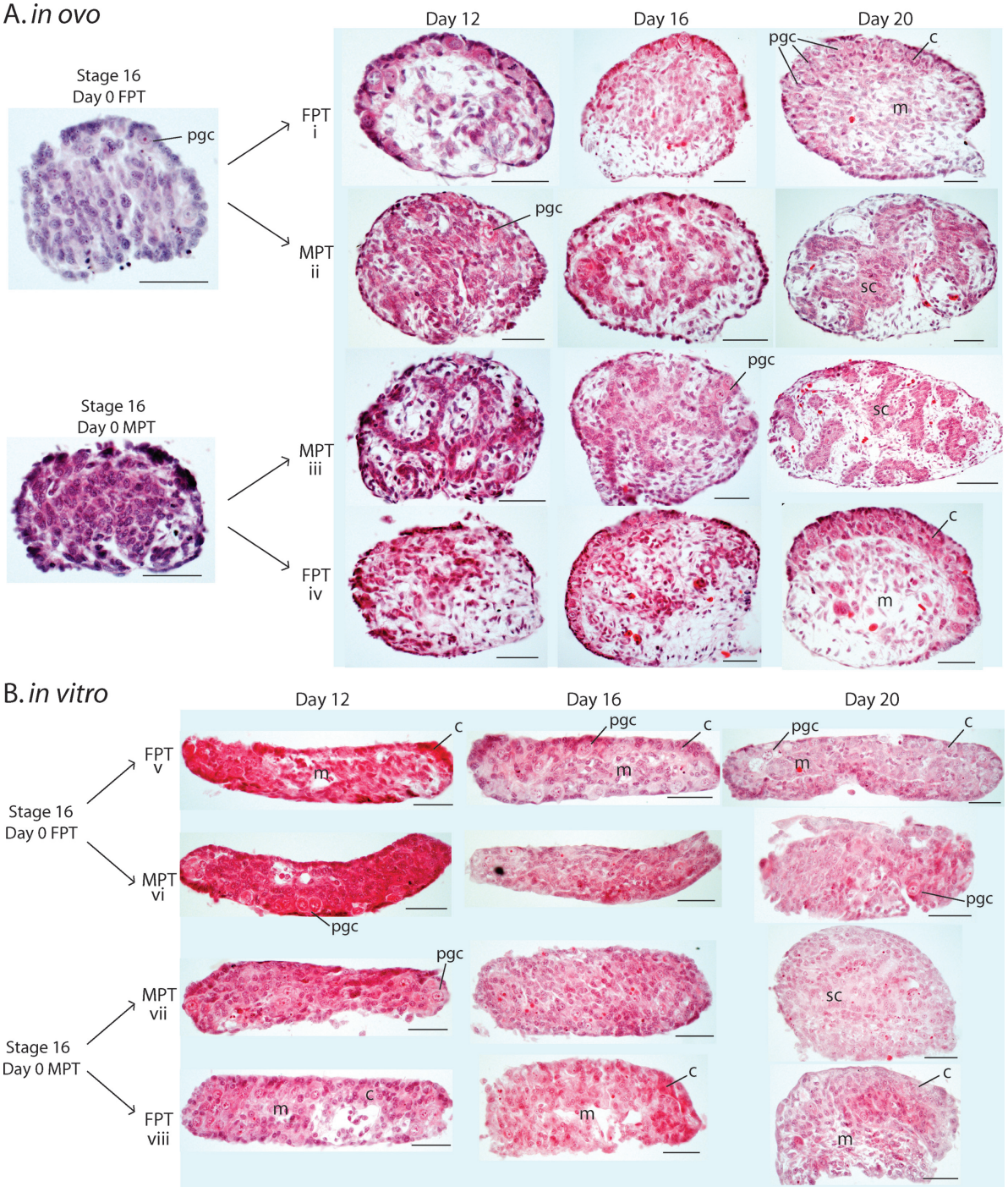


Fig. 3. Morphological characteristics of gonad development in ovo and in vitro. **A**: Developmental progression of embryos incubating in ovo at male-producing temperature (MPT) or female-producing temperature (FPT) was monitored. At Greenbaum's embryonic stage 16 (= Day 0), some eggs remained at constant temperatures (i and iii), while others were shifted to the opposite temperature (ii and iv). After up to 20 days further in ovo incubation, embryos were killed and gonads preserved for standard hematoxylin and eosin (H&E) staining. **B**: Developmental progression of embryos incubating in ovo at MPT or FPT was monitored, and at Greenbaum's embryonic stage 16 (= Day 0), embryos were killed and gonads dissected. Gonads from each temperature were cultured in vitro at either constant temperature (v and vii) or in a shifted regimen (vi and viii) for up to 20 days and preserved for H&E staining. m, medullary region; c, cortex; pgc, primordial germ cell; sc, sex cord. Scale bar = 100 μm.

COLOR

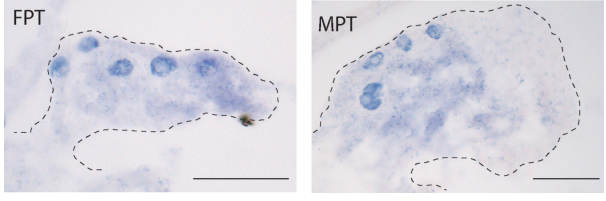


Fig. 4. Localization of *Sox9* expression in individual turtle gonads in ovo. In situ hybridization of *Sox9* at stage 15 in both female-producing temperature (FPT) and male-producing temperature (MPT) embryos shows localization of transcript in clusters of expressing cells that surround nonexpressing cells. For example, typical sections shown here contain n = 5 (FPT) and n = 4 (MPT) clusters of expressing cells. Scale bar = 50 μm.

COLOR

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1066

boundary between cortex and medulla was apparent. These female-typical changes were observable but less apparent in gonads shifted from MPT→FPT for the culture period; a thickened outer cortex began to form next to a disorganized medulla by Day 20 (Fig. 3B, viii).

During gonad culture at MPT, male-typical sex cords began to form after 20 days of incubation (Fig. 3B, vii). At this point, sex cord morphology showed the expected migration of somatic cell nuclei toward the outside of the cord, leaving the cytosol of each somatic cell toward the center. These male-typical structures were not apparent in cultured gonads shifted from FPT→MPT (Fig. 3B, vi).

Localization of *Sox9* Transcripts

To examine a previous finding that *Sox9* expression is sexually dimorphic early in the TSP in the developing gonad at the level of transcript localization, whole-mount in situ hybridization was conducted on stage 15 AKGs at both FPT and MPT. Groups of cells expressing *Sox9* clustered around nonexpressing cells were observed in gonads developing at both
F4 temperatures (Fig. 4). The total number of *Sox9*-expressing cell clusters in the gonad were counted in all sections per AKG and ranged from 0 to 7 clusters/section/AKG for both MPT ($n = 5$) and FPT ($n = 10$). The average number of clusters per section per AKG was also not different between temperatures: FPT mean = 3.7, MPT mean = 3.8 clusters/section/AKG.

Gene Expression Patterns at Constant Incubation Temperature In Ovo

The expression of putative members of the sex-determining network in individual gonads developing in ovo at constant incubation temperatures was analyzed by quantitative real-time polymerase chain reaction
F5 (qPCR; Fig. 5). The conventional use of embryonic stage when examining in ovo gene expression patterns becomes nonsensical in the in vitro culture system, as the morphological characteristics of the embryo used to assess stage no longer exist. Therefore, both in ovo and in vitro expression data are plotted against days of development for comparison.

From embryonic stage 16 (Day 0), *Sox9* expression levels at MPT became significantly higher than FPT at Day 4 and were subsequently maintained (Fig. 5A). *Sox9* expression at FPT was initially higher than at MPT, and decreased to negligible levels by Day 8. Both *Dmrt1* and *Mis* expression at MPT was significantly greater than FPT early in development, beginning at Day 1 and 0, respectively, and continued to increase at MPT throughout gonadogenesis (Fig. 5C,E). A second dramatic increase in *Mis* expression at MPT occurred later in the sex-determining period from Day 12 to Day 20. At FPT, expression of *Dmrt1* and *Mis* remained low, and *Mis* levels dropped below qPCR detectability from Day 4 onward. While *FoxL2* expression levels were similar between MPT and FPT early in the TSP (Day 0–4), expression remained low at MPT and increased significantly at FPT from Day 12 onward (Fig. 5G).

