Cell and Tissue Research (2023) 394:229–241
https://doi.org/10.1007/s00441-023-03814-1

REGULAR ARTICLE

Evidence for RA-dependent meiosis onset in a turtle embryo

Kaiyue Wu[1] · Qiran Chen[1,2] · Fang Li[1] · Jiadong Shen[1] · Wei Sun[1] · Chutian Ge[1]

Received: 22 February 2023 / Accepted: 12 July 2023 / Published online: 1 August 2023

Abstract

Meiotic entry is one of the earliest sex determination events of the germ cell in higher vertebrates. Although advances in meiosis onset have been achieved in mammals, birds and fish, how this process functions in reptiles is largely unknown. In this study, we present the molecular analysis of meiosis onset and the role of retinoic acid (RA) in this process in the red-eared slider turtle. Our results using *Stra8* as a pre-meiosis indicator show that in the female embryonic gonad, meiosis commitment starts around stage 19. Additionally, signals of the meiosis marker *Sycp3* could be detected at stage 19 and become highly expressed by stage 23. No expression of these genes was detected in male embryonic gonads, suggesting the entry into meiosis prophase I was restricted to female embryonic germ cells. Notably, RA activity in fetal gonads is likely to be elevated in females than that in males, as evidenced by the higher expression of RA synthase *Aldh1a1* and lower expression of RA-degrading enzyme *Cyp26a1* in female gonads prior to meiotic entry. In addition, exogenous RA treatment induced the expression of *Stra8* and *Sycp3* in both sexes, whether in vivo or in vitro. Together, these results indicate that high levels of RA in the embryonic female gonads can lead to the initiation of meiosis in the turtle.

Keywords *Aldh1a1* · *Cyp26a1* · Retinoic acid · Meiosis onset · Red-eared slider turtle

Introduction

Meiotic entry is exclusive and essential for germ cells to produce gametes in sexually reproducing animals (Wilkins and Holliday 2009). This process is tightly regulated by multiple sexually dimorphic molecules, resulting in different timing of meiotic entry in different sexes (Anderson et al. 2008; Ishiguro et al. 2020; Koubova et al. 2014). In higher vertebrates, germ cells in the fetal ovary enter meiosis and arrest at prophase I, while those in the testis enter meiosis postnatally (Sou et al. 2021). The main factor that instructs germ cells to undergo the mitotic-meiotic transition has been a mystery for many years. Growing evidence points to a key role for all-*trans*-retinoic acid (RA) in this transition in both sexes (Bowles et al. 2006; Koubova et al. 2006; Li et al. 2016; Smith et al. 2008; Wallacides et al. 2009).

RA is a classical diffusing morphogen that participates in various biological processes critical for development (Cunningham and Duester 2015). However, RA cannot be produced de novo in animal cells. Cells need to first obtain the sources of RA, either as all-*trans*-retinol or as carotenoids, and then catalyze them into retinal by retinol dehydrogenase and beta-carotene oxygenase. Subsequently, retinal is oxidized into RA by RA synthases (including ALDH1A1, ALDH1A2 and ALDH1A3). The synthesized RA acts as a ligand of RA receptors (RARs), which can regulate gene transcription by binding to the RA-responsive elements (RAREs) (Rhinn and Dolle 2012). The RA-degrading enzymes (CYP26A1, CYP26B1, CYP26C1) are produced to catalyze RA to inactive forms. In vertebrates, the correct spatiotemporal distribution of RA is essential for development, as insufficient or excessive RA causes embryonic malformations. Therefore, maintaining RA homeostasis in developing tissues is critically dependent on the balance between production by ALDH1As and degradation by locally produced CYP26s (Niederreither and Dolle 2008).

Kaiyue Wu, Qiran Chen and Fang Li contributed equally to this work.

✉ Qiran Chen
chen.qiran@zwu.edu.cn

✉ Chutian Ge
cge@zwu.edu.cn

[1] College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo 315100, China

[2] MOE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

Until now, three RA-responsive elements have been identified in the regulatory region of the meiosis gatekeeper gene, *stimulated by retinoic acid gene 8* (*Stra8*), in mice, suggesting that RA can trigger the expression of *Stra8*, thereby initiating meiosis (Anderson et al. 2008; Feng et al. 2021). In vitro induction of RA signaling up-regulated *Stra8* expression in cultured mouse gonad-mesonephros complexes (GMCs), while suppression of this signaling down-regulated *Stra8* expression. These results reveal that RA is both necessary and sufficient for meiotic entry in fetal gonads (Koubova et al. 2006). In addition, the RA-degrading enzyme CYP26B1 was found to be suppressed in females but continuously expressed in males. Tissue-specific expressions and knockout of this gene suggested its dimorphic expression caused the sexually dimorphic regulation of meiotic entry in mice, as its continuous expression in males inhibited the RA activity (Bowles et al. 2006; Koubova et al. 2006; MacLean et al. 2007). This regulatory system seems to be conserved in many vertebrates including chickens, amphibians and fish, but it has not been investigated in reptiles yet (Li et al. 2016; Smith et al. 2008; Wallacides et al. 2009; Yu et al. 2013).

The red-eared slider turtle *Trachemys scripta elegans* (*T. scripta*), is a reptile that exhibits temperature-dependent sex determination (TSD), with sex determination occurring during the thermosensitive period (TSP) from stages 15 to 19. In this period, 100% of females are produced under the female-producing temperature (FPT) of 32 °C, while 100% of males are produced under the male-producing temperature (MPT) of 26 °C (Weber et al. 2020; Wibbels et al. 1991). Gonadal differentiation begins around stage 20, with distinct gonadal structures developing in males and females, and observable morphological differences from stage 21 onwards. During sex differentiation (from stage 20 until hatching), there is a remarkable increase in the number of germ cells in the developing gonads, and in female ovaries, germ cells enter meiosis. In the present study, we investigated the possible relationship between retinoic acid signaling and meiotic entry in fetal gonads of *T. scripta*. The expression of meiosis-related markers (*Stra8* and *Sycp3*) and RA metabolism-related genes were examined. In addition, we analyzed the effect of exogenous RA on meiosis initiation of germ cells in vitro and in vivo. Altogether, our study suggests the conservation of RA-dependent meiosis in germ cells in a TSD reptile.

Materials and methods

Experimental animals

Freshly laid eggs of the turtle (*T. scripta*) were obtained from the Hanshou Institute of Turtles (Hunan, China). Fertilized eggs were collected and reared under vermiculite in an incubator controlled at 26 °C (male-producing temperature, MPT) or 32 °C (female-producing temperature, FPT), with the humidity maintained at 85–95%. Developmental stages (stage 15–25) of turtle embryos were determined according to the criteria from Greenbaaum's work (Greenbaum and Carr 2002). The gonad-mesonephros-complexes (GMCs) dissected from embryos were fixed overnight with 4% paraformaldehyde (PFA). Fixed samples were dehydrated in graded methanol concentrations (50%, 75%, 87.5%, 93.75%, 100%) and stored at -20 °C for further analysis. Sections with a thickness of 8 µm were stained with Mayer's hematoxylin and eosin (HE). All experiments involving live turtles were approved by Zhejiang Wanli University.

RNA isolation and RT-qPCR

Embryonic gonads of each developmental stage were carefully separated from GMCs using forceps. The total RNA of the mixed 15 gonads was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was adjusted to the same concentration (1 µg/µl) before reverse transcribed by oligo-p(dT) from RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The relative expression of genes was assessed by RT-qPCR using SYBR Green Fast qPCR Mix (ABclonal) on a Bio-Rad iCycler system. Three biological replicates were carried out in each experiment. $2^{-\Delta\Delta Ct}$ was used for quantifying the gene expression levels (normalized with GAPDH). Significance of differences in the relative ratios was examined by student's t test using GraphPad Prism 8. Primers are listed in Supplementary Table 1.

In situ hybridization

To synthesize riboprobes at 700–1000 bp length, primers were designed according to the cDNA sequence deposited in the NCBI database under the accession numbers shown in Supplementary Table 2. KOD One master mix (Toyobo) was used to synthesize the desired templates. By using TArget Clone plus (Toyobo) or pGEM-T Easy Vector Systems (Promega), recombinant plasmids were produced and transformed into DH5α. Before in vitro transcription, all plasmid inserts were verified by Sanger sequencing. Digoxygenin (DIG)-labeled antisense and sense riboprobes were synthesized using T7, SP6 or T3 RNA polymerase (Roche).

