Regulation of the *Sox3* Gene in an X0/X0 Mammal without *Sry*, the Amami Spiny Rat, *Tokudaia osimensis*

Kohei Washio^a Shusei Mizushima^{a, b} Takamichi Jogahara^{c, d} Asato Kuroiwa^{a, b}

^aReproductive and Development Sciences, Biosystems Science Course, Graduate School of Life Science, Hokkaido University, Sapporo, Japan; ^bDivision of Reproductive and Developmental Biology, Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo, Japan; ^cDivision of Bio-Resources, Frontier Science Research Center, Kiyotake Campus, University of Miyazaki, Kiyotake-cho, Japan; ^dFaculty of Law and Economics, Okinawa University, Naha, Japan

Keywords

Evolution · Sex chromosomes · Sex determination · *Sox3* · *Tokudaia* · X0/X0 system · Y chromosome

Abstract

Two species of spiny rats, *Tokudaia osimensis* and *Tokudaia tokunoshimensis*, show an X0/X0 sex chromosome constitution due to the lack of a Y chromosome. The *Sry* gene has been completely lost from the genome of these species. We hypothesized that *Sox3*, which is thought to be originally a homologue of *Sry*, could function in sex determination in these animals in the absence of *Sry*. *Sox3* was localized in a region of the X chromosome in *T. osimensis* homologous to mouse. A similar testis- and ovary-specific pattern of expression was observed in mouse and *T. osimensis*. Although the sequence of the *Sox3* gene and its promoter are highly conserved, a 13-bp deletion was specifically found in the promoter region of the 2 spiny rat species. Reporter gene assays were performed to examine the effect of the 13-bp deletion in the promoter region on *Sox3* regulation. Although an approximately 60% decrease in activity was observed using the *Tokudaia* promoters with the 13-bp deletion, the activity was recovered using a mutated promoter in which the deletion was filled with mouse sequence. To evaluate whether SOX3 could regulate *Sox9* expression, a reporter gene assay was

carried out using testis-specific enhancer of *Sox9* core (TESCO). Co-transfection with a combination of mouse SF1 and mouse SOX3 or *T. osimensis* SOX3 resulted in a greater than 2-fold increase in activity of mouse and *T. osimensis* TESCO. These results support the idea that the function of SOX3 as a transcription factor, as has been reported in mice and humans, is conserved in *T. osimensis*. Therefore, we conclude that the *Sox3* gene has no function in sex determination in *Sry*-lacking *Tokudaia* species.

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Sex-determining region Y (SRY)-box genes are a large family of genes encoding a group of proteins that carry a DNA-binding high mobility group (HMG) domain and additional domains involved in transcriptional regulation [reviewed in Pevny and Lovell-Badge, 1997; Wegner, 1999]. This domain was first identified in *Sry*/SRY, a sex-determining gene on the Y chromosome of eutherian mammals [Gubbay et al., 1990; Sinclair et al., 1990]. Together with nuclear receptor subfamily 5, group A, member 1 (NR5A1, also referred to as SF1), SRY binds directly to the enhancer and synergistically upregulates the expression of *Sox9* (SRY-box 9) [Sekido and Lovell-Badge, 2008; Gonen et al., 2018]. After SOX9 expression reaches a threshold, SOX9 maintains its own expression via its

enhancer. SOX9 mainly functions in testis differentiation in vertebrates. Specifically, in eutherians, SOX9 is involved in the differentiation of the precursor cells to Sertoli cells in the gonads of XY embryos after sex determination by SRY. SOX9 directly regulates the expression of anti-müllerian hormone (AMH, also known as müllerian inhibitory substance, MIS). After initiation of testes differentiation, AMH expression is induced in the Sertoli cells of eutherian mammals [Vigier et al., 1983; Josso and Picard, 1986; Josso et al., 1993].