Gene Expression Patterns in Response to Sex-Reversing Temperature Shifts In Ovo

The regulation of these four genes in response to changes in temperature is sustained for the duration of gonadogenesis. In gonads developing in ovo at MPT and shifted to FPT at stage 16 (MPT→FPT), *Sox9* expression decreased to baseline levels by Day 8 post-shift (Fig. 5A). In gonads shifted in the opposite direction, FPT→MPT, *Sox9* expression was indistinguishable from FPT-typical levels through Day 2, and were maintained at MPT-typical levels through gonadogenesis, becoming significantly greater than decreasing FPT expression at Day 8.

Dmrt1 expression in gonads shifted MPT→FPT responded rapidly to the new FPT temperature environment; expression was repressed significantly below MPT-typical levels by Day 2, and remained at baseline despite a slight increase at Day 4 (Fig. 5C; Supp. Table S3, which is available online). In the opposite shift (FPT→MPT), *Dmrt1* expression also responded quickly. Increased expression was apparent by Day 1, was significantly greater than FPT levels by Day 4, and continued for the duration of the period examined. While *Dmrt1* expression in FPT→MPT gonads was significantly higher than at FPT, it did not reach MPT-typical levels at the later time points (Days 16 and 20).

Repression of *Mis* in response to a MPT→FPT shift occurred rapidly by Day 2 post-shift and was maintained through the in ovo incubation period, except for a slight increase at Day 4 (Fig. 5E). *Mis* expression in FPT→MPT gonads rose significantly above FPT-typical levels by Day 4, continuing at MPT-typical levels for the duration of gonadogenesis.

FoxL2 expression was also regulated by changes in temperature. In gonads shifted from MPT→FPT, *FoxL2* expression increased significantly above MPT levels by Day 12 and continued to increase (Fig. 5G). In gonads shifted from FPT→MPT, *FoxL2* expression was maintained at low levels typical of MPT for the duration of gonadogenesis, and was significantly lower than increasing FPT expression by Day 8.

Gene Expression Patterns in Isolated Gonads Cultured In Vitro

At embryonic stage 16, gonads were dissected from embryos at both MPT and FPT and incubated in an in vitro culture system for up to 20 days at constant and shifted temperatures. Expression of *Sox9*, *Dmrt1*, *Mis*, and *FoxL2* in these gonads showed strongly sexually dimorphic patterns at constant temperatures and were regulated in response to temperature shifts.

Sox9 expression levels at MPT became significantly higher than FPT by Day 4 and continued to increase through Day 12, reaching a peak expression slightly higher than that seen in ovo (Fig. 5B). Expression after Day 12 declined, returning to levels similar to MPT in ovo gonads. At FPT, *Sox9* expression decreased substantially by Day 1 of in vitro culture, and continued to decrease until reaching baseline levels. Gonads that were grown in ovo at MPT through stage

ID: kumarpr | Black Lining: [ON] | Time: 17:24 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

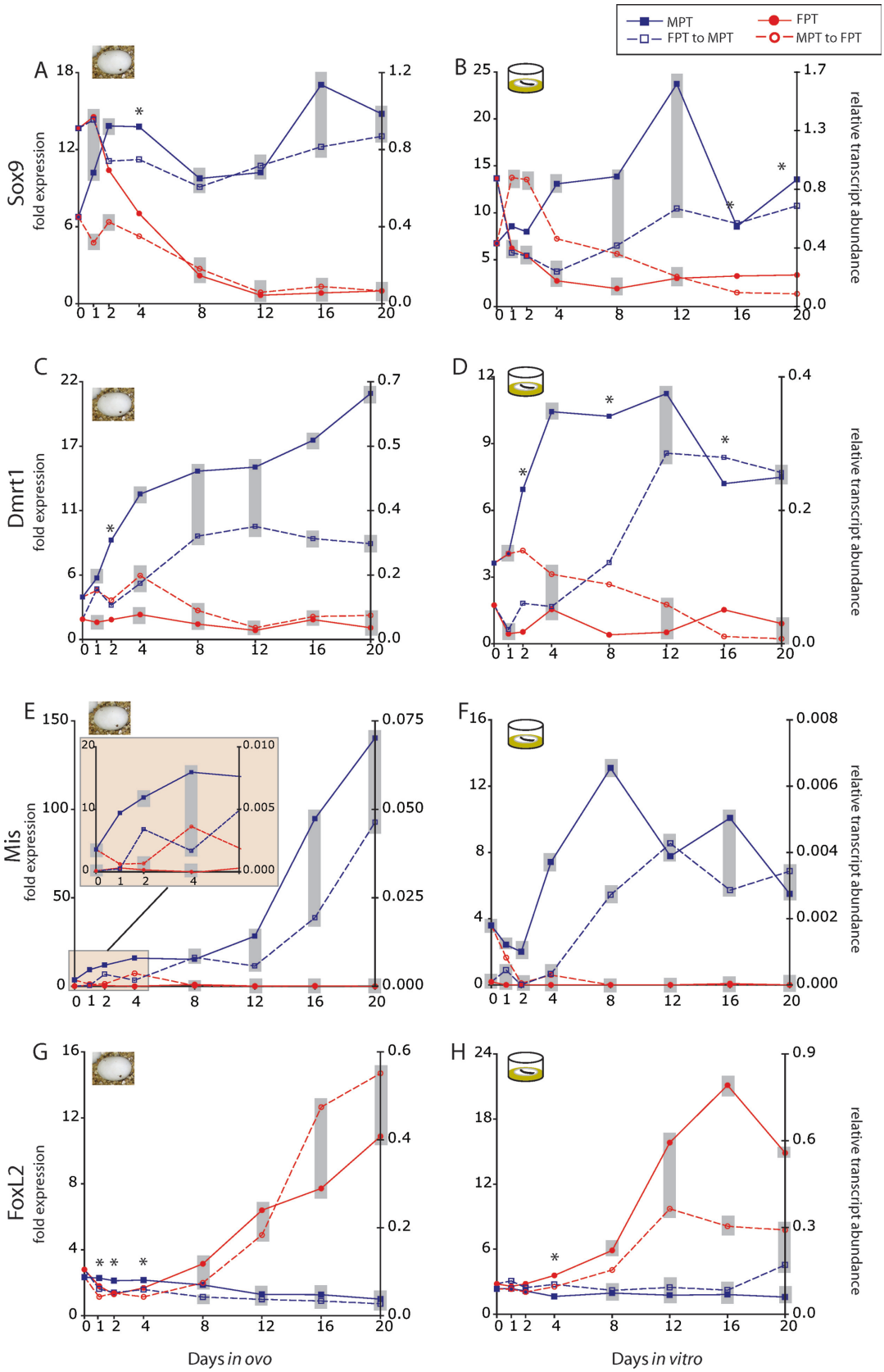
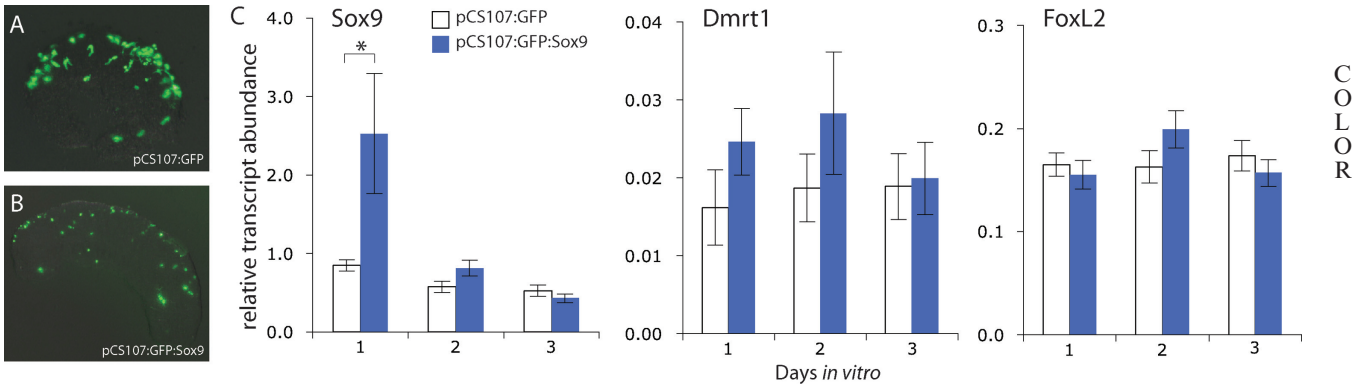