Whole-mount in situ hybridization (ISH) was adapted from the protocol developed for zebrafish embryos with a few modifications (Thisse and Thisse 2008). In brief, rehydrated GMCs were penetrated by 10 µg/ml protease K (Thermo Fisher Scientific) at 37 °C for 15–25 min. After 1-night hybridization with 1 ng/µl probes at 65 °C, the GMCs were washed and then blocked by 1% blocking

reagent (Roche). Anti-DIG-AP antibody (1:5000, Roche) was used for probe detection in situ. Purple precipitates were developed after 1 night with 2% NBT/BCIP substrate (Roche). The samples were dehydrated by methanol and rocked in glycerol at 4 °C. For section ISH, paraffin sections of 8 μm were prepared using HistoCore BIOCUT (Leica). Followed by digestion with 10 μg/ml protease K at 37 °C for 5 min, slices were pretreated with hybridization buffer without tRNA and then hybridized with RNA probe (0.5–1 ng/μl) at 50 °C overnight. After washing with 50% formamide-2×SSC, 2×SSC and 0.2×SSC, sections were blocked and treated with anti-DIG-AP antibody (1:5000) for 2 h at room temperature. The color was developed after 1-night treatment with 2% NBT/BCIP substrate (Roche) in the dark. Sections were dehydrated, cleared, and mounted in Eukitt mounting medium (Sigma-Aldrich) for microscopy. For tyramide signal amplification-based (TSA) ISH, the slides were treated with anti-DIG-POD (1:500, Roche) after blocking. The sections were incubated in Cyanine 3 Plus Amplification Reagent (Akoya) (1:50 dilution in Plus Amplification Diluent (Akoya), followed by 2:1 dilution with distilled water) for 15 min. After washing with phosphate-buffered saline solution with 0.1% Tween 20 (PBST), sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and mounted in 75% glycerol/PBST. At least three samples were prepared for each detection.

In vivo injection of RA

RA (Sigma-Aldrich) solution (20 μg/μl) was freshly prepared in dimethyl sulfoxide (DMSO) before injection. For stage-15 embryos at FPT and stage-21 embryos at MPT, we injected 5 μl RA solution or DMSO into each egg using Hamilton syringes. Then, the gonads and GMCs of day-2 were collected for RNA extraction and 4% PFA fixation, respectively. Two hundred eggs were used for each group.

Ex vivo culture of fetal gonads and GMCs

Gonads and GMCs from stage-18 turtles under MPT were separated and washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco) in petri dishes. The tissues were placed in Polycarbonate Membrane Insert (8.0 μm pore size, Corning) filled with Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco) in an incubator maintained at 26 °C/5% CO_2. Soon, DMSO and 1 mM RA stock solution were supplied 1:1000 to produce a final concentration of 0.1% DMSO and 1 μM RA, respectively. The medium was changed every 2 days. Three replicates were used for each experiment. After 4 days of incubation, gonads and GMCs were collected for RNA extraction and ISH, respectively.

Microscopy

Whole-mount embryos were observed by a Zeiss stereo microscopy system (SteREO Discovery.V20). H&E and chromogenic ISH staining were observed using Eclipse Ni-E (Nikon). Fluorescent photomicrographs were taken using a confocal microscopy system (A1 Plus, Nikon). Images were processed using Adobe Photoshop CS5.

Results

Meiosis onset occurs during embryogenesis in turtles under FPT

The onset of meiosis in *T. scripta* was evaluated using HE staining. At stage 21, clear morphological differences between sexes were observed, with germ cells located in the seminiferous cords of testes and the cortex of ovaries (Fig. 1b, b'). The germ cells that have not yet undergone meiosis are distinguished by an empty vesicle with a small central nucleolus, whereas the meiotic germ cells are identified by chromatin clumps close to the center. In this study, meiotic germ cells were found in the ovaries at stage 21, and no meiotic germ cells were observed in the developing testes of male embryos (Fig. 1).

Next, we used *Stra8* and *Sycp3* to investigate the process of meiosis, which have been respectively used as markers for meiosis onset and initiation of prophase I in many studies of vertebrate animals. First, the amino acid sequences of STRA8 and SYCP3 were compared by maximum likelihood estimation among vertebrates. *T. scripta* STRA8 shows 94% and 82% similarity to that of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and the American alligator (*Alligator mississippiensis*), suggesting that STRA8 is highly conserved among reptiles. This similarity is 71% for the chick (*Gallus gallus*), 57% for human (*Homo sapiens*), 53% for the house mouse (*Mus musculus*), and 30% for the Southern catfish (*Silurus meridionalis*) (Supplementary Fig. 1 and Supplementary Table 3). The conservation of SYCP3 is slightly higher among vertebrates. *T. scripta* SYCP3 shares more than 50% similarity to that of reptiles (90% to *P. sinensis*, 84% to *A. mississippiensis*), avian (84% to *G. gallus*), mammals (64% to *M. musculus*, 70% to *H. sapiens*), and fish (55% to *D. rerio*) (Supplementary Fig. 2 and Supplementary Table 4).

In *T. scripta*, *Stra8* and *Sycp3* were preferentially expressed in the female during embryogenesis. An up-regulation of *Stra8* (P value < 0.001) and *Sycp3* (P value < 0.05) was first observed by RT-qPCR at stage 19 gonads under FPT, whereas the expression levels under MPT were weak. Then *Stra8* maintained a constant expression level in next stages, and the expression level of *Sycp3* increased dramatically (Fig. 2a, b). Unfortunately, our chromogenic in situ

hybridization did not give clear signals for both genes. Thus, we used a TSA-based ISH to increase the sensitivity. This result showed that these meiosis-related genes in gonads were expressed in a remarkably female-specific pattern in developing turtle gonads. Under FPT, *Stra8* was first detected at late stage 19, as confirmed by intense signals in germ cells (Fig. 2c–f, c'–f'). *Sycp3* expression was first observed at stage 19; the signal intensity continued to increase throughout ovary differentiation until stage 24, and then slightly decreased at stage 25 (Fig. 2g–l, g'–l'). Both genes were undetectable in fetal gonads under MPT (Fig. 2c''–f'', c'''–f''', g''–l'', g'''–l'''), indicating the progression of meiosis is restricted to FPT individuals before hatching.

Dynamic expression of RA metabolism-related genes in fetal gonads

The concentration of RA is strictly regulated by synthesizing and degrading enzymes. Therefore, understanding of the spatiotemporal expression of these genes can provide valuable information into dynamic RA activity in fetal gonads. According to the transcriptome data deposited in NCBI, three RA synthases genes (*Aldh1a1*, *1a2*, *1a3*) and three RA-degrading enzymes genes (*Cyp26a1*, *b1*, *c1*) were found in the turtle. Among those synthase genes, *Aldh1a1* showed the highest expression level. *Aldh1a1* was expressed as early as stage 15, which corresponds to the beginning of the gonadal sex determination period. And it was the only one detected by in situ hybridizations in gonads among three synthases. Though clear signals were continuously observed in somatic cells in the gonadal cortex and mesonephric tubules before meiosis onset (stage 19) in both sexes, *Aldh1a1* showed considerably stronger expression under FPT than that under MPT from stage 18 to 23 (Fig. 3a, d–d'''', e'–e'''', f–f'''). *Aldh1a2* was not detected by ISH in gonads though the expression was confirmed by RT-qPCR. Its signals were mainly detected in the mesonephric epithelial cells adjacent to the gonad and the cells in the glomeruli tuft (Fig. 3g–g'''', h–h'''', i–i'''). *Aldh1a3* showed statistically higher expression in MPT gonads than in FPT gonads at stage 16 and 17 (Fig. 3c). Due to its low expression in GMCs, no signal could be detected by ISH. Thus, we suppose *Aldh1a3* may not be critical for meiosis initiation.

