Sox9 is indispensable for testis differentiation in the red-eared slider turtle, a reptile with temperature-dependent sex determination

SOX9 (SRY-related HMG box gene 9) is an essential regulator of male sex determination and testis differentiation in many vertebrate species. However, the functional role of Sox9 in testis differentiation has not yet been identified in any reptilian species. Herein, Sox9 knockdown in the red-eared slider turtle (Trachemys scripta) embryos at a male-producing temperature led to complete male-to-female sex reversal, characterized by the formation of an ovary-like structure, disappearance of male marker AMH, and ectopic expression of ovarian regulator FOXL2, as well as a female distribution pattern of germ cells. Conversely, in-ovo overexpression of Sox9 at a female-producing temperature resulted in partial masculinization of putative female embryos, with the co-existence of AMH and FOXL2. Our study provides the first direct evidence that Sox9 is indispensable for testicular differentiation in a reptilian species, further confirming the conserved role of Sox9 in vertebrate sexual development.

Many egg-laying reptiles without sex chromosomes exhibit temperature-dependent sex determination (TSD), in which sex is established by the incubation temperature during the temperature-sensitive period (Pewphong et al., 2021). Sox9 is a member of the SRY-related HMG box (SOX) gene family and plays a conserved role in vertebrate sexual development. The expression patterns of Sox9 during embryonic gonadal development have been studied in several TSD species, which suggest the presence of Sox9 expression dimorphism between male-producing (MPT) and female-producing temperatures (FPT) (Díaz-Hernández et al., 2020; Moreno-Mendoza et al., 2001; Rhen et al., 2007; Shoemaker et al., 2007b; Spotila et al., 1998; Torres-Maldonado et al., 2001; Torres Maldonado et al., 2002; Western et al., 1999). In the red-eared slider turtle (Trachemys scripta), Sox9 expression in embryonic gonads is higher at the MPT than at the FPT in the later temperature-sensitive period (Shoemaker et al., 2007a; Spotila et al., 1998). The expression of Sox9 is significantly down-regulated after exogenous estradiol treatment at the MPT, while inhibition of estrogen synthesis delays Sox9 down-regulation at the FPT in T. scripta (Barske & Capel, 2010; Matsumoto et al., 2013). In addition, knockdown of the male sex-determining gene Dmrt1 in T. scripta results in the down-regulation of Sox9 expression, while overexpression of Dmrt1 leads to an increase in Sox9 expression (Ge et al., 2017). These studies suggest that Sox9 may have a regulatory effect on sexual differentiation in turtles; however, its functional role has not yet been investigated in any TSD reptilian species.

In the current study, we investigated loss- and gain-of-function of Sox9 in the red-eared slider turtle using lentivirus-mediated genetic manipulation. A lentivirus carrying Sox9 short hairpin RNA (shRNA) or open reading frame (ORF) was injected into T. scripta embryos at stage 15, i.e., very beginning of the temperature-sensitive period (Matsumoto & Crews, 2012). Stage 25 gonads were dissected for quantitative real-time polymerase chain reaction (qRT-PCR), hematoxylin-eosin (H&E) staining, and immunofluorescence. Detailed procedures are described in the Supplementary Materials and Methods.

Immunofluorescence analysis of the control group showed that the SOX9 protein was mainly located in the nucleus of Sertoli precursor cells in MPT embryonic gonads, while no Sox9 signal was detected in the FPT embryonic gonads at stage 25 (Figure 1A). In the gonads of MPT embryos treated with LV-Sox9-shRNA, SOX9 decreased to an almost undetectable level, similar to that in the control FPT embryos (Figure 1A), thus indicating a strong inhibitory effect of LV-
Sox9-shRNA on Sox9 expression. Histological analysis showed that the MPT gonads had a dense medulla with seminiferous cords and a reduced cortex, while the FPT gonads showed a thickened outer cortex and vacuolated medulla (Figure 1A). In contrast, the Sox9 knockdown MPT gonads were strongly feminized, characterized by a highly developed cortical region and a significantly degraded medulla region, similar to that of the FPT gonads (Figure 1A). Immunofluorescence labeling showed that VASA-positive germ cells were distributed in the developed medulla of the MPT gonads (Figure 1B). However, after Sox9 knockdown, the germ cells were mainly enriched in the outer cortex, exhibiting similar female germ cell distribution (Figure 1B). To confirm activation of the female developmental pathway in Sox9-deficient MPT embryos, we analyzed the expression of the male-specific protein AMH and ovarian development regulator FOXL2. Immunofluorescence analysis of the control group showed that AMH was strongly expressed in the Sertoli cells of the control MPT gonads but not in the control FPT gonads, while FOXL2 showed the opposite pattern and