SOX genes are expressed in various phases of embryonic development and cell differentiation in a manner linked to cell speciation. They perform their functions as part of a complex interplay with other transcription factors, highly dependent on cell type and promoter context [reviewed in Kamachi et al., 2000]. SOX3 (SRY-box 3) is a single-exon gene located on the X chromosome [Stevanović et al., 1993; Collignon et al., 1996]. This gene is widely expressed in the central nervous systems of vertebrate embryos and is required for normal brain development. By contrast, its expression is extremely low or absent in the developing gonads of mice and marsupials [Collignon et al., 1996; Pask et al., 2000]. Thus, although SOX3 does not normally function in sex determination, an evolutionary link between SOX3 and SRY is indicated. Of the 20 SOX genes in the mammalian genome, SOX3 encodes a protein that is most similar to SRY, sharing 67% amino acid identity (and 90% similarity) across the HMG-domain [Stevanović et al., 1993; Bowles et al., 2000]. From molecular and cytogenetic studies of X and Y chromosomes, it was hypothesized that SRY and SOX3 originally were homologs and SRY evolved from SOX3 due to a gain-of-function mutation in the lineage of therian mammals [Foster and Graves, 1994; Graves, 2006]. Transgenic mice overexpressing SOX3 showed its ectopic expression in the bipotential gonads, frequently leading to complete XX male sex reversal [Sutton et al., 2011]. This suggests that the 2 SOX genes are functionally interchangeable in sex determination.

All 3 species of the genus *Tokudaia* (Muridae, Rodentia) have uniquely evolved in their sex chromosomes and sex-determining mechanisms. The Okinawa spiny rat (*Tokudaia muenninki*) has an XX/XY sex chromosome constitution as mammals generally have. However, the neo-X and neo-Y chromosome regions were acquired by fusion of a pair of autosomes to X and Y chromosomes in *T. muenninki* [Murata et al., 2012]. Two species of *Tokudaia*, the Amami spiny rat (*T. osimensis*) and the Tokunoshima spiny rat (*T. tokunoshimensis*), show an X0/X0 sex chromosome constitution due to lack of the Y chromosome [Honda et al., 1977, 1978; Kobayashi et al., 2007].

Several Y-linked genes have been rescued by translocation or transposition to the X chromosome and/or autosomes [Kuroiwa et al., 2010]. However, *Sry* has been completely lost from the genome of these species [Sutou et al., 2001; Murata et al., 2010], suggesting that they have acquired a new sex-determining mechanism that functions independent of SRY. SOX9 is also important for the sexual differentiation of this species [Kimura et al., 2014]. Furthermore, *Amh* expression is regulated by SOX9 in *T. osimensis*, similar to the mechanism observed in the mouse [Otake and Kuroiwa, 2016]. These previous studies indicate that a new sex-determining gene that superseded *Sry* regulates the *Sox9* expression in these species, but this putative gene has not yet been identified.

In this study, we investigated the possibility that SOX3 could perform the role of SRY in sex determination in *Sry*-lacking *Tokudaia* species. The coding sequence of the *Sox3* gene was highly conserved in *Tokudaia* species, but a 13-bp deletion was found in the promoter sequence. We were unable to determine the expression of *Sox3* in the embryonic gonads of *Tokudaia* because all species are endangered and protected by the Japanese government [Yamada et al., 2010]. Furthermore, experimental samples of embryonic gonads from *Tokudaia* species are currently not available. Therefore, we analyzed the expression pattern of *Sox3* in adult animals and performed an in vitro reporter gene assay to examine the effect of the 13-bp deletion on *Sox3* regulation.

Materials and Methods

Animals

All 3 species of *Tokudaia* (*T. muenninki*, *T. osimensis*, *T. tokunoshimensis*) are endangered (IUCN Red List of Threatened Species, <https://www.iucnredlist.org/>), and have been protected by the Japanese government as a natural monument since 1972. With permission from the Agency for Cultural Affairs and the Ministry of the Environment in Japan, all animals were released at their capture sites after a small piece was cut from the tip of their tail. Tissues were harvested from animals that died naturally or accidentally. DNA was extracted from the tail tissues as reported previously [Kuroiwa et al., 2011]. Slc:ICR strain mice were used for obtaining the coding sequence (CDS) of *Sox3* and its promoter as a control.