Fig. 2 Expressions of *Stra8* and *Sycp3* mRNA in embryonic turtle gonads. **a** Quantitative real-time PCR (qRT-PCR) analysis of *Stra8* mRNA expression. **b** qRT-PCR analysis of *Sycp3* mRNA expression. Data are exhibited as mean ± SD (standard deviation); N = 3. Expression patterns of *Stra8* by tyramide signal amplification-based (TSA) in situ hybridization (ISH) staining in fetal gonads at FPT (**c–f**, **c'–f'**) and MPT (**c''–f''**, **c'''–f'''**). Expression patterns of *Sycp3* by TSA ISH staining in fetal gonads at FPT (**g–l**, **g'–l'**) and MPT (**g''–l''**, **g'''–l'''**); White arrow indicate the germ cell (GC). Scale bar: 25 μm

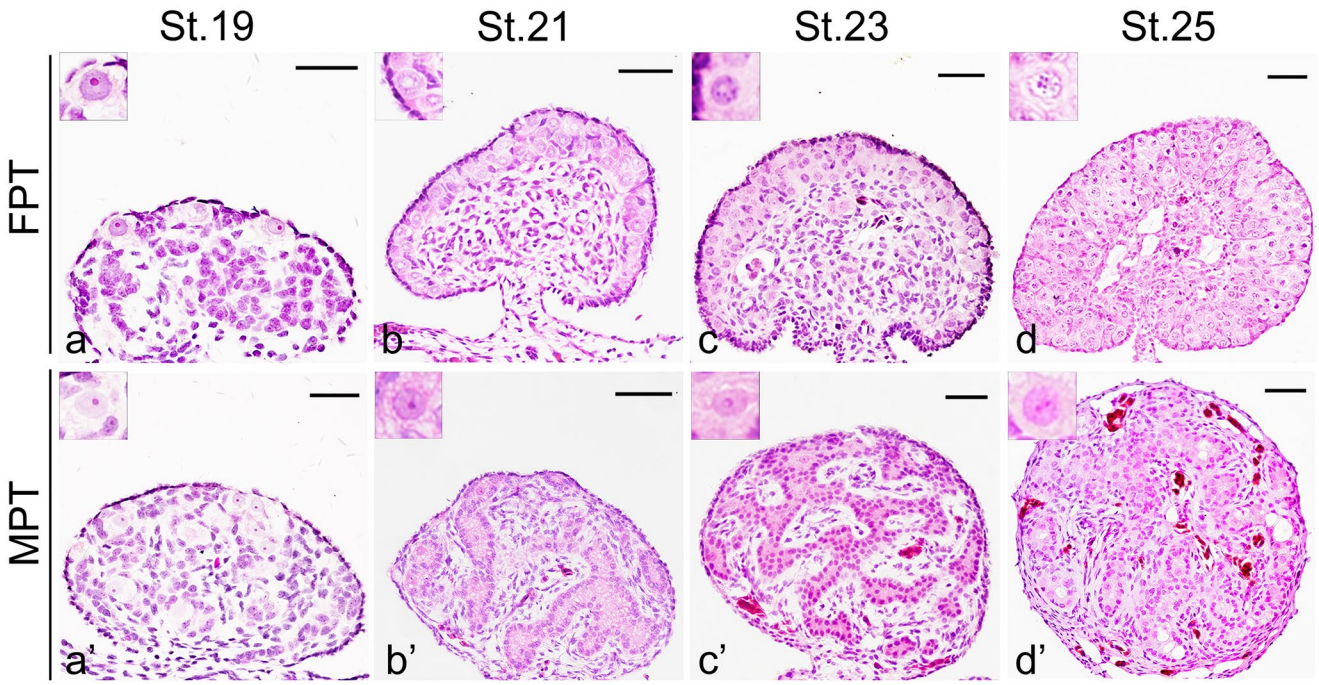
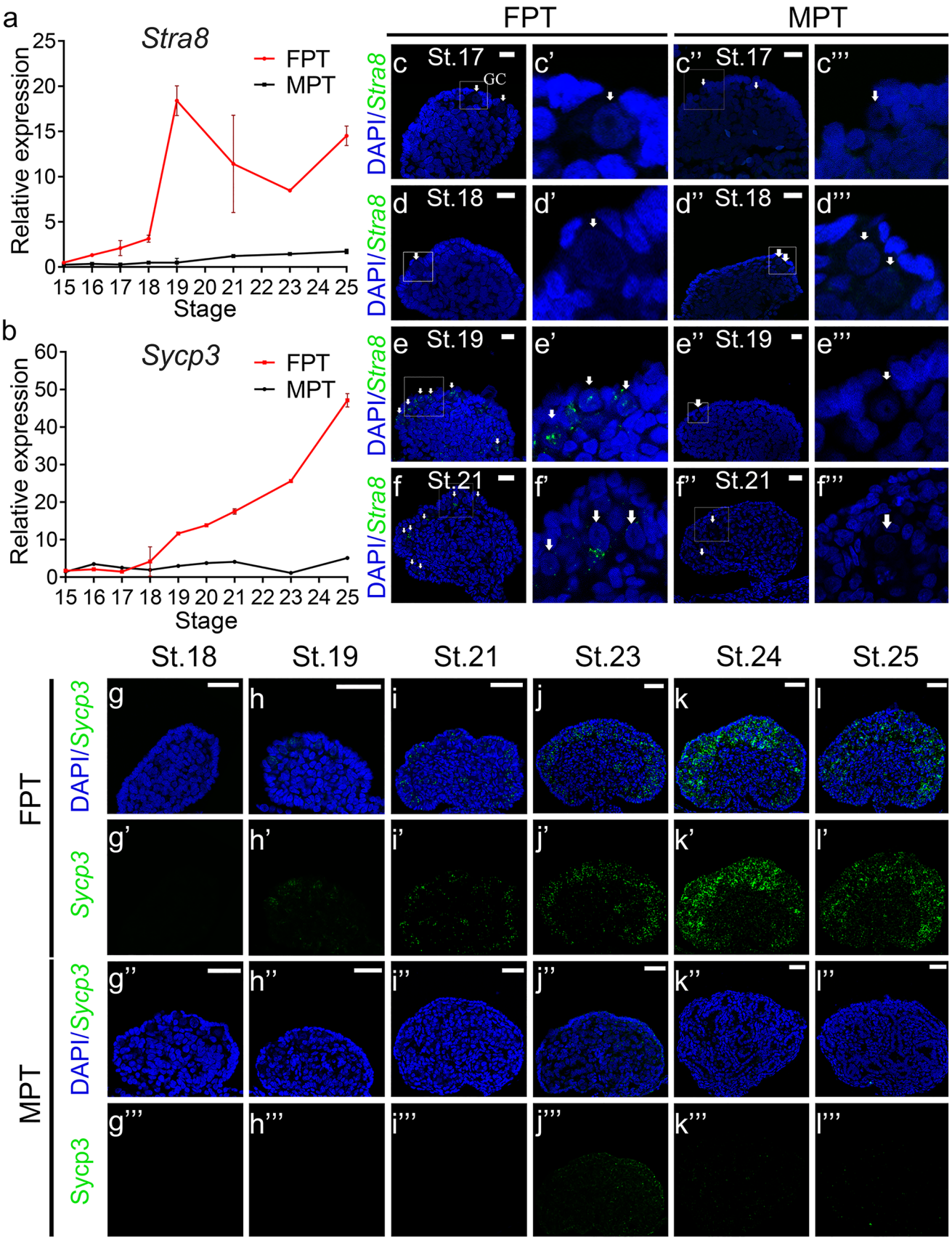


Fig. 1 Histological analysis of meiotic process in developing gonads. Hematoxylin and eosin (HE) staining of gonads under female-producing temperature (FPT) (**a–d**) and gonads (**a'–d'**) under male-producing temperature (MPT) at sex differentiation stages. Enlarged germ cells are indicated in the top left corner of each photo. Morphological changes in nuclei are observed in meiotic germ cells in FPT gonads from stage 21. Scale bar: 50 μm


a
Stra8
FPT
MPT
Relative expression
Stage
b
Sycp3
FPT
MPT
Relative expression
Stage
FPT
MPT
St.17
St.18
St.19
St.21
GC
DAPI/Stra8
St.18
St.19
St.21
St.23
St.24
St.25
FPT
MPT
DAPI/Sycp3
Sycp3

