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Ontogenic and morphological study of gonadal formation in genetically-modified sex reversal XY^{POS} mice

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ABSTRACT. Mammalian sexual fate is determined by the presence or absence of *sex determining region of the Y chromosome* (*Sry*) in the "bipotential" gonads. Recent studies have demonstrated that both male and female sexual development are induced by distinct and active genetic pathways. Breeding the Y chromosome from *Mus m. domesticus poschiavinus* (POS) strains into C57BL/6J (B6J) mice (B6J-XY^{POS}) has been shown to induce sex reversal (75%: bilateral ovary, 25%: true hermaphrodites). However, our B6N-XY^{POS} mice, which were generated by backcrossing of B6J-XY^{POS} on an inbred B6N-XX, develop as males (36%: bilateral testis with fertility as well as bilateral ovary (34%), and the remainder develop as true hermaphrodites. Here, we investigated in detail the expressions of essential sex-related genes and histological features in B6N-XY^{POS} mice from the fetal period to adulthood. The onsets of both Sry and SRY-box 9 (Sox9) expressions as determined spatiotemporally by whole-mount immunohistochemistry in the B6N-XY^{POS} gonads occurred 2–3 tail somites later than those in B6N-XY^{B6} gonads, but earlier than those in B6J-XY^{POS}, respectively. It is possible that such a small difference in timing of the Sry expression underlies testicular development in our B6N-XY^{POS}. Our study is the first to histologically show the expression and ectopic localization of a female-related gene in the XY^{POS} testes and a male-related gene in the XY^{POS} ovaries. The results from these and previous experiments indicate that the interplay between genome variants, epigenetics and developmental gene regulation is crucial for testis development.

KEY WORDS: C57BL/6, *Mus m. musculus poschiavinus,* sex reversal, Sox9, Sry

Mammalian XX individuals normally develop as females with ovaries, and XY individuals develop as males with testes [2]. Mammalian sex determination depends on the presence or absence of *sex determining region of the Y chromosome* (*Sry*), the switch gene of testis development, located in the male-specific region of the Y chromosome [18, 41]. *Sry* encodes a transcriptional factor containing a DNA-binding domain of some high mobility group proteins (HMG box) [37]. The Sry protein binds through its consensus binding site (A/T) AACAAT [12] and activates the TESCO (testis-specific enhancer core sequence) of *SRY-box 9* (*Sox9*) [38]. The Sox9 protein in turn induces somatic precursor cells to develop into Sertoli cells, which orchestrate the development of the gonads as testis with nuclear receptor

subfamily 5, group A, member 1 (Nr5a1) [47]. Nr5a1 is a key transcriptional factor in the formation and development of genital ridges. Because no *Sry* expression is observed in the *Nr5a1*^{−/−} gonad, it has been proposed that Nr5a1 may be one of the upstream regulators of *Sry* [39]. *Sry* is expressed in a dynamic wave that emanates from the central and/or cranial regions in a gonad, then extends to both poles and ends in the caudal pole from 11 tail somites (ts) to 27 ts, and this wave is closely followed by expression of the Sry protein, which starts at 11 ts and ends with the last Sry-positive cells detectable at 30 ts, and ceases entirely by 34 ts [6, 46]. The ability of *Sry* to induce testis development is limited to approximately 11.0–11.25 days post coitum (dpc), which corresponds to a time window of only 6 hr after the normal onset of *Sry* expression in the XY gonad [21]. Reduced or delayed *Sry* expression impairs testis development, highlighting the importance of its accurate spatiotemporal regulation and implying a potential role for *SRY* dysregulation in human intersex disorders [27].

On the other hand, despite its important roles, the Sry protein structure except for the HMG box varies across some mammalian species. Mouse Sry has a particularly unusual structure in comparison with other mammals. The N-

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terminal domain has only 2 amino acids, and the C-terminal domain includes a glutamine (Q)-rich domain that is derived from degenerate CAG repeats. Moreover, there is a "bridge domain" situated between the HMG box and the Q-rich domain [37]. Although it was previously considered that the HMG box is the only functional domain involved in the activation of Sox9, recent studies demonstrated that the Q-rich domain also has important roles in protein stabilization and transcriptional activation, both of which are essential for male sex determination [48].