Figure 1 Complete sex reversal induced by Sox9 knockdown
A: Immunofluorescence of SOX9, representative images of gonad-mesonephros complexes (GMCs) and H&E staining of gonadal sections from control MPT, MPT+Sox9-RNAi, and control FPT embryos at stage 25. Gonads in GMCs are outlined by yellow dotted lines. Med: medulla, Cor: cortex. Scale bar for GMCs: 500 μm, scale bar for immunofluorescence and H&E: 50 μm. B: VASA and CTNNB1 immunostaining of gonadal sections from control MPT, MPT+Sox9-RNAi, and control FPT embryos at stage 25. Scale bar: 50 μm. C: AMH and FOXL2 expression changes in response to Sox9 knockdown at stage 25. Scale bar: 50 μm. D: Knockdown of Sox9 led to a high rate of male-to-female sex reversal in MPT embryos. Phenotype of embryonic gonads was evaluated by gonadal histological analysis and expression of sex-specific proteins (AMH and FOXL2).
primary expression in the cortex region of the FPT gonads (Figure 1C). After Sox9 knockdown, the expression of AMH in most treated gonads was extremely low, while the expression of FOXL2 was robust, similar to that in normal FPT gonads (Figure 1C). Furthermore, knockdown of Sox9 at the MPT led to a high rate (92%, 23/25) of complete male-to-female sex reversal (Figure 1D).

Sox9-overexpressing embryos were generated by an injection of a lentivirus vector carrying the Sox9 ORF into FTP turtle eggs at stage 15. Immunofluorescence showed strong expression of the SOX9 protein in the medulla of FPT gonads after LV-Sox9-OE treatment (Figure 2A). Morphological analysis indicated that the FTP embryos overexpressing Sox9 had thicker, shorter, and obviously masculinized gonads (Figure 2A). The H&E staining of gonadal sections showed typical male characteristics in some treated FPT gonads, including a degenerated cortex and well-developed medulla, although some FPT gonads overexpressing Sox9 still retained a developed cortex (Figure 2A). VASA staining indicated that germ cells were distributed in both the cortex and medulla after Sox9 overexpression (Figure 2B). To confirm activation of the male developmental pathway in the Sox9-overexpressing FTP embryos, we analyzed the expression levels of testicular differentiation markers Dmrt1 and Amh and ovarian development regulators Cyp19a1 and Foxl2. The qRT-PCR results indicated that the mRNA expression levels of Dmrt1 and Amh were significantly increased, while the expression levels of Cyp19a1 and Foxl2 were decreased in response to overexpression of Sox9 (Figure 2D). In addition, based on immunofluorescence analysis, the ectopic expression of AMH was detected in the well-developed medulla in almost all cases, while the expression of female marker FOXL2 was markedly reduced (disappeared or limitedly retained) after overexpression of Sox9 in the FPT.