Cyp26b1 is considered the major RA-degrading enzyme during meiosis onset in mice. However, in the turtle, both *Cyp26b1* and *Cyp26c1* showed low expression in gonads (Fig. 4b, c). During embryogenesis, *Cyp26b1* expression was similar in both sexes other than stage 17 (Fig. 4b), and there is no statistical difference in *Cyp26c1* expression between females and males (Fig. 4c). Interestingly, *Cyp26a1*, the only highly expressed gene for RA-degrading enzymes, showed significantly higher expression levels in males from stage

Fig. 4 Expressions of genes (*Cyp26a1*, *Cyp26b1* and *Cyp26c1*) encoding RA-degrading enzymes in embryonic turtle gonads. **a** qRT-PCR analysis of *Cyp26a1* mRNA expression. **b** qRT-PCR analysis of *Cyp26b1* mRNA expression. **c** qRT-PCR analysis of *Cyp26c1* mRNA expression. Data are exhibited as mean ± SD; N = 3; * $p < 0.05$; *** $p < 0.001$. Expression patterns of *Cyp26a1* in fetal gonads at FPT (**d**–**d’’’’’**) and MPT (**e**–**e’’’’’**) by whole-mount ISH. Signals were constantly detected in gonads at MPT until stage 21. Expression patterns of *Cyp26a1* in fetal gonads at stage 16 (**f**–**f’’’**), 17 (**g**–**g’’’**), 18 (**h**–**h’’’**), 19 (**i**–**i’’’**) by section ISH. Scale bar: 500 μm for whole-mount; 250 μm for GMC sections; 25 μm for gonad sections

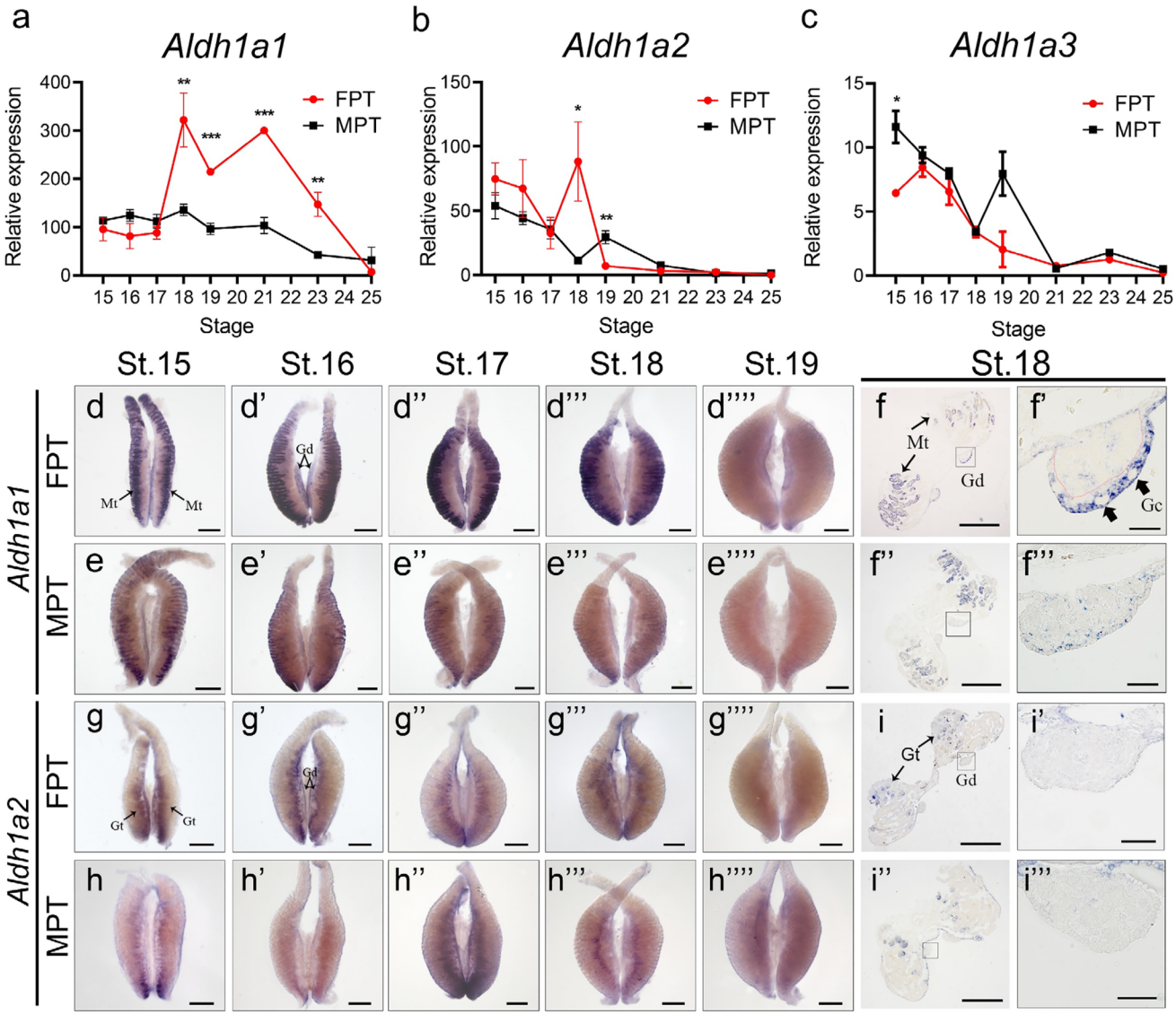
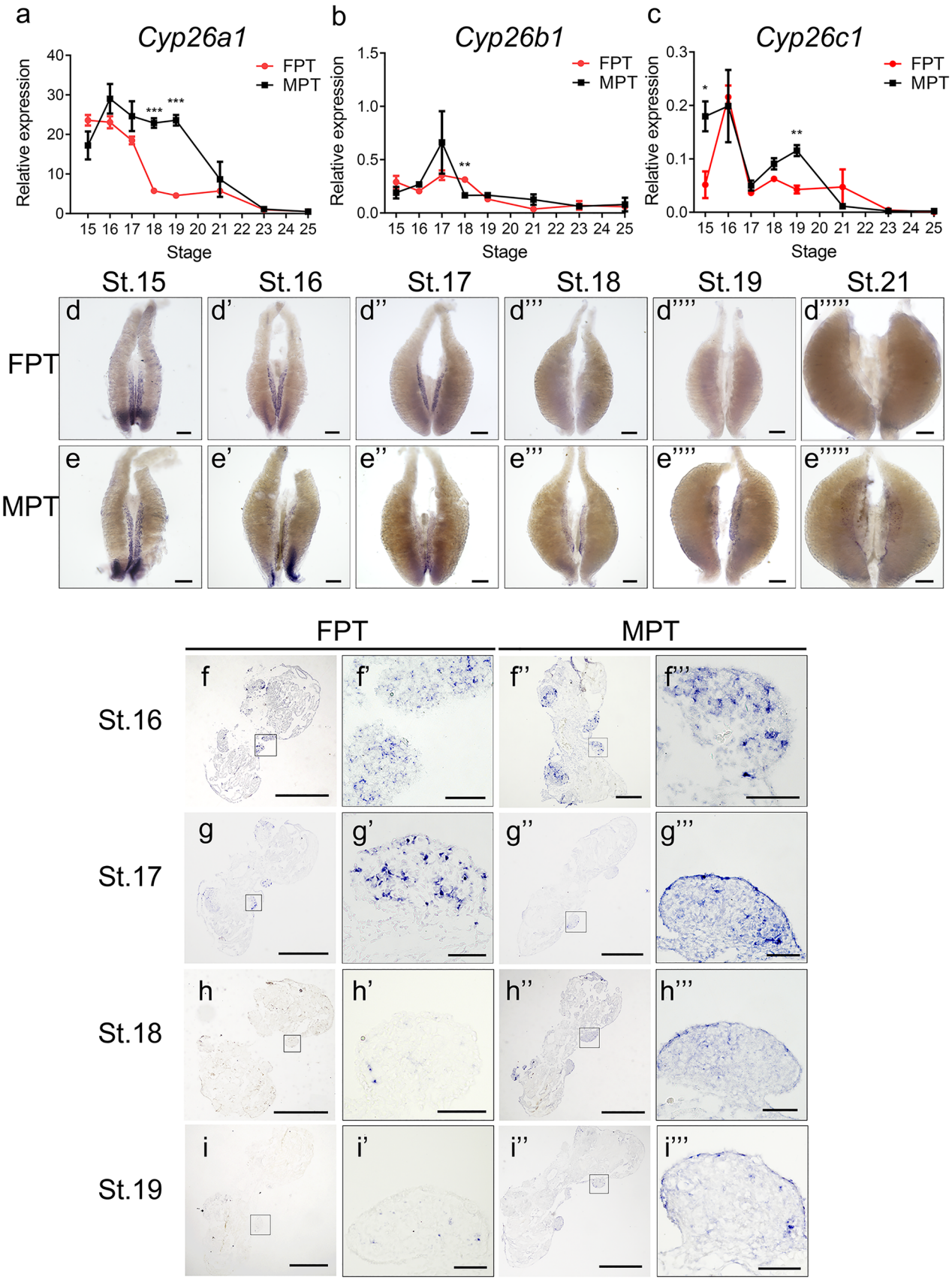