Fig. 5. Expression of *Sox9*, *Dmrt1*, *Mis*, and *FoxL2* in individual turtle gonads in ovo and in vitro. **A–H**: Embryos developed in ovo until Greenbaum's stage 16 (= Day 0), at which point gonads either (A,C,E,G) continued developing in ovo or (B,D,F,H) were dissected and cultured in vitro. Following up to 20 days additional development, gonads were preserved individually for quantitative real-time polymerase chain reaction (qPCR) analysis (n = 7–10 gonads per data point). Right y-axes correspond to relative transcript abundance normalized to constitutive *PP1* expression, and left y-axes correspond to normalized values calibrated to expression level at either (A–F) female-producing temperature (FPT) Day 20 in ovo or (G, H) male-producing temperature (MPT) Day 20 in ovo. X-axes correspond to (A,C,E,G) days of development in the egg or (B,D,F,H) days of development in vitro. Statistical comparisons are all made within gene, within day/stage, between temperatures. Expression points contained within the same gray shaded box are not significantly different from each other, while points in separated boxes within a timepoint are significantly different. Points not contained in any shaded box are not statistically different from any other point within the same timepoint. Asterisks indicate statistical relationships that were too complicated to be accurately described by this shading system and are presented, along with statistical values for all comparisons, in Supp. Table S3.

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1068



COLOR

Fig. 6. Misexpression of *Sox9* in gonads developing in vitro at female-producing temperature (FPT). Gonads were dissected from embryos incubating at FPT at stage 16, electroporated with a plasmid construct and cultured in vitro at FPT for up to three days. **A,B**: Electroporation efficiency evaluated by green fluorescent protein (GFP) expression under fluorescence is shown in two typical gonads at Day 1 for pCS107:GFP (A) and pCS107:GFP:Sox9 (B). **C**: Effect of plasmid electroporation on gene expression was measured by qPCR and average relative transcript abundance is plotted with standard error bars (n = 12 to 20 gonads per data point).

16 (= Day 0) and then shifted to FPT for culture (MPT→FPT) responded immediately to the new temperature environment. After 1 day of in vitro culture, *Sox9* expression increased to Day 0 FPT-typical levels, remained there through Day 2, and then decreased. Expression reached basal levels not significantly different from FPT expression, and significantly lower than MPT levels, by Day 12. In the opposite shift, gonads grown in ovo at FPT through Day 0 and shifted to MPT for culture (FPT→MPT) exhibited a delayed response to the new MPT environment. Through Day 4, *Sox9* expression followed an FPT-typical pattern of decline; from Day 8 onward, expression increased significantly above FPT baseline.

Expression of *Dmrt1* was significantly greater at MPT than FPT for all time points examined following Day 0. *Dmrt1* levels at MPT increased quickly from Day 0 to 4, were maintained through Day 12, and then decreased at the end of the culture period (Fig. 5D). Peak MPT expression in vitro was roughly 2 times lower than peak levels observed in ovo (Fig. 5C,D). Expression at FPT remained low for the duration of the culture period. In MPT→FPT gonads, *Dmrt1* expression was significantly repressed below MPT levels by Day 4, one time point later than the respective in ovo response. In FPT→MPT gonads, an increase in *Dmrt1* expression was also delayed compared with in ovo development. Expression remained at FPT-typical levels through Day 4, increased significantly above FPT by Day 8, and was subsequently maintained at MPT-typical levels (Fig. 5D, Supp. Table S3).

In vitro gonads expressed *Mis* at significantly greater levels at MPT than at FPT for all time points examined (Fig. 5F). MPT gonads showed an initial increase in *Mis* expression from Day 2 through Day 8, which then decreased, diverging from the in ovo pattern. Furthermore, while in vitro *Mis* expression at MPT was consistently significantly greater than at FPT, peak expression was 10 times lower than that observed in ovo. FPT expression in vitro was below the level of qPCR detectability at all time points examined. Gonads shifted MPT→FPT revealed an immediate down-regulation of expression that was significantly less than MPT levels by Day 1 and became undetectable by Day 8. In FPT→MPT gonads, *Mis* expression displayed a strong increase in response to new MPT environment significantly greater than FPT levels by Day 8, delayed from the respective increase in ovo. Expression increased through Day 12, and was then maintained for the duration of culture.

FoxL2 expression in vitro at MPT remained at baseline levels throughout the culture period (Fig. 5H). At FPT, cultured gonads showed a significant increase in expression above MPT levels by Day 4, earlier than in ovo, with a dip in expression at Day 20. Peak *FoxL2* expression at FPT was roughly 1.5 times greater in vitro than that observed in ovo. Following an MPT→FPT shift, *FoxL2* expression remained at MPT-typical levels through Day 8, and then increased significantly by Day 12, at the same time as the respective increase in ovo. Expression then plateaued and was maintained for the duration of the culture period, rather than continuing to increase. In FPT→MPT gonads, expression remained at baseline levels throughout the culture period, with a slight increase observed at Day 20.

Misexpression of *Sox9*

Gonads were dissected from stage 16 embryos incubating at FPT and a plasmid construct (pCS107:GFP or pCS107:GFP:Sox9) was introduced by electroporation. The gonads were subsequently cultured in vitro for up to 3 days at FPT and preserved for gene expression analysis. The efficiency of electroporation was examined by fluorescence microscopy and revealed a mosaic GFP expression pattern throughout the gonad (Fig. 6A,B). F6
Brighter GFP fluorescence was observed in gonads electroporated with control GFP plasmid as compared to fusion GFP:Sox9.

At Day 1 after electroporation, *Sox9* levels were significantly greater in pCS107:GFP:Sox9 gonads as compared to pCS107:GFP gonads (Fig. 6C). This increase was gone by Day 2. At both Days 1 and 2, *Dmrt1* expression in gonads electroporated with pCS107:GFP:Sox9 was increased

ID: kumarpr | Black Lining: [ON] | Time: 17:24 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1069

above control levels. However, variation within these groups was substantial and this increase was not statistically significant. Expression of *FoxL2* was not different between gonads electroporated with each plasmid. Expression of *Mis* was below the level of qPCR detectability for all gonads (data not shown).

DISCUSSION

Sox9, *Mis*, *Dmrt1*, and *FoxL2* Exhibit Sustained Responses to Changes in Temperature During In Ovo Development

The gene expression patterns observed in individual in ovo gonads reveals the ability of the embryo to sense and respond to change in environmental temperature by regulating expression of the putative sex-determining molecular network. We previously investigated the ability of sex-determining genes to respond rapidly to changes in incubation temperature at two early time points of the TSP (Shoemaker et al., 2007b). Because we were interested in the ability of the expression of these genes to change rapidly, the timeframe examined did not facilitate elucidating downstream responses. Here, we extend those results to examine changes throughout the entire sex-determining period and reveal previously undetected sensitivity to changes in temperature in both shift directions in *Dmrt1*, *Mis*, and *FoxL2*. Furthermore, we confirm previous findings in another species that *Sox9* expression is also regulated by temperature shifts in both directions (Moreno-Mendoza et al., 2001).

The regulation of *Sox9* expression by temperature was evident by its repression in MPT→FPT shifted gonads and up-regulation in FPT→MPT shifted gonads. Furthermore, the timing of these responses suggest that in the slider turtle, *Sox9* is not involved early in the opening of the temperature-sensitive window during sex "determination," the initial steps that direct or fate a bipotential gonad to a particular sexual trajectory. Instead, our data are consistent with the hypothesis that *Sox9* may play a role in this species in sex "commitment," the final commitment of the bipotential gonad to a testicular fate. As described above, gonadal sex in TSD organisms remains plastic until the end of the TSP. Therefore, dimorphic expression of genes involved in commitment to a sexual fate would not be necessary in the developing gonad until the end of the TSP when the window of sex-reversibility is closing, as occurs with *Sox9*.