Figure 2 Partial female-to-male sex reversal induced by Sox9 overexpression

A: Immunofluorescence of SOX9, representative images of GMCs and H&E staining of gonadal sections from control FPT, FPT+Sox9-OE, and control MPT embryos at stage 25. Gonads in GMCs are outlined by yellow dotted lines. Med: medulla, Cor: cortex. Scale bar for immunofluorescence and H&E: 50 μm. B: VASA and CTNNB1 immunostaining of gonadal sections from control FPT, FPT+Sox9-OE, and control MPT embryos at stage 25. Scale bar: 50 μm. C: Responses of AMH and FOXL2 expression to Sox9 overexpression at stage 25. Scale bar: 50 μm. D: Expression changes in Dmrt1, Amh, Foxl2, and Cyp19a1 after Sox9 overexpression. Stage 25 gonads were dissected for qRT-PCR analysis. Results were normalized to Gapdh, and expression levels of MPT were defined as 1. Data were expressed as mean±standard deviation (SD) for three biological replicates. **: P<0.01; ***: P<0.001. E: Ectopic expression of Sox9 resulted in partial female-to-male sex reversal.
Sox9 appears later than that of Dmrt1, and knockdown of Dmrt1 in MPT embryos results in down-regulation of Sox9 (Ge et al., 2017). In fish, Dmrt1 positively regulates the transcription of the Sox9b gene (ortholog of tetrapod Sox9) by directly binding to a specific cis-regulatory element within the Sox9b promoter (Wei et al., 2019). These results imply that Sox9 may be involved in testsis differentiation rather than sex determination in red-eared slider turtles and may be directly or indirectly regulated by Dmrt1.

Herein, we showed that knockdown of Sox9 in T. scripta led to complete male-to-female sex reversal, while overexpression of Sox9 caused partial female-to-male sex reversal, thus implicating the indispensable role of Sox9 in testicular differentiation in reptiles. This study confirms the conserved role of Sox9 in male sexual development across different vertebrate species.

SUPPLEMENTARY DATA
Supplementary data to this article can be found online.

COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS
C.T.G., H.B.H., and L.X. conceived and designed the study. H.B.H., L.X., W.S., Y.J.Z., and H.Y.Z. collected the samples and analyzed the data. H.B.H., L.X., and C.T.G. wrote the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES
Bagheri-Fam S, Combes AN, Ling CK, Wilhelm D. 2020. Heterozygous deletion of Sox9 in mouse mimics the gonadal sex reversal phenotype associated with campomelic dysplasia in humans. Human Molecular Genetics, 29(23): 3781–3792.
Bagheri-Fam S, Sreenivasan R, Bernard P, Knower KC, Sekido R, Lovell-Badge R, et al. 2012. Sox9 gene regulation and the loss of the XY/XX sex-determining mechanism in the mole vole Ellobius lutescens. Chromosome Research, 20(1): 191–199.
Barske LA, Capel B. 2010. Estrogen represses SOX9 during sex determination in the red-eared slider turtle Trachemys scripta. Developmental Biology, 341(1): 305–314.
Chaboissier MC, Kobayashi A, Vidal VI P, Lützkendorf S, van de Kant HJG, Wegner M, et al. 2004. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development, 131(9): 1891–1901.
Croll B, Ohresorg T, Hewitt J, Bowles J, Quinn A, Tan J, et al. 2018. Human sex reversal is caused by duplication or deletion of core enhancers upstream of SOX9. Nature Communications, 9(1): 5319.
Cutting A, Chue J, Smith CA. 2013. Just how conserved is vertebrate sex determination? Developmental Dynamics, 242(4): 380–387.

Da Silva SM, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R. 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nature Genetics, 14(1): 62–68.

Díaz-Hernández V, Domínguez-Mora P, Chino-Palomó L, Marmolejo-Valencia A, Harfush M, Merchant-Larios H. 2020. Spatiotemporal Expression of Foxi2 and Dmrt1 before, during, and after Sex Determination in the Sea Turtle Lepidochelys olivacea. Sexual Development, 13(6–6): 280–296.

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.

Gonen N, Fultner CR, Wood S, García-Moreno SA, Salamone IM, Samson SC, et al. 2018. Sex reversal following deletion of a single distal enhancer of Sox9. Science, 360(6396): 1469–1473.

Gonen N, Quinn A, O’Neill HC, Koopman P, Lovell-Badge R. 2017. Normal levels of Sox9 expression in the developing mouse testis depend on the TES/TESCO enhancer, but this does not act alone. PLoS Genetics, 13(1): e1006520.

Hirst CE, Major AT, Smith CA. 2018. Sex determination and gonadal sex differentiation in the chicken model. The International Journal of Developmental Biology, 62(1-3): 153–166.