Fig. 3 Expressions of genes (*Aldh1a1*, *Aldh1a2* and *Aldh1a3*) encoding retinoic acid (RA) synthases in embryonic turtle gonads. **a** qRT-PCR analysis of *Aldh1a1* mRNA expression. **b** qRT-PCR analysis of *Aldh1a2* mRNA expression. **c** qRT-PCR analysis of *Aldh1a3* mRNA expression. Data are exhibited as mean ± SD; N = 3; *$p < 0.05$; **$p < 0.01$ ***$p < 0.001$. Expression patterns of *Aldh1a1* in fetal gonads before meiosis onset at FPT (**d**–**d’’’’**) and MPT (**e**–**e’’’’**) by whole-mount ISH and section ISH (**f**–**f’’’**). Expression patterns of *Aldh1a2* in fetal gonads before meiosis onset at FPT (**g**–**g’’’’**) and MPT (**h**–**h’’’’**) by whole-mount ISH and section ISH (**i**–**i’’’**). Red dot line refers to the boundary between cortex and medullary region. Mt, mesonephric tubules. Gc, germ cell. Gd, gonad. Gt, glomerular tuft. Scale bars: 500 μm for whole-mount; 250 μm for gonad mesonephros complex (GMC) sections; 25 μm for gonad sections


a
Cyp26a1
Relative expression
FPT
MPT
Stage
b
Cyp26b1
c
Cyp26c1
St.15
St.16
St.17
St.18
St.19
St.21
FPT
MPT
d
d'
d''
d'''
d''''
d'''''
e
e'
e''
e'''
e''''
e'''''
St.16
St.17
St.18
St.19
f
f'
f''
f'''
g
g'
g''
g'''
h
h'
h''
h'''
i
i'
i''
i'''

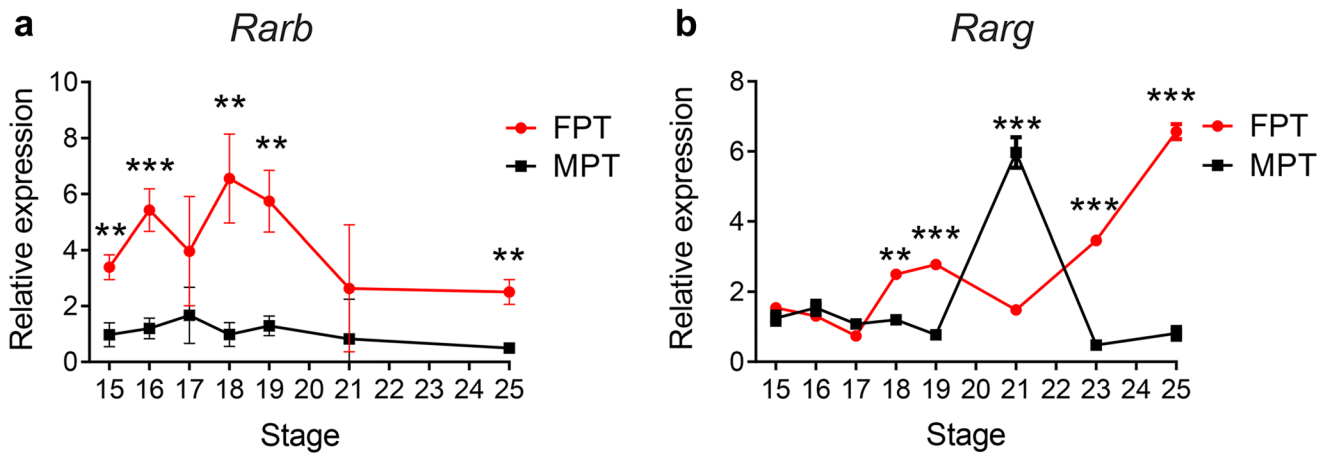


Fig. 5 Expressions of genes (*Rarb* and *Rarg*) encoding RA receptors in embryonic turtle gonads. **a** qRT-PCR analysis of *Rarb* mRNA expression. **b** qRT-PCR analysis of *Rarg* mRNA expression. Data are exhibited as mean ± SD; N = 3; ** $p < 0.01$; *** $p < 0.001$

18 to stage 19. The expression declined significantly after stage 19 in both sexes (Fig. 4a). By whole-mount in situ hybridization, male-specific signals were detected from stage 18 to stage 21 in gonads, suggesting suppression of RA activity during embryogenesis (Fig. 4e–e''''). Additionally, *Cyp26a1* mRNA was detected in somatic cells in medullary and cortical regions in FPT and MPT gonads from stage 16 to 17 (Fig. 4f–f''', g–g'''), with the signals persisting only in MPT gonads after stage 18 (Fig. 4h–h''', i–i'''). No signal was detected for *Cyp26b1* and *Cyp26c1*. Taken together, these results indicate that *Cyp26a1* was the major RA-degrading enzyme in fetal gonads.

We also investigated the expression of *Rar*s in turtle gonads. Both *Rarb* and *Rarg* were expressed in the gonads of both sexes, suggesting that RA signaling may function in these gonads. *Rarb* and *Rarg* exhibited higher expression levels in gonads that incubated at FPT at stage 18 and stage 19 (Fig. 5a, b). However, in situ hybridization failed to detect any signal of these two genes due to their low expression levels.

Exogenous retinoic acid stimulates meiosis in fetal testes in vitro

The bipotential gonads differentiate into testes from stage 20. To examine whether RA can induce meiosis in testes, we performed in vitro culture of testes at stage 21 under MPT conditions. We used Cyp*26a1*, a commonly used indicator for successful ectopic RA stimulation, to verify the gonadal exposure to RA (Adolfi et al. 2016; Balmer and Blomhoff 2002; Chen et al. 2020; Zolfaghari et al. 2019). As suggested by these studies, *Cyp26a1* is directly regulated by RA signaling and induced in the RA-responsive cells to prevent the accumulation of excessive RA. In this study, qRT-PCR analysis revealed that upon RA treatment, the expression of *Cyp26a1*, *Stra8* and *Sycp3* were induced in germ cells on day 2 and day 4, respectively (Fig. 6a, b). Their expressions were also detected in RA-treated gonads (3/3) by ISH (Fig. 6c–c''', d–d'''), indicating that the exogenous RA can trigger meiotic entry in germ cells in vitro.

Exogenous retinoic acid stimulates meiotic entry in the turtle embryo in vivo

To verify the function of RA during meiosis onset, we injected 5 µl of RA solution into eggs at FPT at stage 15, prior to the meiosis onset in female gonads. Significant up-regulation of *Cyp26a1* was observed on day 2 (stage 16) and day 4 (stage 17) (Fig. 7a). Meanwhile, expression levels of meiosis markers *Stra8* and *Sycp3* were also induced in the RA-treated group (Fig. 7b). The expression of *Stra8* was confirmed by ISH as all examined samples (10/10) gave clear signals in fetal gonads in RA treated group on day 2 (Fig. 7c–c'''). We then injected 5 µl of RA into eggs at MPT at stage 21. The expression of *Cyp26a1*, *Stra8* and *Sycp3* was detected on day 2 and day 4 as well (Fig. 7d, e). Expression signals of *Stra8* were also intensively detected in germ cells in RA-treated gonads (10/10) on day 2 (Fig. 7f–f'''). Taken together, exogenous RA induced the expression of meiosis-related genes in germ cells in both sexes in vivo.