We reveal for the first time a significant, rapid repression of *Dmrt1* expression below MPT-typical levels in response to a MPT→FPT temperature shift. Furthermore, we show that the previously demonstrated increase in *Dmrt1* expression in response to a FPT→MPT shift at two early time points during the TSP (Shoemaker et al., 2007b) is maintained through gonad differentiation. These results confirm the temperature-sensitivity of *Dmrt1* and suggest a function in the developing testis of the slider turtle. The data indicate that it may be critical to repress this male-specific function in gonads at FPT to facilitate ovary formation. The sexually dimorphic pattern of *Dmrt1* expression from very early in the TSP may suggest that it plays a role in the upstream process of sex determination in the developing slider turtle testis, in contrast to its downstream role in mammalian testis differentiation.

Previously, a rapid repression of *Mis* in MPT→FPT shifted gonads was demonstrated (Shoemaker et al., 2007b), and here we reveal that this repression is sustained throughout gonadogenesis. Additionally, we show for the first time that *Mis* expression is significantly up-regulated in gonads shifted from FPT→MPT. This increase continues through the sex determining-period and into differentiation. Taken together, these data are consistent with the hypothesis that *Mis* plays a role in the development of the testis, and that this function is suppressed in developing ovaries. It would be productive to investigate whether *Mis* is responsible for the inhibition of Müllerian duct differentiation into female-specific ducts in developing male slider turtles, as it does in mammals (Behringer et al., 1990).

We demonstrate that *FoxL2* expression significantly increases in gonads 12 days following a MPT→FPT shift, supported by a similar finding in the snapping turtle (Rhen et al., 2007). This up-regulation occurs at the same time as the increase in expression observed in unshifted FPT gonads, indicating that the gonad maintains a developmental clock even when shifted between temperatures. Furthermore, *FoxL2* expression in gonads shifted FPT→MPT remains low at MPT-typical levels for the duration of gonadogenesis. The timing of these responses suggests a role for *Foxl2* in the developing ovary of slider turtles either during the commitment of the gonad to an ovarian fate or during ovarian differentiation. In both mammals and fish, *FoxL2* up-regulates *aromatase* expression, which is required for estrogen production (Pannetier et al., 2006; Wang et al., 2007), and inhibiting *aromatase* results in decreased *FoxL2* expression in chicken (Hudson et al., 2005). These results indicate that a positive feedback loop may exist between *FoxL2* and *aromatase*, and it is not unlikely that estradiol plays a role in modulating this interaction (Smith et al., 2008). It was previously shown in the slider turtle that strongly dimorphic *aromatase* expression between FPT and MPT appears by stage 21, concurrent with dimorphic *FoxL2* expression (Ramsey et al., 2007). Taken together with the data presented in the current study, these results are consistent with the hypothesis that a similar mechanism may occur in vertebrates with TSD.

Sex-Determining Response to Temperature Is Regulated Endogenously in the Cells of the Gonad

Here, we show that in the red-eared slider turtle, an isolated gonad endogenously retains the ability to sense changes in environmental temperature and respond by regulation of the gene network governing sexual development. Importantly, individual cultured gonads respond to temperature with gene transcription changes in the absence of other possible temperature-sensing systems (e.g., the brain, Merchant-Larios et al., 1989; Gutierrez-Ospina et al., 1999). While the morphology of gonads developing in vitro is hindered by isolation,

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1070

expression of the sex-determining pathway closely mimics in ovo patterns. Gene expression pathways initiated by either a maintained or a shifted temperature progress normally for some time despite limits placed on gonad development by altered cellular behavior. Specifically, in vitro molecular interactions proceed normally for at least 8 and up to 20 days, while cellular interactions are more strongly hindered by the gonad's isolation from neighboring tissues.

The timing of these gene expression changes was generally similar between in ovo and in vitro samples, although the differences are illuminating. Disparities include a delay in the up-regulation of *Dmrt1* and *Mis* in FPT→MPT shifted gonads in vitro as compared to in ovo. Thus, gonads encountering a new male-producing environment took longer to induce a male-typical gene cascade in isolated gonad tissue than when the gonad was incubating within the embryo. Furthermore, the repression of *Sox9* and *Dmrt1* in MPT→FPT gonads was also delayed in vitro, indicating that FPT repression of the male pathway is delayed in isolated tissue. The exception to this is *Mis*, whose repression in MPT→FPT gonads occurred one day faster in vitro than in ovo. The timing of *FoxL2* expression changes in both shift directions was identical in vitro and in ovo, indicating that the expression of this gene is highly correlated to an internal "developmental clock." It is dangerous to make generalizations based on one marker, but according to our findings of *FoxL2* expression patterns, gonads encountering or leaving a new female-producing environment up-regulate or down-regulate, respectively, the female cascade at the appropriate time.

Throughout the duration of the 20-day culture period, the relative levels of gene expression in vitro compared with in ovo levels varied among the markers examined. At constant temperatures, *Sox9* expression in vitro was consistently similar to in ovo levels throughout culture, except for a brief spike in expression at MPT above what was observed in ovo. In contrast, *Dmrt1* showed slightly decreased in vitro gene expression compared with in ovo beginning at Day 16, and *Mis* decreased from Day 12 onward. Finally, *FoxL2* expression levels were similar in vitro and in ovo through Day 16, at which point expression at FPT began to decline. Thus, the optimal length of culture varies depending on which marker is used for justification. The utility of this culture system may be limited to molecular changes observable in a 2-week time period.

Functional Manipulation of the Sex-Determining Molecular Network

Gonads electroporated with either control GFP or a fusion GFP:Sox9 construct exhibited mosaic GFP fluorescence throughout the tissue. GFP fluorescence was brighter in gonads electroporated with control plasmid than in samples electroporated with GFP:Sox9. This could be due to several reasons: electroporation efficiency may differ between the two plasmids, expression of the two plasmids may differ within gonadal cells, or fusing Sox9 to GFP may inhibit correct folding and thereby decrease fluorescence. We were not able to distinguish between these possibilities in this study, but future examination of protein levels by Western blot or immunocytochemistry as well as analysis of GFP expression by qPCR may elucidate this discrepancy. In any event, it is clear that both constructs entered gonadal cells and were misexpressed.

Sox9 expression was significantly increased in gonads electroporated with GFP:Sox9 over control gonads by 1 day later. Although there was no difference in *FoxL2* or *Mis* expression between gonads treated with either plasmid, the effect of increased SOX9 on endogenous *Dmrt1* expression is unclear. An observable increase in *Dmrt1* expression at 1 and 2 days after electroporation is not statistically significant due to large variation between individuals, and further optimization of the culture technique to decrease variation will clarify this response.

To our knowledge, this is the first time that misexpression has been used to examine the genetic network underlying TSD. The ability to conduct both gain- and loss-of-function studies using in vitro cultured gonads will facilitate elucidating both the functions of specific members of this regulatory network as well as the temporal hierarchy of their action within the developing gonad.

Morphological Development of Gonads Is Slower in Isolated Tissues

Removing the gonad from its embryonic environment slows morphological development in general, and specifically hinders sex cord formation. At FPT and MPT→FPT, it takes longer for ovarian-specific structures to form in vitro, and at MPT and FPT→MPT, sex cord formation is hindered by the lack of supporting tissues. These results may be due to the lack of underlying mesonephric tissue that the gonad is thought to require for full differentiation. In the slider turtle, the mesonephric coelomic epithelium invaginates into the gonad and appears to be required for fully formed sex cords in the developing testis (Yao et al., 2004). When this coelomic epithelium is removed, the formation of sex cords is likely inhibited. Unfortunately, whole adrenal-kidney-gonad complexes cultured with underlying mesonephric tissue intact undergo rapid degradation and cell death, and development does not proceed (N. Moreno-Mendoza, personal communication).

