Changes in gonadal gene network by exogenous ligands in temperature-dependent sex determination

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Abstract

We examined the expression of candidate sex-determining genes in the red-eared slider turtle (Trachemys scripta) during the temperature-sensitive period (TSP). Aromatase and Rspo1 were used as markers of ovarian differentiation and Sox9 was used as a marker of testicular differentiation. Eggs were incubated at a male-producing temperature (26 °C or MPT) and a female-producing temperature (31 °C or FPT). First, eggs at the beginning of the TSP (stage 16) were topically treated with the steroid hormones 17ß-estradiol (E2), testosterone in combination with aromatase inhibitor (AI + T), the E2 antagonist (ICI 182 780), and the androgen antagonist (flutamide). Secondly, gonads were removed at stage 16 and treated in vitro with E2, AI + T, or hormone antagonists. At the FPT, AI + T in ovo suppressed aromatase and Rspo1, while activating Sox9. At the MPT, E2 treatment rapidly increased aromatase and Rspo1, while suppressing Sox9. Treatment with the E2 antagonist in ovo decreased aromatase at the FPT. Treatment with the androgen antagonist in ovo increased aromatase and Rspo1 at early time points at MPT and decreased Sox9 at MPT at later developmental stages. Treatment of isolated gonads cultured in vitro with AI + T at FPT decreased aromatase and Rspo1 and E2 increased the expression of these genes at MPT. In vitro treatment with E2 antagonist suppressed aromatase and Rspo1 expression at FPT.

Overall, our results suggest that exogenous ligands dictate gonadal development by redirecting the expression of candidate sex-determining genes within the genetic cascades induced by temperature.

Introduction

Sex determination in vertebrates is a developmental process where the bipotential embryonic gonads differentiate into ovaries or testes depending on external or internal cues. Based on the susceptibility of the embryonic gonads to specific cues, the sex determination system can be divided into two categories: the genotypic sex determination (GSD) system and the environmental sex determination (ESD) system. Temperature-dependent sex determination (TSD) is a form of ESD, where the sex determination of the embryonic gonad is dictated by the incubation temperature of the eggs. In the red-eared slider turtle (Trachemys scripta), a well-studied TSD species, eggs incubated at 26 °C become males (male-producing temperature or MPT) while eggs incubated at 31 °C become females (female-producing temperature or FPT). The effect of the incubation temperature is only observed midway through development, also known as the temperature-sensitive period (TSP). Exogenous ligands...
(steroid hormones and steroid metabolism enzyme inhibitors) can override the effect of the incubation temperature if applied during this TSP window. Specifically, 17β-estradiol (E2) treatment of the eggs induces ovarian development at the MPT, while testosterone along with aromatase inhibitor (AI) treatment induces testicular development at the FPT (Wibbels et al. 1991, Crews et al. 1996). Although exogenous ligands can override the effect of the incubation temperature, the role of endogenous sex steroid hormones in sex determination is still somewhat controversial in species with TSD. For example, administration of sex steroid hormone antagonists – E2 antagonist applied at the FPT or androgen antagonist applied at the MPT – often fails to affect the gonad phenotype at hatching (Wibbels et al. 1991, Crews et al. 1996).

How environmental cues, such as temperature or exogenous ligands, engage the molecular mechanisms underlying gonadal development is not fully understood, although recent studies show that several genes (or gene networks) involved in the GSD system exhibit typical gonadal expression patterns in response to temperature in the red-eared slider. For example, aromatase, a gene that encodes the enzyme that catalyzes the conversion of androgens to estrogens, is upregulated at the FPT and downregulated at the MPT in the embryonic gonads (Ramsey et al. 2007). Aromatase expression and activity is not directly involved in sex determination in mammals; however, aromatase is sufficient to control gonadal sex during development in some nonmammalian vertebrates including birds and fish (Elbrecht & Smith 1992, Guiguen et al. 2010). R-spondin1 (RSPO1), a gene involved in ovarian differentiation in mammals, is also highly expressed during the TSP in red-eared slider gonads at the FPT but is only expressed at low levels at the MPT (Smith et al. 2008). A transcription factor, sex-determining region on Y chromosome-box 9 (Sox9), a direct target of the transcription factor SRY during testicular differentiation in mammals, is upregulated at the MPT and downregulated at the FPT in embryonic gonads (Shoemaker et al. 2007a). Accumulating evidences suggest that Rspo1 and Sox9 are balancing factors to determine a unidirectional result of gonad phenotype in mammals (Huang et al. 1999, Barrionuevo et al. 2006, Parma et al. 2006, Chassot et al. 2008, Tomizuka et al. 2008). In this study, we examined the expression pattern of three candidate sex-determining genes in TSD, aromatase, Rspo1, and Sox9, in exogenous ligand-mediated sex determination in red-eared slider gonads. Slider eggs were topically treated with exogenous ligands at a corresponding temperature at stage 16, the beginning of the TSP.

