Supplementary Information

Supplementary Materials and Methods

Turtle eggs

All animal experiments were carried out following the guidelines approved by the Institutional Animal Care and Use committee at Zhejiang Wanli University, China. Fresh red-eared slider turtle eggs were collected within 12 h after spawning from Hanshou, Hunan. Fertilized eggs were randomized in trays of moist vermiculite and hatched in a hatching system with constant temperature and humidity. The incubation temperatures were 26 °C (MPT) and 32 °C (FPT) and humidity ranged from 75% to 85%. A total of 2 400 eggs were divided into four groups: i.e., *Sox9* knockdown group (26 °C, 600 eggs), *Sox9* overexpression group (32 °C, 600 eggs), and two control groups (26 °C and 32 °C, 600 eggs each group). Embryonic gonads were collected at different stages according to the embryo development map (Greenbaum, 2002).

Construction of lentivirus expression vector systems and infection of turtle embryos

The lentivirus overexpression vectors used in this experiment were all produced by GenePharma (Shanghai, China). The *Sox9* lentiviral interference vector (LV-*Sox9*-shRNA-GFP) and *Sox9* lentiviral overexpression vector (LV-*Sox9*-OE-GFP) were designed based on the known sequence of the *T. scripta Sox9* gene (GenBank: EU914820.1). High-titer lentivirus vectors (10^9 U/mL) carrying the coding sequence of green fluorescent protein (GFP) as a reporter gene were produced according to previously described methods (Ge et al., 2017).

The high-titer lentiviruses of LV-*Sox9*-shRNA and LV-*Sox9*-OE were injected into stage 15 turtle embryos with incubation temperatures of 26 °C and 32 °C, respectively. We then injected 10 μl of lentivirus into the albumin of each egg using a fine (0–25 μl) metal Hamilton needle, with a total of 600 eggs injected in each group. After injection, the eggs were sealed with 3M Vetbond Tissue Adhesive (USA) and further incubated under the same environment. Embryos showing GFP fluorescence were chosen for further analysis.

Paraffin sections and hematoxylin-eosin (H&E) staining

The gonad-mesonephros complexes (GMCs) of stage 25 *T. scripta* embryos were fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C, then stored in 50% ethanol at 4 °C for tissue sectioning. After dehydration in graded ethanol, the GMCs were embedded in paraffin wax and sectioned (5–6 μm). Paraffin sections were deparaffinized and rehydrated before H&E staining. The stained sections were visualized under light microscopy (Nikon, Japan).

Immunofluorescence staining

Paraffin sections were deparaffinized and rehydrated (xylene for 30 min, xylene:ethanol 1:1 for 10 min, ethanol for 3 min, 90% ethanol for 3 min, 70% ethanol for 3 min, 50% ethanol for 3 min, and water for 3 min) before immersion in 10 mM sodium citrate buffer for 20 min at 95 °C for antigen retrieval. After blocking for 1 h in blocking solution (10% normal donkey serum, 3% bovine serum albumin (BSA), and 0.3% Triton X-100) at room temperature, sections were incubated overnight at 4 °C with primary antibodies (rabbit anti-SOX9 (1: 1 000, Millipore), rabbit anti-VASA (1:50, Abcam, UK), goat anti-FOXL2 (1: 250, Abcam), and mouse anti-CTNNB1 (1: 250, Sigma, USA), rabbit anti-AMH (1: 200, produced privately through Sangon Biotech)). The sections were then washed three times (10 min each time) with PBST (0.3% Triton X-100/phosphate-buffered saline), followed by incubation with secondary antibodies (IgG-594 (1:250, Invitrogen) and IgG-488 (1:250, Invitrogen)) for 2 h at room temperature in a dark environment, and further washing. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Gonadal sections were imaged using a fluorescence microscope (Ti-E, Nikon, Japan) or a confocal microscope (A1Plus, Nikon, Japan).

RNA extraction and qRT-PCR

Total RNA was isolated using a TRIzol kit (Thermo Scientific, USA), and 1 μg of total RNA was digested with DNase I and reverse transcribed into cDNA using a

reverse transcription kit (Thermo Scientific, USA). Quantitative RT-PCR was performed on a BioRad iCycler system using SYBR PrimeScript II (TAKARA, Japan) according to the manufacturer's protocols. The qRT-PCR conditions were as follows: pre-denaturation at 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s, 65 °C for 5 s. After normalization with *Gapdh*, relative RNA levels were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak & Schmittgen, 2001). Primer sequences are listed in Supplementary Table S1.

REFERENCES

Ge CT, Ye J, Zhang HY, Zhang Y, Sun W, Sang YP, et al. 2017. *Dmrt1* induces the male pathway in a turtle species with temperature-dependent sex determination. *Development*, **144**(12): 2222–2233.

Greenbaum E. 2002. A standardized series of embryonic stages for the emydid turtle *Trachemys scripta*. *Canadian Journal of Zoology*, **80**(8): 1350–1370.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, **25**(4):402-408.

Supplementary Table S1 Primer sequence list

Primer name	Primer sequence (5'–3')
Dmrt1-F	ACTACCCTCCTGCCTCCTACCT
Dmrt1-R	CTCCTTTGGTGCTTTCATTGCT
Amh-F	CGGCTACTCCTCCCACACG
Amh-R	CCTGGCTGGAGTATTTGACGG
Foxl2-F	AGAACAGCATCCGCCACAAC
Foxl2-R	CGGGTCCAGCGTCCAGTAG
Cyp19a1-F	AGCACTATGGAAAGAAATTCGACCT
Cyp19a1-R	GGTTTCAATAAGAGTGCTTGCCAA
Gapdh-F	ACTACCCTCCTGCCTCCTACCT
Gapdh-R	CTCCTTTGGTGCTTTCATTGCT