Sox9 Localization Early in the Temperature-Sensitive Period Is Not Sexually Dimorphic

We previously reported a sexually dimorphic localization of *Sox9* early in the TSP (Shoemaker et al., 2007a). At stage 15, *Sox9* transcripts were found to be preferentially organized in gonads developing at MPT in clusters of cells surrounding nonexpressing cells, while expression remained diffuse at FPT (Shoemaker et al., 2007a). Here, we clarify those results and find that while *Sox9* expression does remain diffuse in some gonads at FPT, other gonads at FPT exhibit clusters of *Sox9*-expressing cells surrounding nonexpressing cells similar to the pattern seen at MPT. Overall, there were no differences in the range or average number of clusters found

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1071

in gonads at MPT versus FPT. In light of this new evidence, it is concluded that *Sox9* becomes sexually dimorphic in the gonads of the red-eared slider turtle after dimorphic *Mis* expression, similar to the case in alligator and chicken (Oreal et al., 1998; Smith et al., 1999a, Western et al., 1999). Furthermore, it confirms previous evidence in the snapping turtle that dimorphic *Dmrt1* expression also appears before dimorphic *Sox9* expression (Torres-Maldonado et al., 2002). This expression pattern is consistent with the hypothesis that *Sox9* plays a role in the commitment of the developing gonad (Shoemaker and Crews, 2009), and does not appear to be involved early in the opening of the temperature-sensitive window in the slider turtle.

Evidence *Sox9* May Not Initially Up-regulate *Mis* in the Slider Turtle Gonad

Sox9 directly up-regulates the initial expression of *Mis* in the developing mammalian testis, and this relationship appears not to be conserved throughout all taxa. In chicken, alligator and slider turtle, sexually dimorphic expression of *Mis* in developing gonads precedes dimorphic *Sox9*, indicating that the initial up-regulation of *Mis* has likely been coopted by other molecule(s) (Fig. 4; Oreal et al., 1998; Western et al., 1999; Smith et al., 1999a; Takada et al., 2004; Shoemaker et al., 2007b). While any possible functional relationship between these two molecules in organisms with TSD is as yet undescribed, the data here continue to support the hypothesis that *Mis* is initially up-regulated by something other than *Sox9* in the red-eared slider turtle. It must be noted that although gene expression patterns do not dictate a hierarchy of gene action (presence of transcript does not mean transcript is functional, that it is spliced consistently in different cellular environments, or that protein splice forms are functionally equivalent), they can be useful as a predictive model.

First, in ovo gonads shifted FPT→MPT exhibit significant up-regulation of *Mis* above FPT levels by Day 4, while up-regulation of *Sox9* became significantly greater than FPT by Day 8. Second, in vitro gonads shifted MPT→FPT also reveal that *Mis* expression responds more rapidly to the new FPT environment than *Sox9* expression. *Mis* is immediately down-regulated by a shift to FPT, exhibiting statistically lower expression by Day 1. The transient increase in *Mis* expression in MPT→FPT gonads at Day 4 is driven by one individual gonad (n = 8 total, data not shown). In contrast, *Sox9* expression in MPT→FPT gonads showed an initial increase at Days 1 and 2, followed by a slow descent to baseline levels by Day 8. These data indicate that *Mis* has a strong and rapid temperature sensitivity in both directions (MPT→FPT and FPT→MPT), and that regulation of *Mis* is not due to a simple increase in or lack of *Sox9* transcript. In fact, if *Sox9* was responsible for the initial up-regulation of *Mis*, the up-regulation in vitro of *Sox9* in MPT→FPT gonads would be predicted to cause a corresponding increase in *Mis*, which is not observed.

Third, when exogenous *Sox9* is misexpressed in gonads developing at FPT in vitro, *Mis* expression remains undetectable in all cases (data not shown). Because *Sox9* up-regulation is transient and only lasts for 1 day, it is possible that an effect on *Mis* is not observed due to timing of the experiment, and given stable expression of *Sox9* at FPT for a longer period, *Mis* would be up-regulated. In this initial study, however, the repression of *Mis* by FPT was not altered by a significant increase in SOX9 levels. Therefore, although our data do not rule out the possibility that *Sox9* may help to maintain the expression of *Mis*, they do support the hypothesis that some other factor upstream of *Mis* controls its initial expression in the gonad of the slider turtle. To further confirm this, sustained misexpression is a goal of future experiments.

In all likelihood, there are multiple mechanisms regulating the expression of *Mis*. For example, *Mis* expression may be initially up-regulated by an unknown male-typical factor, maintained by *Sox9*, repressed by estrogen, and repressed directly by female-producing temperature or indirectly by means of a female-typical factor. It is possible that *Mis* directly senses temperature, or more likely is being regulated by means of a separate temperature-sensing molecule; both hypotheses would be fruitful to examine, beginning with a functional analysis of the *Mis* promoter and misexpression of various components of the sex-determining network.

Interaction of the Genetic and Hormonal Networks Underlying Gonadogenesis

The pattern of expression of the genes studied here do not conflict with the hypothesis that their expression is hormone-sensitive, and examining the hierarchy of action of these various players (temperature, hormones, and genes) is critical to understanding patterning of the vertebrate gonad. It is now known that, in contrast to the case in mammals, estradiol is critical to early patterning of the ovary in the red-eared slider turtle (Pieau and Dorizzi, 2004; Ramsey and Crews, 2009). Estradiol treatment to the eggshell of developing embryos overrides the effect of MPT in ovo, feminizing the resulting gonad (Crews et al., 1991). The interactions between estradiol and the molecular network underlying gonadogenesis have begun to be explored in studies examining *ER*α, *ER*β, *aromatase*, *AR*, and *Sf1* (Fleming and Crews, 2001; Ramsey et al., 2007; Ramsey and Crews, 2007). It is probable that estradiol-induced sex-reversal is modulated in part by the repression of gene products required for testis development, and these repressed factors may include *Sox9*, *Mis*, and *Dmrt1*.

The ability of estradiol to regulate expression of these four genes in an organism with TSD has been explored in only a few cases. When estrogen is applied to slider turtle eggshells incubating at MPT before the TSP, expression of *Dmrt1* is down-regulated to FPT-typical levels; effect on other genes were not examined (Murdock and Wibbels, 2006). In a teleost fish, treatment of an aromatase inhibitor to embryos at a temperature that produces mixed sex ratios caused an increase *Mis* expression (Fernandino et al., 2008b). The modulation of these interactions remains to be elucidated but, as indicated by our data (Supp. Fig. S1), it would be revealing to expand hormone treatment studies of

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1072

these components of the sex-determining pathway. In fact, during the publication of this manuscript, Barske and Capel demonstrated the regulation of *SOX9* by estradiol in the slider turtle (2010). It will be of critical importance for future research on the TSD molecular network to examine these interactions downstream of temperature between endogenous steroid hormones and gene products. Understanding the complex interactions of these two systems, hormonal and genetic, is necessary to fully explain how a gonad is patterned in organisms with TSD.

In summary, we demonstrate in the red-eared slider turtle that expression of *Sox9*, *Dmrt1*, *Mis*, and *FoxL2* exhibit sustained changes in expression in response to sex-reversing temperature shifts that last through embryonic gonadogenesis. We use a whole-organ in vitro culture system and show that when gonads are separated from other embryonic tissues, expression patterns of these four genes mimic in ovo patterns at both constant and shifted temperature regimes. Isolated gonads retain the ability to sense and respond to changes in temperature and endogenously regulate the putative molecular network governing sexual development, demonstrating that this process occurs within the cells of the gonad of the slider turtle. Furthermore, we demonstrate the ability to misexpress fusion plasmid constructs introduced by electroporation into gonadal cells, allowing functional manipulation experiments that are classic in more traditional model systems. These types of experiments open the door to future studies elucidating the function and temporal hierarchy of action of the molecular cascade underlying gonadogenesis in organisms exhibiting temperature-dependent sex determination.