As previous studies of the red-eared slider show that treatment with aromatase inhibitor or testosterone alone during development produces limited or no male hatchlings (Wibbels et al. 1991, Crews & Bergeron 1994, Crews 1996), we used combined treatment with aromatase inhibitor and testosterone at the FPT to ensure mostly male hatchlings in the current study. The expression patterns were then examined during the following developmental time periods including hatching. Furthermore, we examined the effect of hormone antagonists on candidate sex-determining gene expression to understand the role of endogenous sex steroid hormones in gonadal determination. The effect of exogenous ligands and hormone antagonists on candidate sex-determining genes was also examined in isolated cultured gonads.

**Materials and methods**

**Animals**

Freshly laid red-eared slider turtle eggs were purchased from Clark Turtle Farms (Hammond, LA, USA) and maintained in accordance with humane animal practices under IACUC protocol # AUP-2011-00149. Eggs were collected daily within 24 h of laying so that all individual eggs in the shipment were roughly of equal developmental stages. Eggs were mixed, so clutches were equally represented in each experimental group. Eggs were stored at room temperature for 10 days, at which time all eggs were candled to assess viability. Viable eggs were randomly placed in trays with moistened vermiculite and incubated at either 26 °C (MPT) or 31 °C (FPT). Incubator temperatures were checked daily with thermometers, and temperature fluctuations were monitored with HOBO data loggers (Onset Computer Corp., Bourne, MA, USA). Embryos were allowed to develop until stage 16, the early TSP when all embryos are sensitive to temperature and exogenous ligands (Wibbels et al. 1991). Staging was according to external morphological characteristics according to the Greenbaum’s staging series (Greenbaum 2002).

**In ovo exogenous ligand treatment**

At stage 16, eggs were treated as follows, after which the eggs were returned to their designated incubation temperature; at the FPT, eggs were treated with i) 10 µl EtOH as a vehicle control, ii) 100 µg testosterone (Sigma) combined with 75 µg non-steroid aromatase inhibitor, fadrozole (AI: Sigma), iii) 100 µg E2 antagonist...
ICI 182 780 (Sigma), or iv) 500 µg ICI 182 780. At the MPT, eggs were treated with i) 10 µl EtOH as a vehicle control, ii) 10 µg E₂ (Sigma), iii) 1 mg androgen antagonist, flutamide (Sigma), or iv) 2.5 mg flutamide. All chemicals were dissolved in 10 µl 100% EtOH and topically applied to the surface of the eggs. The dosages used were based on previous studies and on the solubility of the specific agents (Crews & Bergeron 1994, Wibbels & Crews 1994, Wu et al. 2009, Barske & Capel 2010). These eggs were incubated until the embryos reached stages 17, 19, 21, 23, 25, or hatching, at which time gonads were collected to be processed individually (n=6–8 gonads, i.e. three to four individuals/stage/treatment). Gonads from stage 16 embryos incubating at the FPT and MPT were collected before the treatments to determine a basal gene expression level. Gonads were placed individually into tubes containing 800 µl Trizol (Life Technologies), vortexed, and stored at −80 °C until RNA extraction. Sex was macroscopically diagnosed in hatching turtles before processing for gene expression (Crews & Bergeron 1994). All treatments and sex diagnosis were coded and so blind to the investigators.