Screening of the BAC Clone and Cloning and Sequencing of the *Sox3* Gene and Promoter

A BAC library derived from a single male *T. osimensis* was constructed previously [Kimura et al., 2014]. The BAC library was screened using a 2-step 3-dimensional PCR screening system, as described [Kimura et al., 2014]. The primer sequences used are shown in Table 1. The experimental number of the isolated BAC clone is TOB1-256I18. CDS and promoter sequence of *T. osimen-*

Table 1. Primer list

Experiment	Forward primer, 5'→3'	Reverse primer, 5'→3'
Screening of BAC clone	TGA AAA CGG ATT TTC CAA GG	CCT TCA CTT TYT CGC TCT GG
Cloning		
CDS of <i>Sox3</i>	ATG CGA CCA GCT CGA GAG A	TCA GAT GTG GGT CAG CGG
<i>Sox3</i> promoter	TGA AAA CGG ATT TTC CAA GG	GTA AAG GCC GGG GGA CTC
RT-PCR		
<i>Sox3</i>	GCC TTG CTG TTT AGC TTT GC	CSG GAC TTC TYG CTT TTG TA
<i>Actb</i>	AAG GCC AAC GCN GAR AAG ATG	TGC GCA AGT TAG GTT TTG TCA AA
Reporter gene assay		
SOX3 expression vector	GCA AGC TGC GAA TGC GA	TCA GAT GTG GGT CAG CGG
<i>Sox3</i> promoter of mouse	CCG AGC TCC CAG GTG GGC CAT GGG ATG GGG CG	CCA AGC TTG GCT TCT CTC ACC TGA TGC GTT
<i>Sox3</i> promoter of <i>Tokudaia</i>	CCG AGC TCC CAG GCC GGC CAC GGG ATG GGG CG	CCA AGC TTG GCT TCT CTC ACC TGA TGC GTT
Overlap extension PCR	TGA AAA CGG ATT TTC CAA GG	CGC AGC TTG CCC GGG AAG CTT GCC CGG CCT
	AGC TTC CCG GGC AAG CTG CAA TGC GCG AC	GTA AAG GCC GGG GGA CTC

sis Sox3 were determined by sequencing of the BAC clone. The *Sox3* promoter region in *T. osimensis* female, *T. tokunoshimensis* male and female, and *T. muenninki* male and female was amplified by PCR using genomic DNA as a template, and the sequence was determined.

Total RNA was extracted from mouse and *T. osimensis* tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total RNA was reverse transcribed using SuperScript III (Invitrogen) and oligo(dT) primers. The synthesized cDNA was used as a template for CDS amplification. A primer pair was designed to amplify the CDS (Table 1). The GenBank accession numbers are as follows: LC431772 for *Sox3* CDS in *T. osimensis*, LC431773 for the *Sox3* promoter in *T. osimensis*, and LC431774 for the *Sox3* promoter in *T. tokunoshimensis*.

Preparation of Chromosomes and FISH Mapping of the BAC Clone

Preparation of R-banded chromosomes and direct R-banding FISH analyses were performed as described previously [Kobayashi et al., 2007; Kimura et al., 2014]. The BAC clone containing *Sox3* was used as a probe.

Reverse Transcription-PCR

Total RNA was extracted from liver, kidney, heart, whole brain, testis, and ovary of mouse and *T. osimensis* using the RNeasy Kit (Invitrogen, CA) according to the manufacturer's instructions. RNA was treated with DNase I and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primers. The primers are listed in Table 1.

Construction of Plasmids and Reporter Gene Assays

Forward primers and reverse primers with *SacI* and *HindIII* restriction sites, respectively (Table 1), were designed to amplify the *Sox3* promoter regions of mouse, *T. osimensis*, and *T. tokunoshimensis*. The PCR products were cloned into the *SacI/HindIII* sites of the PGL3-Basic vector (Promega, WI). A reporter construct containing the *Sox3* promoter of *T. osimensis* in which the 13-bp deletion was filled with the mouse sequence was generated by overlap extension PCR, as described previously [Ho et al., 1989;

Kimura et al., 2014]. The sequences of a set of complementary primers covering the filled deletion are shown in Table 1. The reporter activity was measured using NT2/D1 cells as reported by Kovacevic Grujicic et al. [2005] and Kimura et al. [2014].