EXPERIMENTAL PROCEDURES

Embryos

Freshly laid red-eared slider eggs purchased from Clark Turtle Farms (Hammond, LA) were maintained as previously described (Wibbels et al., 1991), in accordance with humane animal practices under IACUC protocol #03102301. Briefly, viable eggs were randomized in trays of moistened vermiculite and placed in incubators (Precision, Chicago, IL) at 26.0°C or 31.0°C. Incubator temperatures were monitored daily with HOBO data loggers (Onset Computer Corp., Bourne, MA) and verified with calibrated thermometers. For temperature shift experiments, multiple trays of 30 eggs/tray were shifted at developmental stage 16 from incubators held at 26.0°C to 31.0°C and vice versa. Progression of development was monitored by staging external morphological characteristics according to Greenbaum's staging series (Greenbaum, 2002), with the addition of stage "16.5" as described previously (Shoemaker et al., 2007b). Greenbaum's staging series of *Trachemys scripta* embryos is similar to Yntema's staging series of *Chelydra serpentina*, and the reader is referred to the original papers for differences in specific stage comparisons (Yntema, 1968). In this study, the terms "early" and "late" were used to describe slight stage variations observed between individual eggs laid on the same day. For example, an "early" stage 16 embryo (quantified as 15.75 in Fig. 2) had younger morphology as compared to an exact stage 16 embryo (16.0), but an older phenotype than a late stage 15 embryo (15.25). Individual stage data collected throughout the study are presented in Supp. Table S1.

Selection of Shift Timepoint: Stage 16

In the temperature-shift studies, development was allowed to proceed at the initial incubation temperature through stage 16, at which point the embryo is in the middle of the TSP, the gonad has begun to follow a particular sexual trajectory (testicular or ovarian), and molecular differentiation has begun. Gonads (in ovo or in vitro) were then shifted to the opposing temperature regime and analyzed at various subsequent time points to assess altered gene expression patterns. Shifting eggs at stage 16, therefore, represents a true change from one initiated developmental trajectory to the other, and is the latest known stage at which 100% of shifted embryos will respond to the new temperature and undergo complete sex-reversal in both directions (MPT→FPT or FPT→MPT; Wibbels et al., 1991).

In Ovo Tissue Collection

Eggs were incubated at 26.0°C (MPT) or 31.0°C (FPT) until Greenbaum's stage 16 (= Day 0), at which point some eggs were allowed to continue developing at constant temperature, while other eggs were shifted to the opposite temperature regime (MPT→FPT or FPT→MPT). Following 1, 2, 4, 8, 12, 16, or 20 days of in ovo growth post-Day 0, embryos were removed from the egg, staged, and killed by rapid decapitation. Adrenal-kidney-gonad complexes (AKGs) were immediately dissected and floated in sterile phosphate buffered saline (PBS). Gonads were carefully separated from underlying mesonephric tissues using fine scissors and a dissecting microscope and preserved immediately for histology or gene expression analysis (Fig. 1A).

Whole-Gonad In Vitro Organ Culture

Eggs were incubated at 26.0°C (MPT) or 31.0°C (FPT) until Greenbaum's stage 16, at which point embryos were removed from the egg and killed by rapid decapitation. AKGs were immediately dissected and floated in sterile PBS. Gonads were separated from underlying mesonephric tissues and grown individually in culture on a sterile, low protein-binding 0.4 μm Biopore Millicell membrane (Millipore, Billerica, MA) floating on 2 ml culture medium. Culture medium initially contained Leibovitz's L-15 medium (+L-Glutamine, phenol red; Gibco) supplemented with 10% unstripped fetal bovine serum (FBS; Sigma) and 0.2% Antibiotic/Antimycotic (Gibco). However, gonads cultured at MPT in the presence of phenol red and fetal serum hormones exhibited dramatic repression of *Sox9*, *Dmrt1*, and *Mis* and slight upregulation of *FoxL2* mRNA expression (Supp. Fig. S1). Thus, a component of this culture medium (likely phenol red, a known estrogen mimic; Berthois and Katzenellenbogen, 1986; Welshons et al., 1988) repressed MPT-typical patterns and induced FPT-typical patterns of expression. Subsequent studies used phenol red-free culture medium containing Leibovitz's L-15 medium (+L-Glutamine; Gibco) supplemented with 10%

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1073

charcoal-stripped FBS (Sigma) and 0.2% Antibiotic/Antimycotic (Gibco).

Gonads were cultured in sterile culture plate wells (Corning) positioned in a self-contained growth chamber unit placed in large incubators maintained at 26.0°C or 31.0°C (Precision, Chicago, IL). Incubator temperatures were monitored daily with HOBO data loggers (Onset Computer Corp., Bourne, MA) and verified with calibrated thermometers. Culture medium was refreshed by replacing 1 ml every 2 days for the duration of culture. Gonads were grown at either constant temperatures (the same temperature as before dissection) or shifted temperatures (FPT→MPT and MPT→FPT). Following 1, 2, 4, 8, 12, 16, or 20 days of culture, gonads were preserved for histology or gene expression analysis (Fig. 1B).

Histology

Gonads collected for histology were stored in 1 mL of Bouin's solution (71% picric acid, 24% formaldehyde, 5% acetic acid) and subsequently washed in 70% EtOH containing several drops of ammonium hydroxide. Gonads were dehydrated through an increasing EtOH series, cleared in xylene, and embedded in paraffin. Tissue was sectioned on an American Optical Company microtome at 6 μm, stained using standard hematoxylin and eosin (H&E) procedures, and photographed on a Nikon microscope.

RNA Extraction and cDNA Synthesis

Following dissection from the egg or in vitro culture, individual gonads were placed immediately in 800 μl of RNA Denaturing Solution (Promega), vortexed for 1 min to dissociate, and stored at −80°C. Total RNA was extracted using the RNAgents Total RNA Isolation kit (Promega) and treated with DNA-Free Turbo DNase I (Ambion, Austin, TX). All RNA extracted from each individual gonad was reverse-transcribed into cDNA using the SuperScript First-Strand Synthesis for reverse transcriptase-polymerase chain reaction system (Invitrogen, Carlsbad, CA) with both oligo-(dT) and random hexamer primers and stored at −20°C for gene expression analysis by qPCR.

Quantitative Real-Time PCR

Relative gene expression levels were quantified using SYBR Green I dye technology (Invitrogen) and an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.2.1 software) as described previously (Shoemaker et al., 2007a,b). Briefly, samples were each run in triplicate and the median value was adjusted for gene-specific PCR efficiency and normalized to constitutive expression of *protein phosphatase 1* (*PP1*). To examine gene expression fold changes both in ovo and in vitro, values were calibrated to the lowest in ovo expression value. These time points were as follows: for *FoxL2*, in ovo Day 20 MPT; for *Sox9* and *Dmrt1*, in ovo Day 20 FPT; for *Mis*, the value of 0.00001. *Mis* expression at Day 20 FPT was below the level of detectability; 0.00001 is the threshold of detection for *Mis* expression as determined by the ABI 7900, and, therefore, is used to approximate the lowest point of detectable expression.

Whole-Mount In Situ Hybridization

Turtle embryos were harvested from eggs at Greenbaum's stage 15 and AKGs were dissected and fixed overnight in 4% paraformaldehyde/PBS at 4°C (n = 10 at FPT and n = 5 at MPT). Whole-mount ISH was performed as by Andrews et al. (1997) with modifications described previously (Shoemaker et al., 2007a). AKGs were subsequently embedded in OCT freezing medium (Fisher Scientific, Hampton, NH), sectioned on a 2800 Reichert-Jung cryostat and photographed on a Nikon microscope. Clusters of expressing cells were counted in a blind experimental design by two independent researchers for every readable section per gonad and averaged. The total number of cell clusters per section were then analyzed to give a range and mean for each gonad.

Plasmid Construction

An *Eco*RI restriction site was inserted in frame by PCR into the 5' UTR of slider turtle *Sox9* (GenBank accession #EU914820). The full-length coding region (1604 base pairs) was then subcloned into pCS107:GFP:3stop containing a simian CMV IE94 promoter by restriction enzyme digest. The plasmid was a kind gift from John Wallingford, originally derived by the Harland lab (http://mcb.berkeley.edu/labs/harland/links.html) from Dave Turner's pCS2+ multipurpose expression vector (http://sitemaker.umich.edu/dlturner.vectors).