**In vitro exogenous ligand treatment in tissue culture**

Red-eared slider embryonic gonads were isolated and cultured as described previously (Shoemaker-Daly et al. 2010). Briefly, at stage 16 (Day 0), embryonic gonads were dissected under sterile conditions. The isolated gonads were immediately transferred to a floating 0.4 µm Millicell membrane (Millipore, Billerica, MA, USA) placed on the surface of a 24-well plate (BD Falcon, Franklin Lakes, NJ, USA) filled with cell culture media composed of Leibovitz’s L-15 medium (+10% FBS; Sigma) and 1% antibiotic/antimycotic (Life Technologies). Isolated gonads from eggs incubated at the FPT were treated with i) 10 µM AI combined with 1 µM testosterone or ii) 10 µM ICI 182 780 and allowed to develop at the FPT. Gonads from eggs incubated at the MPT were treated with i) 100 nM E₂ or ii) 10 µM flutamide and allowed to develop at the MPT. All chemicals were dissolved in EtOH and adjusted to a final concentration of 0.03%. Both incubation temperatures included two negative control conditions: i) vehicle control of 0.03% EtOH and ii) negative control gonads without any treatment to reinforce the validity of EtOH as a proper negative control for subsequent gene expression analysis. The dosages used were chosen based on previous *in vitro* studies performed using other cell types (Yano et al. 1995, Bhattacharryya et al. 2006, Jolly et al. 2006, La Sala et al. 2010, Yilmaz et al. 2011). Culture plates were incubated in a self-contained growth chamber under sterile condition. On the day of the tissue culture at stage 16 (Day 0), gonads were also extracted and immediately placed in individual tubes containing 800 µl Trizol solution, vortexed, and stored at −80 °C until RNA extraction (baseline control). The cell culture media were changed every 2 days, and the same concentration of exogenous ligands or controls was added to the fresh media in each well. Gonads were collected in 800 µl Trizol on day 1, 4, 8, 12, 16, or 20, vortexed, and placed at −80 °C until RNA extraction.

**Total RNA extraction, cDNA synthesis, and quantitative real-time PCR**

Total RNA was extracted with Trizol by following the manufacturer’s protocol (Life Technologies). Subsequently, total RNA was treated with DNA-Free Turbo DNase I (Life Technologies) and reverse transcribed into cDNA using the iScript Kit (Bio-Rad). Relative gene expression was quantified using SYBR green (Life Technologies) on the ABI PRISM 7900HT real-time PCR cycler (Life Technologies). Samples were run in triplicate in quantitative real-time PCR (qPCR), and the median values were used for analysis. Relative gene expressions were measured using previously published aromatase, Rspo1, and Sox9 primers specific to sliders and normalized to the gene expression of protein phosphatase 1 (PPT), a housekeeping gene expressed constitutively relative to the genes of interest in slider gonads (Ramsey et al. 2007, Shoemaker et al. 2007a, Smith et al. 2008). The specificity of qPCR was validated by melting curve analysis. The obtained qPCR data were analyzed using the ACT method and the gene expression fold changes between control and treatment groups were calculated by setting the lowest values of the group as onefold. For functional landscape analysis, qPCR data were represented as a percentage maximum in each gene and plotted by Matlab (MathWorks, Natick, MA, USA).

**Statistical analysis**

Because of the asymmetric distribution, the nonparametric test was used to analyze all data. Gonad sex ratio and qPCR data were analyzed using χ² (Fisher’s exact) and Wilcoxon rank-sum test respectively by JMP 8.0 (SAS Institute, Cary, NC, USA). All P values acquired by qPCR data are shown in Supplementary Table 1, see section on supplementary data given at the end of this article. Functional landscape
analysis was performed using MultiDimBio package in R (Scarpino et al. 2013). A P value <0.05 was considered to be statistically significant.

Results

Exogenous ligands, not steroid hormone antagonists, can override the incubation temperature on gonad sex ratio at hatching

In the red-eared slider, a single treatment of exogenous ligands at the beginning of TSP, stage 16, is sufficient to override the ambient temperature and produce a specific gonad phenotype (Wibbels et al. 1991, Crews et al. 1996).