Discussion

The role of RA in inducing the first meiosis in germ cells has been well-established in mammals, but its involvement in reptiles remains unclear. In this study, we show that meiosis was specifically initiated in *T. scripta* embryos under FPT. Using ISH, sexually dimorphic expression patterns of genes

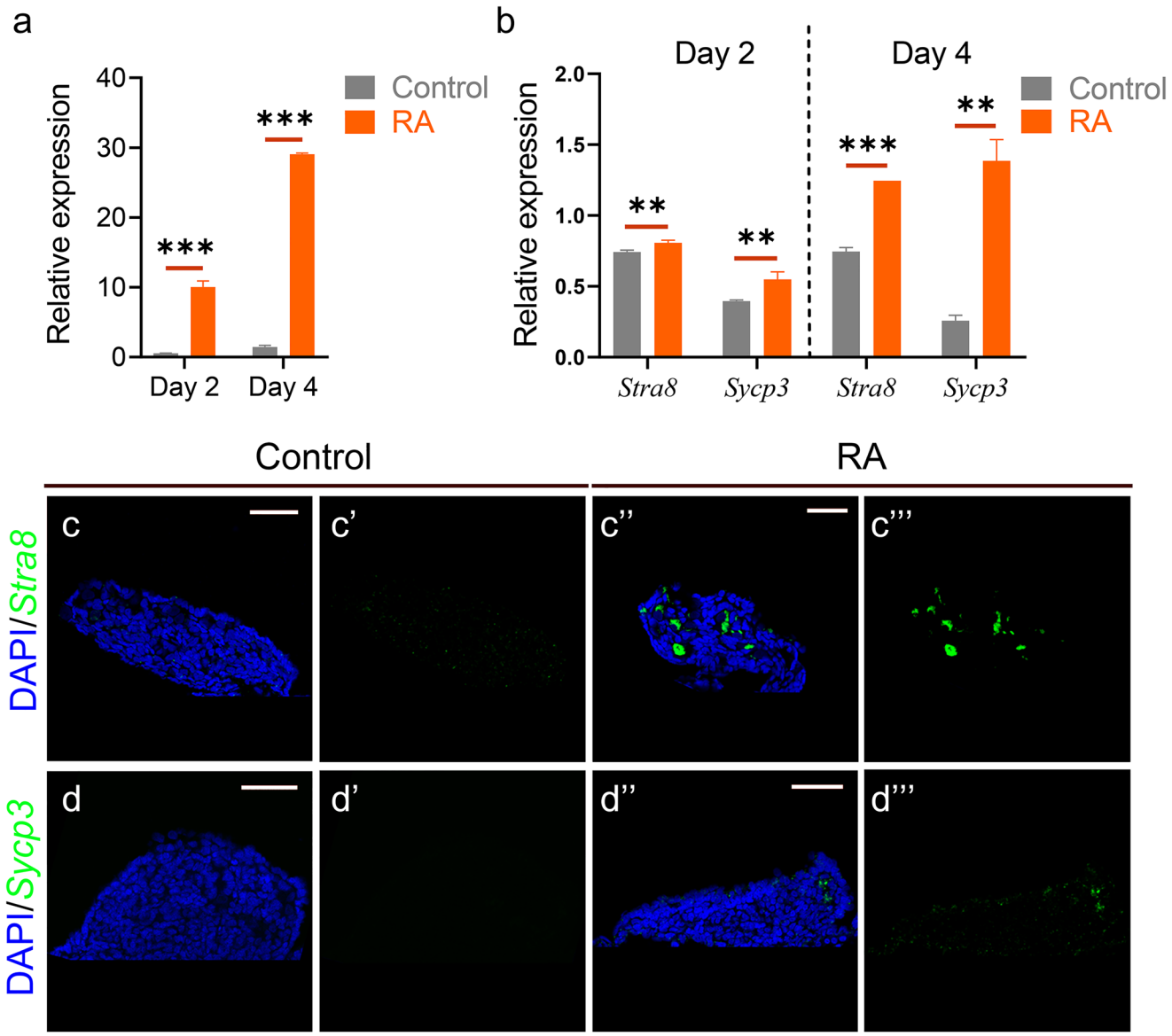
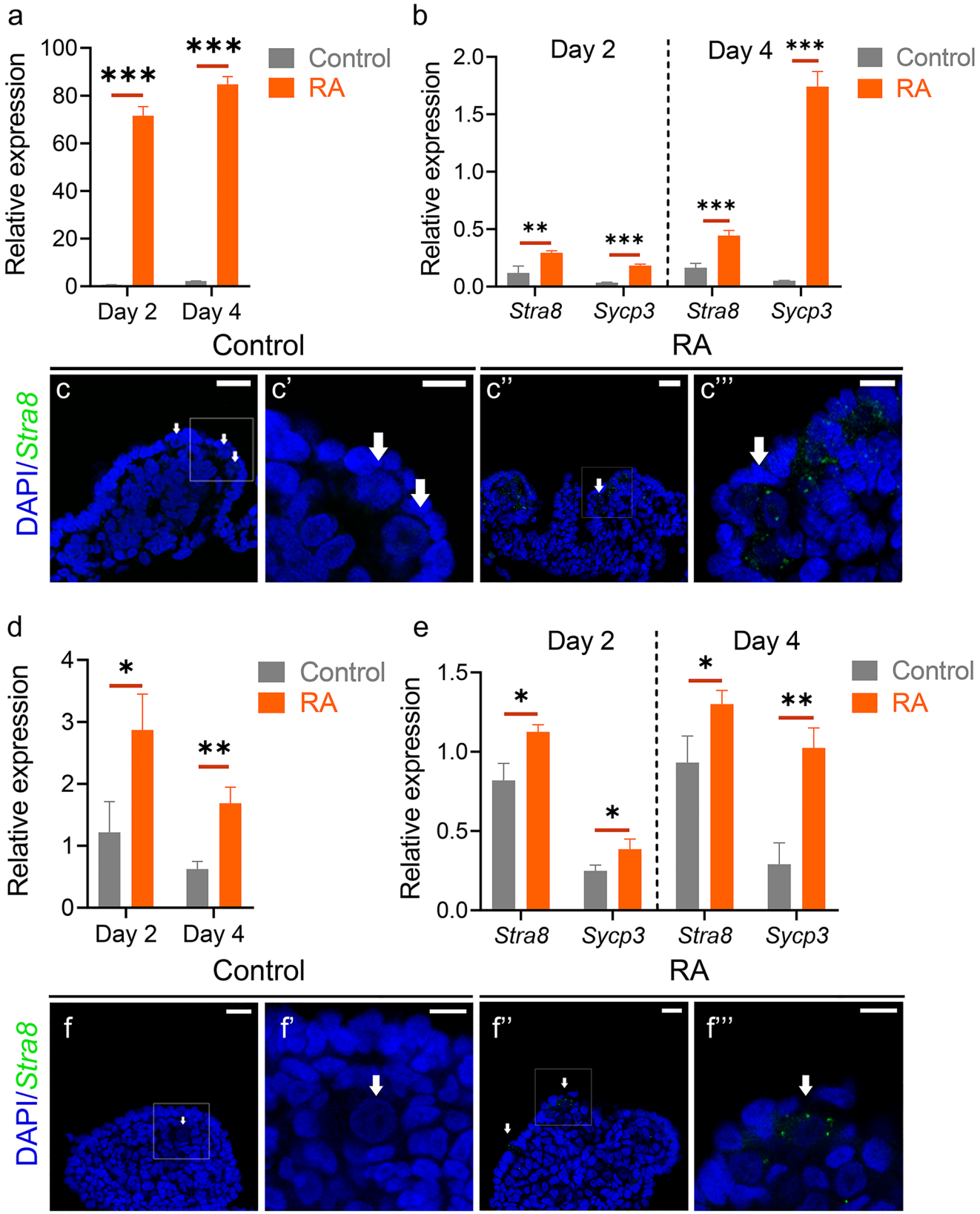


Fig. 6 Exogenous retinoic acid (RA) triggers premature meiotic entry in stage-21 males in vitro. **a** Expression changes of *Cyp26a1* after 2 days and 4 days of RA treatment in cultured gonads under MPT. **b** Expression of *Stra8* and *Sycp3* after 2 days and 4 days of RA treatment in cultured gonads under MPT. Expression patterns of *Stra8* (**c**–**c'''**) and *Sycp3* (**d**–**d'''**) in DMSO (left panel) or RA-treated (right panel) at MPT on day 4. Scale bar: 50 µm

which encode RA synthases and degrading enzymes during meiosis initiation were observed, suggesting a higher concentration of RA in embryonic ovaries. Both in vitro and in vivo treatments with exogenous RA resulted in an advanced onset of meiosis in ovaries and testes. Overall, these findings suggest a conserved role for RA in regulating entry into meiosis in fetal gonads.

