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Letter to the editor Open Access

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-

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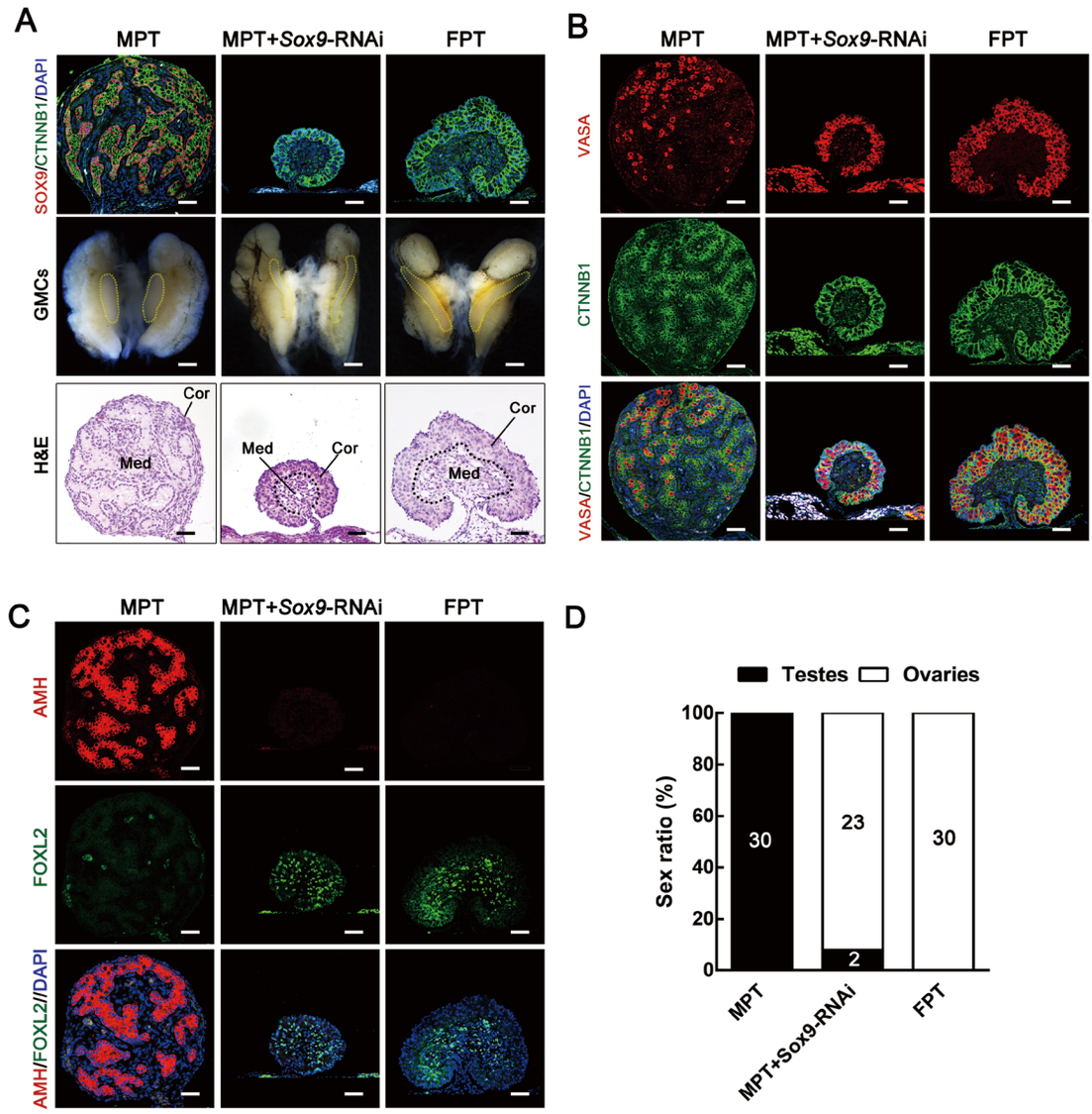

Figure 1 Complete sex reversal induced by *Sox9* knockdown

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).

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

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 FPT 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