In accordance with this finding, the treatments of exogenous ligands were able to override the temperature effect. AI + T at the FPT mostly produced testis phenotypes except one exhibited ovary-like structure with an oviduct (Fig. 1A). Blocking endogenous E2 with a low dosage of ICI 182 780 at the FPT produced all ovarian phenotypes except for two eggs that exhibited testes with oviducts; with high doses, all eggs displayed ovarian phenotypes except one that had testes without an oviduct (Fig. 1A). E2 at the MPT produced 100% ovarian phenotypes at hatching (Fig. 1B). Blocking endogenous testosterone with a low dose of flutamide at the MPT did not have any effect on gonad phenotype, whereas a high dose of flutamide produced three eggs that exhibited ovarian phenotypes with oviduct formation (Fig. 1B). None of the individuals in control groups failed to show the predicted gonad sex according to the ambient temperature. Further, no intersex gonads (e.g. ovotestes) were observed in the treatment groups.

Treatment with testosterone and aromatase inhibitor at FPT reversed the temperature-specific expression patterns of candidate sex-determining genes in ovo

The expression of candidate sex-determining genes in the control group exhibited a typical temperature-specific expression pattern at FPT: i) an ovarian marker, aromatase exhibited a gradual increase at the beginning of stage 17, which peaked at stage 21 and gradually decreased until hatching; ii) another ovarian marker, Rspo1, began to increase after stage 23 and peaked at hatching; and iii) a testicular marker, Sox9, exhibited a low expression throughout development at the FPT (Fig. 2A). These observations are in agreement with previous findings (Ramsey et al. 2007, Shoemaker et al. 2007a,b, Smith et al. 2008), although the current study examines an extended developmental period including hatching. When eggs were topically treated with AI + T at stage 16, the expression of aromatase and Rspo1 decreased as early as stage 17, and they remained at low levels throughout development (Fig. 2A). The expression of Sox9 in the AI + T-treated group exhibited a gradual increase throughout development as observed typically at the MPT (Fig. 2A). Next, we performed functional landscape analysis to examine a functional change in the relationship among genes. In this analysis, a transcriptional change in three genes within an individual was considered as one unit, i.e. a functional landscape, and compared between treatments at specific developmental time points (Scarpino et al. 2013). Such analysis will provide us with biologically different aspects of gene functionality.
The effect of testosterone and aromatase inhibitor on the expression of aromatase, Rspo1, and Sox9 at the FPT. (A) Changes in gene expression in exogenous ligand-treated slider gonads during development in ovo. Eggs at the FPT were topically treated with AI+T at stage 16. Gonads were collected at stage (St) 17, 19, 21, 23, 25, and hatching. The relative gene expression levels of aromatase, Rspo1, and Sox9 were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, PP1, and are represented as a fold change. Each time point included n = 6–8 gonads/treatment/time point. Data represented as the mean ± S.E.M. Grey boxes indicate no statistical difference between the two treatments within a developmental stage by Wilcoxon rank sum test. Points without the boxes were significantly different. All P values from statistical analysis are shown in Supplementary Table 1. (B) Landscape analysis for a functional change in the relationship among aromatase, Rspo1, and Sox9. Values from qPCR were represented as a percentage maximum in each gene and plotted by developmental time points. Difference represents a subtraction of a treatment group (AI+T effect) from a control group (temperature effect) in each stage.

Treatment with E$_2$ at the MPT reversed the temperature-specific expression patterns of candidate sex-determining genes in ovo

Next, we examined the effect of E$_2$ at the MPT on the expression patterns of candidate sex-determining genes, aromatase, Rspo1, and Sox9, in red-eared slider gonads. In the control group, aromatase, Rspo1, and Sox9 genes exhibited typical expression patterns found in MPT: suppressed expression of aromatase and Rspo1 and a corresponding increase in the expression of the testicular marker Sox9 (Fig. 3A). The topical addition of E$_2$ at stage 16 reversed the gene expression pattern typically observed at the MPT. The expression of the ovarian marker aromatase increased in response to E$_2$ at stage 23, which was a later developmental time point and a weaker intensity than the one normally observed at the FPT (Fig. 3A). The expression of Rspo1 was increased as early as stage 17 in rapid response to E$_2$ with an exception of stage 21 (Fig. 3A). Sox9 expression was high at stage 17 in the E$_2$-treated group; however, it decreased throughout the rest of the developmental stages (Fig. 3A). Functional landscape analysis
revealed that a functional relationship of three genes at the MPT was completely altered by a treatment with E2 from the early developmental stages. In E2-treated gonads, Rspo1 expression gradually increased while Sox9 expression was suppressed, as typically observed at the FPT (Fig. 3B). However, unlike a typical landscape at the FPT, it occurred without a spike of aromatase expression (Figs 3B compared to 2B).