In Vitro Electroporation

Eggs were incubated at 31.0°C (FPT) until Greenbaum's stage 16, at which point embryos were removed from the egg and killed by rapid decapitation. AKGs were immediately dissected and floated in sterile PBS. Gonads were separated from underlying mesonephric tissues and placed on solidified Sylgard in a sterile Petri dish in 7.5 μl of 0.8 μg /μl plasmid DNA (either pCS107:GFP or pCS107:GFP:Sox9). Gonads were electroporated (2 × 20 volts × 50 msec × 3 pulses with 100 msec interval) with 0.002″ bare platinum wires (A-M Systems, Inc., Carlsborg, WA) attached to a BTX Electro Square Porator ECM 830 while floating in the solution of oligonucleotides. Following electroporation, the tissue was immediately transferred to a fresh droplet of culture medium for several minutes. Gonads were then transferred to a 0.4-μm Millicell membrane (Millipore) and cultured for 1, 2, or 3 days as above. Electroporation efficiency was assessed by examining GFP fluorescence at day 1 on an Olympus SZX12 microscope. After 1, 2, or 3 days of culture, gonads were preserved for gene expression analysis (Fig. 1C).

Statistical Analysis

For each gene, expression values measured by qPCR relative to the housekeeping gene (*PP1*) were tested for normality and variance assumptions. Raw data were log-transformed to meet the normality assumption and a heterogeneous variance model was selected that allows for variation across groups (sex/day combinations) within the model. For *Mis*, values below the level of qPCR detectability (= 0.0) were replaced with threshold value 0.00001 (see above); maximum likelihood did not converge with a heterogeneous variance model and so a simpler model was chosen. A two-factor Mixed model (proc mixed) on all

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

J_ID: Z7H Customer A_ID: DVDY22266 Cadmus Art: DVDY22266 Ed. Ref. No.: 09-0462 Date: 8-March-10 Stage: Page: 1074

values tested for sex or day main effects and a sex by day interaction within each gene (SAS software program, SAS Institute, Inc., Cary, NC). Sex by day interactions were found to be statistically significant for all genes (Supp. Table S2). Simple pairwise comparison tests examined differences within gene between sex within day, and was corrected for multiple tests (43 contrasts per model) using a stepwise Bonferroni adjustment (Supp. Table S3). Contrasts were considered statistically significant at the $\alpha = 0.05$ level. For electroporated gonads, expression data were normalized to the housekeeping gene *PP1*, and a general linear model (proc glm) was conducted analyzing effect of treatment for each day for each gene (SAS software program).

ACKNOWLEDGMENTS

We thank Raymond Porter and Robbie Bayly for assistance in electroporation technique; John Wallingford for the kind gift of pCS plasmid; Molly Cummings for the use of her microscope; Tom Juenger and Nate Marti for statistical assistance; Norma Moreno-Mendoza and Horacio Merchant-Larios for assistance in setting up in vitro culture technique; John Wallingford, Jim Bull, and Mary Ramsey for helpful review of the manuscript; as well as several reviewers whose perceptive and astute critiques were invaluable.

REFERENCES

Andrews JE, Smith CA, Sinclair AH. 1997. Sites of estrogen receptor and aromatase expression in the chicken embryo. Gen Comp Endocrinol 108:182–190.

Arango NA, Lovell-Badge R, Behringer RR. 1999. Targeted mutagenesis of the endogenous mouse Mis gene promoter, in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409–419.

Baroiller JF, D'Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G. 2009. Tilapia sex determination: where temperature and genetics meet. Comp Biochem Physiol A Mol Integr Physiol 153:30–38.

Barske L, Capel B. 2010. Estrogen represses SOX9 during sex determination in the red-eared slider turtle Trachemys scripta. Dev Biol in press.

Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL. 1990. Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. Nature 345:167–170.

Benayoun BA, Caburet S, Dipietromaria A, Bailly-Bechet M, Batista F, Fellous M, Vaiman D, Veitia RA. 2008. The identification and characterization of a FOXL2 response element provides insights into the pathogenesis of mutant alleles. Hum Mol Genet 17: 3118–3127.

Berthois Y, Katzenellenbogen JA. 1986. Phenol red in tissue culture media is a weak estrogen, implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83: 2496–2500.

Blecher SR, Erickson RP. 2007. Genetics of sexual development, a new paradigm. Am J Med Genet A 143:3054–3068.

Brennan J, Capel B. 2004. One tissue, two fates, molecular genetic events that underlie testis versus ovary development. Nat Rev Genet 5:509–521.

Bull JJ, Wibbels T, Crews D. 1990. Sex-determining potencies vary among female incubation temperatures in a turtle. J Exp Zool 256:339–341.

Burtis K, Baker B. 1989. Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56:997–1010.

Cederroth CR, Pitetti JL, Papaioannou MD, Nef S. 2007. Genetic programs that regulate testicular and ovarian development. Mol Cell Endocrinol 265–266:3–9.

Crews D. 1993. The organizational concept and vertebrates without sex chromosomes. Brain Behav Evol 42:202–314.

Crews D, Bull JJ, Wibbels T. 1991. Estrogen and sex reversal in turtles, a dose-dependent phenomenon. Gen Comp Endocrinol 81:357–364.

Crews D, Bergeron JM, Bull JJ, Flores D, Tousignant A, Skipper JK, Wibbels T. 1994. Temperature-dependent sex determination in reptiles, proximate mechanisms, ultimate outcomes, and practical applications Dev Genet 15:297–312.

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G. 2001. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166.

De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulat F, Berta P. 1998. Direct interaction of SRY-related protein SOX9 and Steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol 18:6653–6665.

DiNapoli L, Capel B. 2008. SRY and the standoff in sex determination. Mol Endocrinol 22:1–9.

Fernandino JI, Hattori RS, Shinoda T, Kimura H, Strobl-Mazzulla PH, Strüssmann CA, Somoza GM. 2008a. Dimorphic expression of dmrt1 and cyp19a1 (ovarian aromatase) during early gonadal development in pejerrey, Odontesthes bonariensis. Sex Dev 2:316–324.

Fernandino JI, Hattori RS, Kimura H, Strussmann CA, Somoza GM. 2008b. Expression profile and estrogenic regulation of antiMullerian hormone during gonadal development in pejerrey Odontesthes bonariensis, a teleost fish with strong temperature-dependent sex determination. Dev Dyn 237:3192–3199.

Fleming A, Crews D. 2001. Estradiol and incubation temperature modulate regulation of steroidogenic factor 1 in the developing gonad of the red-eared slider turtle. Endocrinology 142:1403–1411.

Fleming A, Vilain E. 2004. The endless quest for sex determination genes. Clin Genet 67:15–25.

Greenbaum E. 2002. A standardized series of embryonic stages for the emydid turtle Trachemys scripta. Can J Zool 80:1350–1370.

Gutierrez-Ospina G, Jimenez-Treho F, Favila R, Moreno-Mendoza NA, Granados Rojas L, Barrios FA, Diaz-Cintra S, Merchant-Larios H. 1999. Acetylcholinesterase-positive innervation is present at undifferentiated stages of the sea turtle Lepidochelys olivacea embryo gonads, Implications for temperature-dependent sex determination. J Comp Neurol 410:90–98.

Hudson QJ, Smith CA, Sinclair AH. 2005. Aromatase inhibition reduces expression of FoxL2 in the embryonic chicken ovary. Dev Dyn 233:1052–1055.

Kettlewell JR, Raymond CS, Zarkower D. 2000. Temperature-dependent expression of turtle Dmrt1 prior to sexual differentiation. Genesis 26:174–178.

Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Suzuki A, Saito N, Nakamoto M, Shibata N, Nagahama Y. 2004. Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, Oryzias latipes. Dev Dyn 231:518–526.

Loffler KA, Zarkower D, Koopman P. 2003. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome, FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144:3237–3243.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M. 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559–563.

McCue MD. General effects of temperature on animal biology. 2004. In: Valenzuela N, Lance VA, editors. Temperature-dependent sex determination in vertebrates. Washington: Smithsonian Books. p 71–78.