Meiotic entry in fetal gonads of *T. scripta*

In female amniotes, germ cells enter meiotic prophase I during embryogenesis (Sou et al. 2021). In *T. scripta*, the signals of *Stra8* and *Sycp3* were observed in female gonads from stage 19, indicating that meiosis initiates in females before hatching. These findings are consistent with the female-specific expression of SYCP3 observed at stage 25 by immunofluorescence (Ge et al. 2018). In this study, we detected the signals of *Stra8* and *Sycp3* in female gonads at the end of sex determination by ISH, and confirmed the entrance of meiosis at the beginning of sex differentiation through histological analysis. Therefore, meiosis in the turtle appears to begin almost simultaneously with gonadal sex differentiation, similar to that observed in mice (Byskov 1986).


a
Relative expression
100
80
60
40
20
0
***
***
Control
RA
Day 2
Day 4
Control
b
Relative expression
2.0
1.5
1.0
0.5
0.0
Day 2
Day 4
**
***
***
***
Stra8
Sycp3
Stra8
Sycp3
RA
DAPI/Stra8
c
c'
c''
c'''
d
Relative expression
4
3
2
1
0
*
**
Control
RA
Day 2
Day 4
Control
e
Relative expression
1.5
1.0
0.5
0.0
Day 2
Day 4
*
*
*
**
Control
RA
Stra8
Sycp3
Stra8
Sycp3
RA
DAPI/Stra8
f
f'
f''
f'''

◂**Fig. 7** Exogenous retinoic acid (RA) triggers premature meiotic entry in males and females in vivo. **a** Expression changes of *Cyp26a1* after 2 days and 4 days of RA injection at stage 15 under FPT. **b** Expression changes of *Stra8* and *Sycp3* after 2 days and 4 days of RA injection at stage 15 under FPT. Expression patterns of *Stra8* in female gonads on day 2 after DMSO (control, **c**–**c'**) and RA (**c''**–**c'''**) treatment. **d** Expression changes of *Cyp26a1* after 2 days and 4 days of RA injection at stage 21 under MPT. **e** Expression changes of *Stra8* and *Sycp3* after 2 days and 4 days of RA injection at stage 21 under MPT. Expression patterns of *Stra8* in male gonads on day 2 after DMSO (**f**–**f'**) and RA (**f''**–**f'''**) treatment. Arrow indicates the germ cell. Data are exhibited as mean ± SD; N = 3. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bar: 25 μm for the original size photo; 10 μm for the enlarged photo

In mouse embryos, meiotic entry occurs in an anterior-to-posterior manner in the ovary (Menke et al. 2003). However, this was not observed in the turtle (Supplementary Fig. 3). Meanwhile, no anterior-to-posterior patterning of *Aldh1a1* and *Cyp26a1* was detected in gonads, indicating that RA is distributed uniformly throughout the gonads. Hence, it is reasonable to consider that RA triggers meiosis in germ cells located across different areas of the gonads, instead of initiating meiosis in a gradient-like fashion from anterior to posterior. Furthermore, unlike in mice whose *Stra8* expression declined dramatically before birth (E18.5) (Zhou et al. 2008), the expression of *Stra8* in female turtles maintained at a high level until stage 25. It may be caused by the asynchronous meiosis onset of germ cells, as observed in chicken (Smith et al. 2008), which means while some germ cells undergo proliferation (1–2 layers of germ cells proliferate up to 3–4 layers of germ cells in the cortex from stage 21 to stage 25), others undergo meiosis simultaneously (Fig. 1).

Coordination of *Aldh1a1* and *Cyp26a1* regulates RA levels and meiotic entry during embryogenesis in *T. scripta*

Abundant evidence indicates that RA induces the expression of *Stra8* and the subsequent processes of meiotic prophase (Bowles et al. 2006; Koubova et al. 2006; Wang et al. 2016; Yu et al. 2013). In *T. scripta*, both *Stra8* and *Sycp3* were induced in germ cells by exogenous RA treatment in vitro and in vivo, suggesting that RA acts directly on germ cells as the target to express genes that are related to meiosis. The expressions of two RA receptors, *Rarb* and *Rarg,* are detected in fetal gonads under FPT and MPT (Fig. 5),

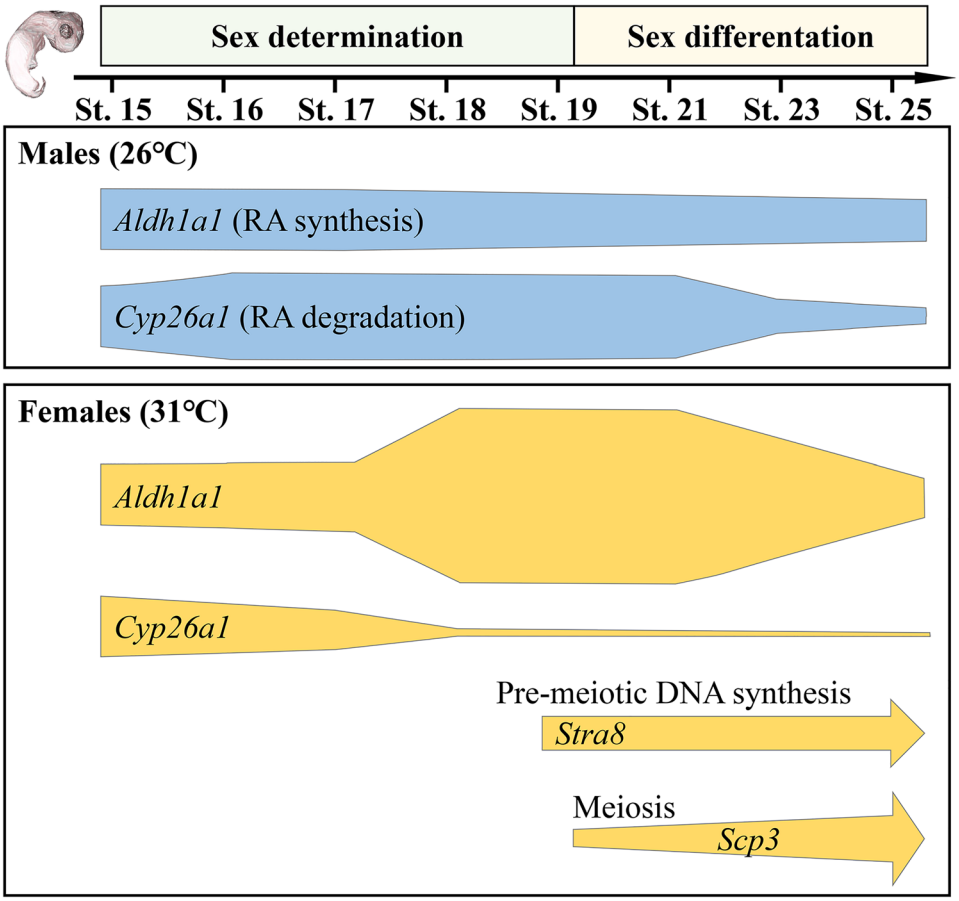


Fig. 8 Scheme of RA metabolism and meiosis during gonadal development in turtle embryos. Gonadal sex determination in red-eared slider turtles lasts from stage 15 to stage 19, followed by gonadal differentiation at stage 20. In male gonads, *Aldh1a1* and *Cyp26a1* are expressed constantly until stage 21. In female gonads, the expression of *Aldh1a1* is up-regulated after stage 17, while *Cyp26a1* is significantly down-regulated. At late stage 18, *Stra8* was up-regulated. At stage 19, *Sycp3* was up-regulated, indicating the entry of meiosis. Blue, males; orange, females. The width represents the rough expression level of the genes. The greater width indicates a stronger expression; the lesser width indicates a weaker expression

which helps to explain why our exogenous RA treatment can induce meiosis in male germ cells at embryonic stages.