Exogenous ligands shape ovary, not testis, phenotype differently from incubation temperature

We further examined whether a certain phenotype shaped by exogenous ligands followed the same landscape pathway as the phenotype induced by temperature. Eggs incubated at the FPT and those treated with E2 at the MPT produce an ovarian phenotype at hatching (Fig. 1). A functional relationship of three candidate sex-determining genes showed no difference between the two treatments within a developmental stage by Wilcoxon rank sum test. Points without the boxes were significantly different. All P values from statistical analysis are shown in Supplementary Table 1. (B) Landscape analysis for a functional change in relationship among aromatase, Rspo1, and Sox9. Values from qPCR were represented as a percentage maximum in each gene and plotted by developmental stages. Difference represents a subtraction of a treatment group (E2 effect) from a control group (temperature effect) in each stage.

Figure 3

The effect of E2 on the expression of aromatase, Rspo1, and Sox9. (A) Changes in gene expression in E2-treated slider gonads during development in ovo. Eggs at the MPT were topically treated with E2 at stage 16. Gonads were collected at stage (St) 17, 19, 21, 23, 25, and hatching. The relative gene expression levels of aromatase, Rspo1, and Sox9 were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, Pp1, and represented as a fold change. Each time point included n = 6-8 gonads/treatment/time point. Data are represented as the mean ± S.E.M. Grey boxes indicate no statistical difference between the two treatments within a developmental stage by Wilcoxon rank sum test. Points without the boxes were significantly different. All P values from statistical analysis are shown in Supplementary Table 1. (B) Landscape analysis for a functional change in relationship among aromatase, Rspo1, and Sox9. Values from qPCR were represented as a percentage maximum in each gene and plotted by developmental stages. Difference represents a subtraction of a treatment group (E2 effect) from a control group (temperature effect) in each stage.
Figure 4

Landscape analysis compared within a gonad phenotype, ovary (A) and testis (B). The value of qPCR was represented as a percentage maximum in each gene and plotted by developmental stages (St). Difference was calculated by subtraction of a treatment group (E₂ at MPT or AI+T at FPT) from a control temperature (FPT or MPT respectively) and statistical differences were analyzed using MultiDimBio package in R (Scarpino et al. 2013). NS, not statistically significant.
Treatment with steroid hormone antagonists altered the temperature-specific expression patterns of candidate sex-determining genes in ovo

Next, we examined the role of endogenous sex steroid hormones in the expression of candidate sex-determining genes at a specific temperature, i.e. a role of E2 to induce ovaries at the FPT or testosterone to induce testes at the MPT. Eggs were topically treated with E2 antagonist at the FPT or testosterone antagonist at the MPT at stage 16. These treatments did not significantly alter the gonad sex ratio or phenotype from control group at hatching (Fig. 1). However, the expression levels of several genes were affected during development. The addition of low doses of the E2 antagonist ICI 182 780 at FPT delayed the peaking of aromatase expression; however, the overall expression pattern of aromatase was similar to the control group (FPT in Fig. 5). High doses of ICI 182 780 significantly lowered the expression level of aromatase throughout development at the FPT. A low dose of ICI 182 780 decreased the expression of Rspo1 at stage 17 at the FPT; however, the expression patterns during the rest of the developmental stages were not altered. In contrast to our prediction, a high dose of ICI 182 780 increased Rspo1 expression at stages 17 and 21 at the FPT (FPT in Fig. 5). The expression of Sox9 significantly decreased with the treatment of low and high doses of ICI 182 780 at stages 21, 23, 25, and hatching and at stages 19 and 25 at the FPT respectively; the overall expression pattern of Sox9 during gonadal development, however, remained consistent to control at both doses (FPT in Fig. 5). Next, we examined the role of endogenous testosterone in the expression of candidate sex-determining genes at the MPT by topically applying the androgen antagonist flutamide on the eggs at stage 16. A low dose of the androgen antagonist at MPT did not affect aromatase and Rspo1 expression. With high doses of flutamide at the MPT, there was an increased expression of aromatase at stage 19 and Rspo1 at stage 17, though these effects diminished in subsequent stages (MPT in Fig. 5). The expression of Sox9 was increased at stage 17 and decreased at stage 25 and hatching by low doses of flutamide, while high doses led to decreased expression at stages 21, 25, and hatching (MPT in Fig. 5).