Merchant-Larios H, Villalpando FI, Centeno UB. 1989. Gonadal morphogenesis under controlled temperature in sea turtle Lepidochelys olivacea. Herpetol Monogr 3:43–61.

Moreno-Mendoza N, Harley VR, Merchant-Larios H. 2001. Temperature regulates SOX9 expression in cultured

ID: kumarpr | Black Lining: [ON] | Time: 17:25 | Path: N:/Wiley/3b2/DVDY/Vol23904/100059/APPFile/C2DVDY100059

gonads of Lepidochelys olivacea, a species with temperature sex determination. Dev Biol 229:319–326.
Morrish BC, Sinclair AH. 2002. Vertebrate sex determination, many means to an end. Reproduction 124:447–457.
Murdock C, Wibbels T. 2003. Expression of Dmrt1 in a turtle with temperature-dependent sex determination. Cytogenet Genome Res 101:302–308.
Murdock C, Wibbels T. 2006. Dmrt1 expression in response to estrogen treatment in a reptile with temperature-dependent sex determination. J Exp Zool B Mol Dev Evol 306:134–139.
Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Schartl M. 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc Natl Acad Sci 99:11778–11783.
Oreal E, Pieau C, Mattei MG, Josso N, Picard JY, Carre-Eusebe D, Magre S. 1998. Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. Dev Dyn 212:522–532.
Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, Nedorezov T, Cao A, Forabosco A, Schlessinger D. 2007. Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. Hum Mol Genet 16:2795–2804.
Pannetier M, Fabre S, Batista F, Kocer A, Renault L, Jolivet G, Mandon-Pepin B, Cotinot C, Veitia R, Pailhoux E. 2006. FoxL2 activates P450 aromatase gene expression, toward a better understanding of the early steps of mammalian ovarian development. J Mol Endocrinol 36:399–413.
Pieau C, Dorizzi M. 2004. Oestrogens and temperature-dependent sex determination in reptiles, all is in the gonads. J Endocrinol 181:367–377.
Place AR, Lance VA. 2004. The temperature-dependent sex determination drama, Same cast, different stars. In: Valenzuela N, Lance VA, editors. Temperature-dependent sex determination in vertebrates. Washington: Smithsonian Books. p 99–110.
Ramsey M, Crews D. 2007. Steroid signaling system responds differently to temperature and hormone manipulation in the red-eared slider turtle (Trachemys scripta elegans), a reptile with temperature-dependent sex determination. Sex Dev 1:181–196.
Ramsey M, Crews D. 2009. Steroid signaling and temperature-dependent sex determination – reviewing the evidence for early action of estrogen during ovarian determination in turtles. Semin Cell Dev Biol 20:283–292.
Ramsey M, Shoemaker CM, Crews D. 2007. Gonadal expression of Sf1 and aromatase during sex determination in the red-eared slider turtle (Trachemys scripta), a reptile with temperature-dependent sex determination. Differentiation 75:978–991.
Raymond CS, Shamu CE, Shen M, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D. 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391:691–695.
Raymond CS, Murphy MW, O'sullivan MG, Bardwell VJ, Zarkower D. 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev 14:2587–2595.
Rhen T, Metzger K, Schroeder A, Woodward R. 2007. Expression of putative sex-determining genes during the thermosensitive period of gonad development in the snapping turtle, Chelydra serpentina. Sex Dev 1:255–270.
Ross AJ, Capel B. 2005. Signaling at the crossroads of gonad development. Trends Endocrinol Metab 16:19–25.
Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M. 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131:933–942.
Sekido R, Lovell-Badge R. 2008. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 453:930–934.
Shen M, Hodgkin J. 1998. mab-3, a gene required for sex-specific yolk protein expression and a male-specific lineage in C. elegans. Cell 54:1019–1031.
Shoemaker C, Crews D. 2009. Analyzing the coordinated gene network underlying temperature-dependent sex determination in reptiles. Semin Cell Dev Biol 20:290–303.
Shoemaker C, Ramsey M, Queen J, Crews D. 2007a. Expression of Sox9, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. Dev Dyn 236:1055–1063.
Shoemaker C, Queen J, Crews D. 2007b. Response of candidate sex-determining genes to changes in temperature reveals their involvement in the molecular network underlying temperature-dependent sex determination. Mol Endocrinol 21:2750–2763.
Smith CA, Sinclair AH. 2004. Sex determination: insights from the chicken. Bioessays 26:120–132.
Smith CA, Smith MJ, Sinclair AH. 1999a. Gene expression during gonadogenesis in the chicken embryo. Gene 234:395–402.
Smith CA, McClive PJ, Western PS, Reed KJ, Sinclair AH. 1999b. Conservation of a sex-determining gene. Nature 402: 601–602.
Smith CA, Shoemaker CM, Roeszler KN, Queen J, Crews D, Sinclair AH. 2008. Cloning and expression of R-spondin1 in different vertebrates suggests a conserved role in ovarian development. BMC Dev Biol 8:72–88.
Sreenivasulu K, Ganesh S, Raman R. 2002. Evolutionarily conserved, DMRT1, encodes alternatively spliced transcripts and shows dimorphic expression during gonadal differentiation in the lizard, Calotes versicolor. Mech Dev 119(suppl):S55–S64.
Takada S, DiNapoli L, Capel B, Koopman P. 2004. Sox8 is expressed at similar levels in gonads of both sexes during the sex determining period in turtles. Dev Dyn 231:387–395.
Torres Maldonado LC, Landa Piedra A, Moreno-Mendoza N, Marmolejo Valencia A, Meza Martínez A, Merchant Larios H. 2002. Expression profiles of Dax1, Dmrt1, and Sox9 during temperature sex determination in gonads of the sea turtle Lepidochelys olivacea. Gen Comp Endocrinol 129:20–26.
Udar N, Yellore V, Chalukya M, Yelchits S, Silva-Garcia R, Small K; BPES Consortium.. 2003. Comparative analysis of the FoxL2 gene and characterization of mutations in BPES patients. Hum Mutat 22:222–228.
Veitia R, Nunes M, Brauner R, Doco-Fenzy M, Joanny-Flinois O, Jaubert F, Lortat-Jacob S, Fellous M, McElreavey K. 1997. Deletions of distal 9p associated with 46,XY male to female sex reversal, definition of the breakpoints at 9p23.3-p24.1. Genomics 41:271–274.
Vidal VP, Chaboissier MC, de Rooij DG, Schedl A. 2001. Sox9 induces testis development in XX transgenic mice. Nat Genet 28:216–217.
Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y. 2007. FoxL2 upregulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/Steroidogenic factor 1 Mol Endocrinol 21:712–725.
Welshons WV, Wolf MF, Murphy CS, Jordan VC. 1988. Estrogenic activity of phenol red. Mol Cell Endocrinol 57:169–178.
Western PS, Harry JL, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination in the American alligator, AMH precedes SOX9 expression. Dev Dyn 216:411–419.
Wibbels T, Bull JJ, Crews D. 1991. Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381.
Wilhelm D, Palmer S, Koopman P. 2007. Sex determination and gonadal development in mammals. Phys Rev 87:1–28.
Wilhelm D, Washburn LL, Truong V, Fellous M, Eicher EM, Koopman P. 2009. Antagonism of the testis- and ovary-determining pathways during ovotestis development in mice. Mech Dev 126:324–336.
Yao HH. 2005. The pathway to femaleness: Current knowledge on embryonic development of the ovary. Mol Cell Endo 230:87–93.
Yao HH, Capel B. 2005. Temperature, genes, and sex, a comparative view of sex determination in Trachemys scripta and Mus musculus. J Biochem 138:5–12.
Yao HH, DiNapoli L, Capel B. 2004. Cellular mechanisms of sex determination in the red-eared slider turtle, Trachemys scripta. Mech Dev 121:1393–13401.
Yntema CL. 1968. A series of stages in the embryonic development of Chelydra serpentina. J Morphol 125:219–251.
Zhao Y, Lu H, Yu H, Cheng H, Zhou R. 2007. Multiple alternative splicing in gonads of chicken DMRT1. Dev Genes Evol 217:119–126.