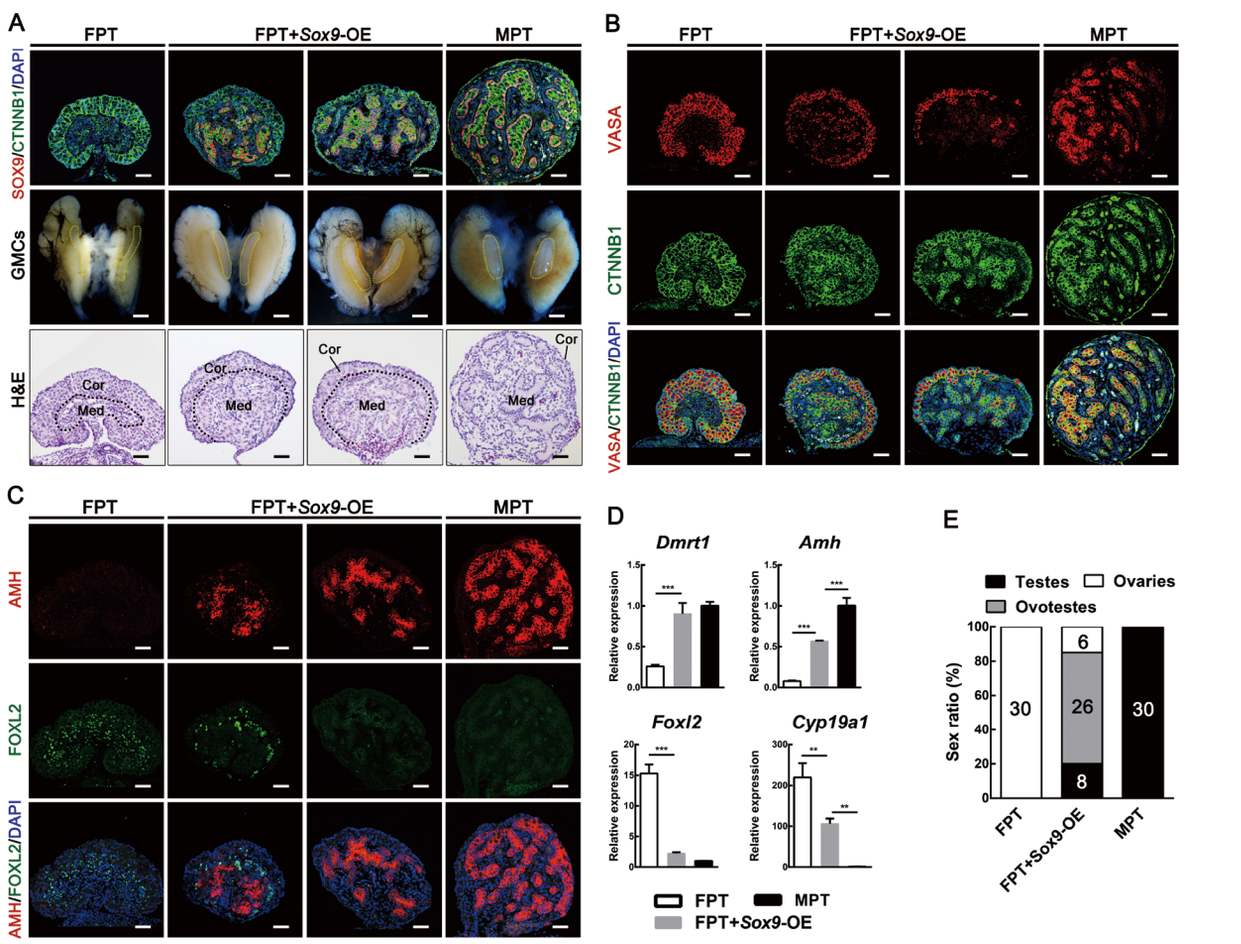

Figure 2 Partial female-to-male sex reversal induced by *Sox9* overexpression

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 GMCs: 500 μm, 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.

gonads (Figure 2C). Gonads showing both AMH and FOXL2 expression were considered as ovotestes. In addition, 85% (34/40) of FPT embryos overexpressing *Sox9* underwent varying degrees of sex reversal, with eight individuals showing complete sex reversal and 26 individuals showing partial sex reversal (Figure 2E).

The sequences of the HMG and C-terminal reactivation domains of SOX9 are highly homologous across humans, chickens, alligators, and fish, suggesting a conserved functional role of *Sox9* in vertebrate gonadal differentiation (Da Silva et al., 1996; Vining et al., 2021). *Sox9* is essential for Sertoli cell differentiation and testis formation in mice (Chaboissier et al., 2004; Kent et al., 1996). In XY mice, the deletion or a 77% reduction of *Sox9* leads to complete male-to-female sex reversal, while 50% reduction causes partial sex reversal (Bagheri-Fam et al., 2020; Gonen et al., 2017; Lavery et al., 2011). Moreover, ectopic expression of *Sox9* in XX gonads causes male development in the absence of *Sry* (Huang et al., 1999; Vidal et al., 2001). In addition, the duplication or deletion of *Sox9* enhancers results in sex reversal in XX or XY individuals, respectively (Croft et al., 2018; Gonen et al., 2018), thus demonstrating its critical role in testis determination in mammals. *Sox9* is highly expressed in MPT gonads during sex differentiation in red-eared slider turtles (Barske & Capel, 2010; Shoemaker et al., 2007b). This, together with our results showing that knockdown or overexpression of *Sox9* can lead to partial or complete sex reversal in red-eared turtles, confirms that the conserved role of *Sox9* in testis differentiation is also present in reptilian species. Interestingly, gonad size decreased in the *Sox9*-deficient embryos, consistent with previous results reported in medaka (Nakamura et al., 2012). This may be due to the *Sox9* gene not only affecting sex determination, but also other biological processes such as cell proliferation and germ cell maintenance.

Mammalian *Sox9* is directly regulated by the master sex determining gene *Sry*, and its initial expression occurs immediately after that of *Sry* (Sekido & Lovell-Badge, 2008). SRY and NR5A1 (nuclear receptor subfamily 5 group A member 1, also named steroidogenic factor 1) together bind to the *Sox9* gonad-specific enhancer and cooperatively up-regulate *Sox9* expression (Bagheri-Fam et al., 2012; Sekido & Lovell-Badge, 2008). In mammals, *Sox9* is reported to activate *Amh* (anti-Müllerian hormone) during testicular development (Cutting et al., 2013; Da Silva et al., 1996; Yamashita et al., 2019). Unlike mammals, *Sox9* expression in chickens (*Gallus gallus domesticus*) and American alligators (*Alligator mississippiensis*) occurs later than that of *Amh*, indicating that *Amh* expression is not regulated by SOX9, and *Sox9* may be located downstream of the sex differentiation pathway in non-mammalian vertebrates (Hirst et al., 2018; Smith & Sinclair, 2004; Western et al., 1999). In teleosts, *Sox9* plays a role in ovary-testis transition and germ cell maintenance (Nakamura et al., 2012; Sun et al., 2013). These studies suggest that *Sox9* neofunctionalization likely occurred during vertebrate evolution, such that the specific regulatory position of SOX9 in the male sex differentiation pathway differs among vertebrates with different evolutionary statuses (Cutting et al., 2013). In the red-eared slider turtle, the sexually dimorphic expression of *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 testis 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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