The expression pattern of aromatase and Rspo1 dictated by exogenous ligands and steroid hormone antagonists in vitro mimics the expression pattern observed in ovo

In previous studies, we showed that the expression pattern of candidate sex-determining genes in isolated, cultured gonads closely mimicked the expression pattern in ovo in response to incubation temperatures; this finding suggests that the gonad itself has the ability to dictate its own gene expression pattern according to environmental cues (Shoemaker-Daly et al. 2010). In this study, we determined whether the observed gene expression profiles elicited by exogenous ligands and hormone antagonists also occurred in isolated gonads. The expression of the testicular marker Sox9 was not included in our data analysis because it showed a significant change in expression by the control vehicle treatment (0.03% EtOH) when compared with non-EtOH treatment (data not shown). The expression levels of the two ovarian markers were not affected by the control vehicle treatment and were subsequently analyzed. Treatment with AI+T repressed aromatase expression at days 1, 12, and 20 at the FPT in vitro (FPT...
Mean cultured gonads/treatment/time point. Data are represented as the mean ± S.E.M. An asterisk indicates statistically significant difference (s) from control group analyzed by Wilcoxon rank-sum test. All P values are shown in Supplementary Table 1.

Discussion

Sex determination in vertebrates takes place as a gradual process and can be categorized into three phases, onset by genetic or environmental triggers, differentiation into testis or ovary, and maintenance by male- or female-specific molecules. At which phase of the sex determination process the sex steroid hormones come into play remains elusive in nonmammalian vertebrates. However, studies demonstrate that disturbance of sex steroid hormone balance during gonadogenesis often results in redirecting the gonad development against genetic or environmental triggers in nonmammalian vertebrates (Elbrecht & Smith 1992, Crews & Bergeron 1994, Kitano et al. 2000). In this study, we investigated the effect of exogenous ligands, i.e. sex steroid hormones and a steroid metabolism enzyme inhibitor, and steroid hormone antagonists on the process of gonad sex determination by examining the patterns of sex-determining gene expression in the red-eared slider turtle. It is known that exogenous ligands can modify the trajectory of gonadal sex determination set by ambient temperature if applied during development in the red-eared slider and other species (Bull & Vogt 1981, Wibbels et al. 1991, Crews et al. 1996). In the hatchlings with AI+T treatment, the typical developmental expression pattern of ovarian markers aromatase and Rspo1 was suppressed and the testicular marker Sox9 was increased (Fig. 2). By contrast, E2 in Fig. 6). The expression of Rspo1 was decreased by AI+T treatment at day 20 at the FPT, but its expression was not affected at any other time points (FPT in Fig. 6). E2 treatment increased the aromatase expression at days 12 and 20 and Rspo1 expression at days 1, 4, 12, and 20 respectively at the MPT (MPT in Fig. 6). As observed in ovo, the treatment with E2 antagonist ICI 182 780 repressed aromatase expression at days 8, 12, and 20 at the FPT in vitro (FPT in Fig. 7). The expression of Rspo1 was also decreased by ICI 182 780 treatment at days 8 and 20 at the FPT (FPT in Fig. 7). The androgen antagonist flutamide altered the expression of aromatase at days 1 and 16 and Rspo1 at days 4, 8, and 12 at the MPT (MPT in Fig. 7).