To determine the time course of RA synthesis/degradation in embryonic gonads, we present a detailed molecular analysis of genes responsible in RA metabolism in turtles. Unlike in mice and avian models, where CYP26B1 are major enzymes involved in RA metabolism (Griswold et al. 2012; Smith et al. 2008), genes that play the same role in the turtle are *Aldh1a1* and *Cyp26a1*. The expression of *Aldh1a1* was detected in somatic cells surrounding germ cells. Interestingly, strong expressions of *Aldh1a1* and *Aldh1a2* were observed in embryonic kidneys. Apart from its known role in renal development (Rosselot et al. 2010), RA synthesized in these regions may also diffuse to the gonads. Figure 8 summarizes the expression of *Aldh1a1* and *Cyp26a1* in relation to the initiation of meiosis in turtle embryos. In developing testis, both enzymes are strongly co-expressed, leading to simultaneous RA synthesis and degradation, thereby preventing excessive RA accumulation and the onset of meiosis. In the female, *Aldh1a1* expression is sustained in the cortical region throughout embryogenesis, while *Cyp26a1* is down-regulated after stage 17. This leads to an accumulation of RA prior to and during the time of *Stra8* induction (Fig. 8). These findings demonstrate a possible regulatory system of meiosis onset in *T. scripta*.

In the present study, we provide evidence supporting the coordination between the RA synthase ALDH1A1 and the degrading enzyme CYP26A1, which is essential for RA metabolism in turtle gonads, resulting in sexually dimorphic RA concentrations and meiotic onset during embryogenesis. However, it remains unclear whether inhibiting RA signaling would prevent meiotic entry in turtles, and the exact function of *Cyp26a1* and *Aldh1a1* require further elucidation. Hence, further studies are needed to clarify the regulatory system, which will shed light on germ cell differentiation in reptiles during embryogenesis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00441-023-03814-1.

Acknowledgements We thank to members of Ge laboratory for their support.

Authors contributions Conceptualization: Q.C., C.G.; methodology: Q.C., C.G.; formal analysis: K.W., Q.C., C.G.; investigation: K.W., Q.C., F.L., J.S., W.S., C.G; resources: C.G.; data curation: Q.C., C.G.; writing—original draft: K.W., Q.C.; writing—review and editing: Q.C., F.L., W.S., C.G.; visualization: K.W., Q.C., W.S., J.S.; supervision: C.G.; project administration: Q.C., C.G.; funding acquisition: Q.C., C.G.

Funding This work was supported by National Natural Science Foundation of China [31922084, 31872960, U22A20529], Natural Science Foundation of Zhejiang Province for Distinguished Young Scholars [LR19C190001] to C.G. China Postdoctoral Science Foundation [2022M722982], Ningbo Natural Science Foundation [2022J192] and the Zhejiang Provincial Top Key Discipline of Biological Engineering [1741000592] to Q.C. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval Not applicable.

Informed consent Not applicable.

Conflict of interest The authors declare no competing interests.

References

Adolfi MC, Herpin A, Regensburger M, Sacquegno J, Waxman JS, Schartl M (2016) Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. Sci Rep 6:34281

Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci U S A 105:14976–14980

Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid Res 43:1773–1808

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. Science 312:596–600

Byskov AG (1986) Differentiation of mammalian embryonic gonad. Physiol Rev 66:71–117

Chen Q, Sato K, Yokoi H, Suzuki T (2020) Developmental regulatory system of ocular-side-specific asymmetric pigmentation in flounder: Critical role of retinoic acid signaling. J Exp Zool B Mol Dev Evol 334:156–167

Cunningham TJ, Duester G (2015) Mechanisms of retinoic acid signalling and its roles in organ and limb development. Nat Rev Mol Cell Biol 16:110–123

Feng CW, Burnet G, Spiller CM, Cheung FKM, Chawengsaksophak K, Koopman P, Bowles J (2021) Identification of regulatory elements required for Stra8 expression in fetal ovarian germ cells of the mouse. Development 148:dev194977

Ge C, Ye J, Weber C, Sun W, Zhang H, Zhou Y, Cai C, Qian G, Capel B (2018) The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. Science 360:645–648

Greenbaum E, Carr JL (2002) Staging criteria for embryos of the spiny softshell turtle, Apalone spinifera (testudines: Trionychidae). J Morphol 254:272–291

Griswold MD, Hogarth CA, Bowles J, Koopman P (2012) Initiating meiosis: the case for retinoic acid. Biol Reprod 86:35

Ishiguro KI, Matsuura K, Tani N, Takeda N, Usuki S, Yamane M, Sugimoto M, Fujimura S, Hosokawa M, Chuma S, Ko MSH, Araki K, Niwa H (2020) MEIOSIN directs the switch from mitosis to meiosis in mammalian germ cells. Dev Cell 52:429–445

Koubova J, Hu YC, Bhattacharyya T, Soh YQ, Gill ME, Goodheart ML, Hogarth CA, Griswold MD, Page DC (2014) Retinoic acid activates two pathways required for meiosis in mice. PLoS Genet 10:e1004541

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci USA 103:2474–2479

Li M, Feng R, Ma H, Dong R, Liu Z, Jiang W, Tao W, Wang D (2016) Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. Gen Comp Endocrinol 232:191–198

MacLean G, Li H, Metzger D, Chambon P, Petkovich M (2007) Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148:4560–4567

Menke DB, Koubova J, Page DC (2003) Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. Dev Biol 262:303–312

Niederreither K, Dolle P (2008) Retinoic acid in development: towards an integrated view. Nat Rev Genet 9:541–553

Rhinn M, Dolle P (2012) Retinoic Acid Signalling during development. Development 139:843–858

Rosselot C, Spraggon L, Chia I, Batourina E, Riccio P, Lu B, Niederreither K, Dolle P, Duester G, Chambon P, Costantini F, Gilbert T, Molotkov A, Mendelsohn C (2010) Non-cell-autonomous retinoid signaling is crucial for renal development. Development 137:283–292

Smith CA, Roeszler KN, Bowles J, Koopman P, Sinclair AH (2008) Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. BMC Dev Biol 8:85

Sou IF, Pryce RM, Tee WW, McClurg UL (2021) Meiosis initiation: a story of two sexes in all creatures great and small. Biochem J 478:3791–3805

Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 3:59–69

Wallacides A, Chesnel A, Chardard D, Flament S, Dumond H (2009) Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. Dev Dyn 238:1389–1398

Wang S, Wang X, Ma L, Lin X, Zhang D, Li Z, Wu Y, Zheng C, Feng X, Liao S, Feng Y, Chen J, Hu X, Wang M, Han C (2016) Retinoic acid is sufficient for the in vitro induction of mouse spermatocytes. Stem Cell Reports 7:80–94

Weber C, Zhou Y, Lee JG, Looger LL, Qian G, Ge C, Capel B (2020) Temperature-dependent sex determination is mediated by pSTAT3 repression of Kdm6b. Science 368:303–306

Wibbels T, Bull JJ, Crews D (1991) Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381

Wilkins AS, Holliday R (2009) The evolution of meiosis from mitosis. Genetics 181:3–12

Yu M, Yu P, Leghari IH, Ge C, Mi Y, Zhang C (2013) RALDH2, the enzyme for retinoic acid synthesis, mediates meiosis initiation in germ cells of the female embryonic chickens. Amino Acids 44:405–412

Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C, Griswold MD (2008) Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol Reprod 79:35–42

Zolfaghari R, Mattie FJ, Wei C-H, Chisholm DR, Whiting A, Ross AC (2019) CYP26A1 gene promoter is a useful tool for reporting RAR-mediated retinoid activity. Anal Biochem 577:98–109