Mouse and *T. osimensis Sox3* expression vectors were constructed as described previously [Kimura et al., 2014]. The primer sequences used to amplify *Sox3* of each species are shown in Table 1. The PCR product was cloned into the PMD20-T vector (Takara, Kusatsu, Japan). The DNA fragment digested by *Bam*HI and *Xba*I was cloned into the *Bam*HI/*Xba*I sites of the expression vector, pcDNATM3.1(+) (Invitrogen). Luciferase vectors containing the SV40 promoter and mouse testis-specific enhancer of *Sox9*, testis-specific enhancer of *Sox9* core (TESCO) or *T. osimensis* TESCO, and the expression vectors containing mouse *Sf1* (mSF1), mouse *Sry* (mSRY), or *T. osimensis Sox9* (SOX9) were constructed previously [Kimura et al., 2014]. The amino acid sequences of mouse and *T. osimensis* SF1 are identical. The reporter activity was measured as described for COS7 cells [Kimura et al., 2014].

Statistical Analysis

The data are presented as the mean ± SD. Statistical comparisons were performed using Tukey range tests.

Results

The *Sox3* Gene Is Conserved with the Exception of a 13-bp Deletion in the Promoter Region

To determine the chromosomal location of *Sox3* in *T. osimensis*, FISH mapping was performed. The *T. osimensis* BAC clone containing *Sox3* was used as a probe. *Sox3* was mapped to Xq23.2 by BAC FISH (Fig. 1). The fluorescent signal was found at a single locus in a single X chromosome. This locus was within a homologous region on the mouse and rat X chromosomes [Kobayashi et al., 2008; Murata et al., 2012].

Fig. 1. Chromosomal localization of *Sox3* in *Tokudaia osimensis*. The BAC clone containing *Sox3* was mapped to Xq23.2 in male (A) and female (B) specimens. Arrows mark the hybridization signals. A, C Propidium iodide-stained R-banding patterns. C, D DAPI staining. Scale bar, 10 μ m.

Fig. 2. Expression of *Sox3* in mice and *Tokudaia osimensis*. The expression pattern of *Sox3* in male and female mice and *T. osimensis* was examined by RT-PCR. L, liver; K, kidney; H, heart; B, whole brain; T, testis; O, ovary. *Actb* was used as internal control. For the negative control, distilled water (dH₂O) was used as template.

The expression of *Sox3* was examined by RT-PCR (Fig. 2). A similar testis- and ovary-specific pattern of expression was observed in mouse and *T. osimensis*. Testis-specific expression in adult male mice was previously shown by Northern blot analysis [Collignon et al., 1996]. Based on the band density, *Sox3* expression was higher in mice than in *T. osimensis*.

The size of the *Sox3* CDS in *T. osimensis* was 1,356 bp. The nucleotide and amino acid sequences showed 94.8 and 95.8% identity to mouse, respectively. The

HMG-box of *T. osimensis* SOX3 was completely identical to that of mouse (Fig. 3A). The *Sox3* promoter sequences in *T. osimensis* and *T. tokunoshimensis* were determined (Fig. 3B). Although all of the putative transcription factor-binding sites previously identified in mouse using MatInspector program [Kovacevic Grujicic et al., 2005] were conserved in the 2 *Tokudaia* species, a 13-bp deletion (–1 to –13) was observed in these (gray shading in Fig. 3B). The promoter sequence (+1 to –28) of mice, rats, *T. muenninki* (XX/XY), *T. osimensis* (X0/X0), and *T. tokunoshimensis* (X0/X0) are shown in Figure 3C. A 13-bp deletion was only observed in the 2 X0/X0 *Tokudaia* species. There were no sex differences in the promoter sequences of all *Tokudaia* species.