Figure 6
The effect of exogenous ligands on the expression of aromatase and Rspo1 in cultured slider gonads during development. Embryonic gonads at the FPT and MPT were isolated at stage 16, treated with exogenous ligands, and continuously cultured at the same temperature for up to 20 days. The control vehicle group was treated with a final concentration of 0.03% EtOH. Gonads were collected on days (D) 1, 4, 8, 12, 16, and 20. Relative gene expression levels of aromatase and Rspo1 during development were measured by qPCR and analyzed using the ΔCt method. All values were normalized to a housekeeping gene, Ppt, and represented as a fold change. The same sample was used for the values at stage 16 in both a control and a treatment group. Each time point included n=8–10 cultured gonads/treatment/time point. Data are represented as the mean ± S.E.M. An asterisk indicates statistically significant difference (s) from control group analyzed by Wilcoxon rank-sum test. All P values are shown in Supplementary Table 1.

Figure 7
The effect of steroid hormone antagonists on the expression of aromatase and Rspo1 in cultured slider gonads during development. Embryonic gonads at the FPT and MPT were isolated at stage 16, treated with steroid hormone antagonists (E2 antagonist at the FPT and androgen antagonist at the MPT), and continuously cultured at the same temperature for up to 20 days. The control vehicle group was treated with a final concentration of 0.03% EtOH. Gonads were collected on days (D) 1, 4, 8, 12, 16, and 20. Relative gene expression levels of aromatase and Rspo1 during development were measured by qPCR and analyzed using the ΔCt method. All values were normalized to a housekeeping gene, Ppt, and represented as a fold change. The same sample was used for the values at stage 16 in both a control and a treatment group. Each time point included n=8–10 cultured gonads/treatment/time point. Data are represented as the mean ± S.E.M. An asterisk indicates statistically significant difference (s) from control group analyzed by Wilcoxon rank-sum test. All P values are shown in Supplementary Table 1. ICI, E2 antagonist ICI 182 780; Flut, androgen antagonist flutamide.
treatment at the MPT during TSP increased aromatase and Rspo1 and decreased Sox9 expression, which resulted in the production of all female hatchlings (Figs 1 and 2). E2-treated gonads at the MPT exhibited a missing peak of aromatase expression while typical Rspo1 and Sox9 expression patterns at the MPT were observed. It suggests that Rspo1 activation is a key signal to initiate ovarian differentiation, and exogenous E2 plays a role in triggering this process when aromatase expression is absent at the MPT. Previous studies also showed that exogenous ligands modify the expression of candidate sex-determining genes, Rspo1 and Sox9. For example, embryonic exposure to DHT increases Sox9 and suppresses Rspo1 mRNA levels in developing mouse prostates (Schaeffer et al. 2008).

In the red-eared slider, multiple treatments of eggs with E2 completely suppress the expression of Sox9 by embryonic stage 19 (Barske & Capel 2010). Moreover, Sox9 mRNA is increased in adult ovaries of mice lacking the presence of estrogen receptors (Couse et al. 1999, Dupont et al. 2003). In chickens, the inhibition of estrogen synthesis by aromatase inhibitors results in a decrease in RSPO1 expression in embryonic gonads of genetic females (Smith et al. 2008). Although the hierarchy of genetic cascade of these genes during sex determination has not been established in TSD species, we applied a new analytic method to observe relationship of three genes, a snapshot of genetic interaction at a particular developmental time point. In the functional landscape analysis, the expression pattern of candidate sex-determining genes within an individual is considered as one network or landscape and compared to the networks/landscapes induced by various environmental cues (Scarpino et al. 2013). Interestingly, when the landscape is compared within a phenotype, E2-induced ovarian development exhibits a different developmental pattern of the landscape network from FPT-induced ovarian development (Fig. 4A). This observation confirms that the gonads at the FPT and E2-treated gonads at the MPT follow different gonad differentiation pathways. In testicular development, however, the difference in the gene network between the MPT and AI+T treatments was not observed (Fig. 4B). It is worth noting that the phenotype of ovary induced by E2 was slightly different from the ovary induced by the FPT, smaller in size with less evidence of primordial germ cells as noted in several turtle species (Merchant-Larios et al. 1997, Barske & Capel 2010, Matsumoto & Crews 2012). The phenotypic difference in testis, however, is less noticeable between the two triggers, the MPT and AI+T (Matsumoto & Crews 2012). This observation may be attributed to a different pattern of landscape pathway during a mid period of development in the current study.