Huang B, Wang SB, Wang AN, Lamb AN, Barley J. 1999. Autosomal XX sex reversal caused by duplication of SOX9. American Journal of Medical Genetics, 87(4): 349–353.

Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. 1996. A male-specific role for SOX9 in vertebrate sex determination. Development, 122(9): 2813–2822.

Lavery R, Lardenois A, Ranc-Jiannotamedi F, Pauper E, Gregoire EP, Vigier C, et al. 2011. XY Sox9 embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes. Developmental Biology, 354(1): 111–122.

Matsumoto Y, Crews D. 2012. Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Molecular and Cellular Endocrinology, 354(1–2): 103–110.

Matsumoto Y, Yatsu R, Taylor C, Crews D. 2013. Changes in gonadal gene network by exogenous ligands in temperature-dependent sex determination. Journal of Molecular Endocrinology, 50(3): 389–400.

Moreno-Mendoza N, Harley VR, Merchant-Larios H. 2001. Temperature regulates SOX9 expression in cultured gonads of Lepidochelys olivacea, a species with temperature sex determination. Developmental Biology, 223(2): 319–326.

Nakamura S, Watakei I, Nishimura T, Toyoda A, Taniguchi Y, Tanaka M. 2012. Analysis of medaka sox9 orthologue reveals a conserved role in germ cell maintenance. PLoS One, 7(1): e29982.

Pewphong R, Kitana J, Kitana N. 2021. Thermosensitive period for sex determination of the tropical freshwater turtle Malayemys macrocephala. Integrative Zoology, 16(2): 160–169.

Rhen T, Metzker 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. Sexual Development, 1(4): 255–270.

Sekido R, Lovell-Badge R. 2008. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature, 453(7197): 930–934.

Shoemaker CM, Ramsey M, Queen J, Crews D. 2007a. Expression of Sox9, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. Developmental Dynamics, 236(4): 1055–1063.

Shoemaker CM, 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. Molecular Endocrinology, 21(11): 2750–2763.

Smith CA, Sinclair AH. 2004. Sex determination: insights from the chicken. BioEssays, 26(2): 120–132.

Spottis L, Spottis JR, Hall SE. 1998. Sequence and expression analysis of W71 and Sox9 in the red-eared slider turtle. Trachemys scripta. Journal of Experimental Zoology, 281(5): 417–427.

Sun D, Zhang Y, Wang C, Hua X, Zhang XA, Yan J. 2013. Sox9-related signaling controls zebrafish juvenile ovary–testis transformation. Cell Death & Disease, 4(11): e930.

Torres-Maldonado L, Moreno-Mendoza N, Landa A, Merchant-Larios H. 2001. Timing of SOX9 downregulation and female sex determination in gonads of the sea turtle Lepidochelys olivacea. Journal of Experimental Zoology, 290(5): 496–503.

Torres-Maldonado LC, Landa Piedra A, Moreno Mendoza N, Marmolejo Valenza A, Meza Martinez A, Merchant Larios H. 2002. Expression profiles of Dax1, Dmrt1, and Sox9 during temperature sex determination in gonads of the sea turtle Lepidochelys olivacea. General and Comparative Endocrinology, 129(1): 20–26.

Vidal VPI, Chaboisser MC, de Rooij DG, Schedi A. 2001. Sox9 induces testis development in XX transgenic mice. Nature Genetics, 28(3): 216–217.

Vining B, Ming ZH, Bagheri-Fam S, Harley V. 2021. Diverse regulation but conserved function: SOX9 in vertebrate sex determination. Genes, 12(4): 486.

Wei L, Li XY, Li MH, Tang YH, Wei J, Wang DS. 2019. Dmrt1 directly regulates the transcription of the testis-biased Sox8b gene in Nile tilapia (Oreochromis niloticus). Gene, 687: 109–115.

Western PS, Harris JY, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination in the American alligator: AMP precedes SOX9 expression. Developmental Dynamics, 216(4–5): 411–419.

Yamashita S, Kataoka K, Yamamoto H, Kato T, Hara S, Yamaguchi K, et al. 2019. Comparative analysis demonstrates cell type-specific conservation of SOX9 targets between mouse and chicken. Scientific Reports, 9(1): 12560.