Decreased Expression Level Caused by a 13-bp Deletion in the Tokudaia Sox3 Gene

Kovacevic Grujicic et al. [2005] performed a chloramphenicol acetyltransferase reporter assay in NT2/D1 cells and reported that the region spanning –219 to +67 bp of the human SOX3 gene was the core promoter region, and positive *cis*-regulatory elements were located within the region –427 to –219 bp. Therefore, we examined *Sox3* promoter activity using constructs containing the regions including the mouse (–423 to +68), *T. osimensis* (–429 to +55) or *T. tokunoshimensis* (–429 to +55) promoter. The luciferase reporter construct containing each promoter

Fig. 4. Reporter gene assay in the *Sox3* promoter. Comparison of the activities of the mouse, *T. osimensis* (TOS), and *T. tokunoshimensis* (TTO) *Sox3* promoters, and a *T. osimensis Sox3* promoter in which the 13-bp deletion was filled with the mouse sequence, in NT2/D1 cells. The fold-change in activity was calculated relative to the luciferase activity obtained from transfection with mouse promoter alone. Data are represented as mean ± SD of at least 3 experiments. * $p < 0.01$.

COS7 cells. mSF1 alone induced a greater than 6-fold increase in mouse TESCO activity compared with the activity observed when only empty expression vectors were added, whereas mouse TESCO was not significantly activated by mSRY or mSOX9 alone (dark gray bars in Fig. 5). Mouse TESCO showed a greater than 8-fold increase in activity when it was co-expressed with a combination of mSF1 and mSRY or mSOX9, reflecting the synergistic regulation of *Sox9* expression by SRY, SOX9, and SF1 [Sekido and Lovell-Badge, 2008]. Unlike mouse TESCO, *T. osimensis* TESCO did not show significant activity in response to the expression of mSRY and mSOX9 in combination with mSF1 (white bars in Fig. 5). This result was consistent with the findings of our previous study [Kimura et al., 2014].

Mouse and *T. osimensis* TESCO were not significantly activated by mSOX3 or *T. osimensis* SOX3 (SOX3) alone (Fig. 5). Co-transfection with a combination of mSF1 and mSOX3 or SOX3 resulted in a greater than 2-fold increase in activity of mouse and *T. osimensis* TESCO. This result is consistent with that of a previous study [Sutton et al., 2011]. However, this activity was lower than the activity observed when only mSF1 expression vector was added to mouse TESCO.

Discussion

The sequence of the *Sox3* gene in *T. osimensis* shows a high degree of similarity with that of the mouse. Specially, the HMG-box, which is a DNA-binding domain that is important for the function of SOX3 as a transcription factor, is completely identical between the 2 species. *Sox3* was localized in a region of the X chromosome in *T. osimensis* homologous to mouse. Furthermore, the expression patterns of the *Sox3* gene in adult *T. osimensis* and mice (testis- and ovary-specific expression) were almost identical. These results indicate that the function of the *Sox3* gene reported in mice and humans is conserved in the *Sry*-lacking species, *T. osimensis*.

All of the putative binding sites for previously described transcription factors are conserved in the promoter regions of the 2 *Tokudaia* species. However, a 13-bp deletion was found in the promoters of both X0/X0 *Tokudaia* species. Mouse, rat, and *T. muenninki*, which have a Y chromosome and the *Sry* gene, have no deletion (Fig. 3C). In the molecular phylogenetic tree previously reported by us, *T. muenninki* first diverged from the genus *Tokudaia* [Murata et al., 2010]. These results indicate that the deletion specifically occurred in the ancestral common species of the 2 X0/X0 *Tokudaia* species. To examine the effect of this deletion on *Sox3* regulation, reporter gene assays were performed using the promoter sequences of mouse, both *Tokudaia* species with the deletion, and a mutated promoter in which the 13-bp deletion was filled with the mouse sequence. The *Tokudaia* promoters with the 13-bp deletion showed an approximately 60% decrease in activity compared with the activity of the mouse promoter. However, this decrease in activity was recovered by filling the deletion, resulting in an approximately 22% decrease in activity compared with the mouse sequence. These results suggest that the 13-bp deletion leads to a downregulation of *Sox3* expression in *Tokudaia* species. The functional consequence of this downregulation of *Sox3* expression was not evaluated in this study. However, the observed downregulation of *Sox3* does not support our hypothesis that the *Sox3* gene functions in sex determination instead of *Sry*. Therefore, we concluded that the *Sox3* gene has no function in sex determination in *Sry*-lacking species of *Tokudaia*.