The experiment with steroid hormone antagonists revealed hormone–gene interactions, specifically a role of endogenous steroid hormones in the TSD system. Previous studies have shown that steroid hormone antagonists interrupt normal gonadal development, occasionally producing abnormal or intersex gonads in other species (Kang et al. 2006, Katoh et al. 2006). In our study, although steroid hormone antagonists resulted in little change in sex ratio and gonad morphology at hatching, the expression of candidate sex-determining genes has changed. For example, blocking endogenous E2 with a low dosage of E2 antagonist delayed the expression of aromatase at the FPT and a high dosage of E2 antagonist significantly suppressed aromatase (Fig. 5). This indicates that endogenous E2 may be responsible for the manifestation of aromatase expression by a positive feedback mechanism at the FPT. Interestingly, despite the low levels of aromatase expression during development in the E2 antagonist-treated group, the ovaries at hatching did not exhibit any morphological or histological differences from control ovaries at FPT (data not shown). In reptiles and birds, aromatase is thought to be a key enzyme in determining the activation of male vs female pathways by balancing the production of estrogen (Elbrecht & Smith 1992, Jeyasuria et al. 1994). Aromatase is still a candidate of the upstream factor to determine gonad sex in TSD species; however, our data suggest that the expression of aromatase may not be a sole determinant for an ovarian development. Similarly, the lack of an observable effect of the androgen antagonist flutamide on aromatase and Rspo1 gene expression at the MPT suggests that endogenous androgen may not be a primary factor for the suppression of typical ovarian markers at this temperature. Furthermore, Sox9 expression was not prominently affected by antagonist treatments either. These observations, along with hatching phenotypes, lead to the hypothesis that endogenous hormones may not participate in onset of the gene network of the sex-determining genes directed by temperature, but rather are involved in the gonad differentiation that follows. We cannot rule out the possibility that the dosage and timing of the application of antagonist in this study may not have been sufficient to completely block the endogenous steroid hormones. A previous study shows that blocking endogenous E2 with multiple treatments of ICI 182 780 leads to a delay in Sox9 downregulation in slider gonad at the FPT (Barske & Capel 2010), which suggests that
endogenous E2 has, at least to some extent, a suppressive effect on Sox9 expression.

We extended our investigations to elucidate whether the extraembryonic environment was involved in ligand-induced patterns of gene expression. Previous studies have shown that isolated embryonic gonads had the ability to sense and respond to the ambient temperature in TSD species (Moreno-Mendoza et al. 2001, Pieau & Dorizzi 2004, Shoemaker-Daly et al. 2010). In the current study, the isolated gonads also exhibited the expression pattern of the candidate sex-determining genes similar to the one observed in ovo when treated with exogenous ligands and steroid hormone antagonists (Figs 6 and 7). However, the timing of the response of gene expression occurred late in the developmental stage with less intensity. For example, Rspo1 expression at FPT is not suppressed by AI+T treatment until 20 days after treatment (Fig. 6). In E2-treated gonads, both aromatase and Rspo1 expression increased, but with less intensity than the increase observed in ovo. One possible explanation is that isolated gonads lack the supporting cells normally migrated from underlying mesonephric tissue, resulting in a slow development and a lack of a morphological structure (Yao et al. 2004, Shoemaker-Daly et al. 2010). The antagonist treatments in vitro also followed the observation found in ovo: the E2 antagonist ICI 182 780 suppressed the ovarian markers at the FPT, whereas the androgen antagonist flutamide lacked the consistent effect on the expression of these genes at the MPT (Fig. 7). It confirms the speculation in ovo that endogenous E2 positively affects ovarian markers at the FPT while endogenous androgen is not responsible for suppression of these genes at the MPT.

Overall, the current study shows that exogenous ligands modify the gonad phenotype by redirecting (suppress or activate) the expression of candidate sex-determining genes. In vitro data further suggest that exogenous ligands, as well as temperature, affect the expression of candidate sex-determining genes in gonads independently of other embryonic organs during development. Although the factors responsible for the regulation of these candidate sex-determining genes yet remain elusive, this study demonstrates further insights into the role of endogenous/exogenous steroid hormones in the process of sex determination in the TSD system.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y M designed and performed the study. R Y and C T assisted animal dissection and organ cultures. D C contributed to the research design and supervised the project and Y M wrote the manuscript.

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