To determine whether *Sox3* could regulate *Sox9* expression instead of *Sry* in *T. osimensis*, reporter gene assays were performed using mouse and *T. osimensis* TESCO. *T. osimensis* SOX3 stimulated a greater than 2-fold increase in mouse and *T. osimensis* TESCO activity in combination with mSF1, compared with the activity ob-

Fig. 5. Reporter gene assay in TESCO. Comparison of the enhancer activities of mouse TESCO and *Tokudaia osimensis* TESCO in COS7 cells expressing a reporter construct and co-transfected with or without mSF1, mSRY, mSOX9, mSOX3, and *T. muenninki* SOX3. The fold-change in activity was calculated relative to the luciferase activity obtained from transfection with mouse TESCO or *T. osimensis* TESCO alone. Data are represented as mean $\pm$ SD of at least 3 experiments. * $p < 0.01$.

served when only the empty vector was added. A similar increase in activity was observed when mSOX3 and mSF1 were co-expressed. These results support the idea that the function of SOX3 as a transcription factor, as has been reported in mice and humans, is conserved in *T. osimensis*. The activity of *T. osimensis* TESCO induced by mSOX3 or *T. osimensis* SOX3 with mSF1 was lower than the activity observed when only mSF1 expression vector was added to mouse TESCO (Fig. 5). We could not determine the reason; however, physical interaction of SRY and SF1 proteins on the DNA-binding sites was indicated in a previous report [Sekido and Lovell-Badge, 2009]. An interaction of SOX3 and SF1 might suppress the SF1 activity to TESCO.

TESCO was first reported as a regulatory element that induces the expression of *Sox9* by binding SRY together with SF1 [Sekido and Lovell-Badge, 2008]. The TESCO sequence contains several SRY- and SF1-binding sites, which are highly conserved between mice, rats, dogs, and humans [Sekido and Lovell-Badge, 2008]. However, we previously found that there were nucleotide substitutions in 2 out of 3 SRY-binding sites and in 5 out of 6 SF1-binding sites in TESCO in all *Tokudaia* species [Kimura et al., 2014]. The activity was restored with a mutated version of TESCO in which the nucleotide substitutions found in the SRY- and SF1-binding sites were replaced with the mouse sequence. Although *T. osimen-*

sis SOX3 could stimulate an approximately 2-fold increase in *T. osimensis* TESCO activity with mSF1, this was a very low level compared to the level of synergistic regulation of *Sox9* expression by SRY, SOX9, and SF1 in mouse. Therefore, we hypothesized that, if *T. osimensis* SOX3 has a role in sex determination, it regulates *Sox9* expression through an enhancer other than TESCO. A new enhancer for *Sox9* regulation in the mouse, Enh13, a 557-bp element located 565 kb upstream of *Sox9*, has been recently reported [Gonen et al., 2018]. Enh13 is thought to be an early-acting enhancer; without it, *Sox9* transcription fails to increase to a level at which other enhancers can act before the gene is silenced. Actually, several enhancer elements upstream of *Sox9* were found in mice and humans [Croft et al., 2018; Ogawa et al., 2018]. These findings indicate a redundancy in *Sox9* regulation that involves several enhancers. In *T. osimensis*, an enhancer other than TESCO might be important for sex determination independent of SRY. Further research to identify other candidate genes that replace *Sry* in sex determination is needed.

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Statement of Ethics

All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee of National University Corporation, Hokkaido University, and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Hokkaido University.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

K.W. and S.M. performed experiments and analyzed the data. T.J. performed sampling. A.K. conceived and designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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