The Q-rich domain is known to be less conserved in mice than in other species, and its sequence and length are different. For example, three main subspecies of the genus *Mus.* (*Mus. m. musculus*: *e.g.* 129, B6, DBA/2J; *Mus. m. domesticus*: *e.g.* POS, TIR, CD-1, AKR; *Mus. m. castaneus*: *e.g.* Southeast Asian house or wild mouse) exist, and the length of the Q-stretch in *Mus. m. domesticus* is approximately half of that in *Mus. m. musculus*, due to a premature stop codon [37]. Moreover, the transfer of the Y chromosome from the mouse species *Mus. m. domesticus poschiavinus* (XY^{POS}) to the genome of the C57BL/6J (B6J) inbred strain causes disruption of the normal testis determination process and results in the partial or complete sex reversal of XY individuals. These XY^{POS} mice have been produced using a standard backcrossing method. After several generations of backcrossing, half of the XY individuals developed as females with ovarian tissue and half developed as hermaphrodites. Some hermaphrodites were fertile, having functional testicular tissue to masculinize their internal and external genitalia and produce sufficient numbers of sperm to fertilize eggs [13, 16].

The progeny of our XY^{POS} mice, which were originally obtained from the Jackson Laboratory [16], also express various combinations of females with ovaries and ambiguous individuals, such as hermaphrodites (unilateral or bilateral ovotestis) or individuals with ovotestes and males with bilateral testes. However, the frequency of male progeny was higher than that of the other phenotypes, and these males appeared normal with fertility. From the above, it is possible that our mice were slightly different from those reported in 1982. However, if this is true, what causes the formation of testes in our mice? Otherwise, what protects against XY^{POS} sex reversal?

Here, we investigated in detail the expressions of certain sex-related genes and histological features of our XY^{POS} mice from the fetal stage to adulthood.

MATERIALS AND METHODS

Animals: Five genetically modified mice, B6J-XY^{POS} Tg4, were generated from the Jackson Laboratory [15] by way of the National Institute for Basic Biology in Okazaki, Japan (Prof. K. Morohashi). Except for the *Sry* allele from *Mus m. domesticus poschiavinus* (*Sry^{POS}*), the *Sry* allele from the 129 inbred strain (*Sry^{129}*) is inserted as a transgene (TG) into an ectopic position in the genome. A 14.6 kb DNA fragment containing the *Sry^{129}* gene was injected into the male pronucleus in fertilized eggs obtained from a B6J-XX mated to B6J-XY^{POS} hermaphrodites [15]. B6J-XY^{POS} Tg (*Sry^{129}*)

mice on an inbred C57BL/6N (B6N) (purchased from Japan SLC, Inc., Hamamatsu, Japan) background were generated by backcrossing for more than 11 generations. The phenotypes have been produced by the same ratio during these generations. Their offspring having only the *Sry* allele from *Mus m. domesticus poschiavinus* (*Sry^{POS}*) were selected and are the subject of this paper. All mice were maintained in individual $40.5 \times 20.5 \times 18.5$-cm ventilated cages (Sealsafe Plus Mouse; Tecniplast S.p.A, Buguggiate, Italy) under specific pathogen-free conditions with controlled temperature ($23 \pm 2°C$) and humidity ($50 \pm 10\%$) on a 12-hr light/dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to water and a pellet diet (DC-8; Clea Japan, Inc., Tokyo, Japan). This study was approved by the Institutional Animal Care and Use Committee (Permission #25-06-03) and carried out according to the Kobe University Animal Experimental Regulation.

Dissection, staging and genotyping of fetal mouse gonads: Timed matings were used for all embryonic experiments. Noon of the day of vaginal plug detection was designated as 0.5 dpc. The gonads and mesonephroi that were removed from the embryos between 11.5 and 12.5 dpc were precisely staged by counting the number of ts: 11.5 dpc is ~18 ts, and 12.5 dpc is ~30 ts [19]. The sexual phenotype of weaning-age mice was determined by examining the distance from the anus to the genitalia (anogenital distance: AGD). Fetal sex and the presence of the *Sry^{POS}* and *Sry^{129}* alleles were determined by multiplex PCR (polymerase chain reaction). The chromosomal sex of each fetus was confirmed by PCR using the primer pair 5′-GGG ACT CCA AGC TTC AAT CA and 5′-TGG AGG AGG AAG AAA AGC AA to detect the *interleukin-3* (*IL-3*) region as an internal control, and the primer pair 5′-CCC CTT TCT AAG CAG ACA TC and 5′-TCA CAC TAA CCT CAC AGG CC to detect *Y-specific B10-derived sequences* (*YB-10*), a region located on the long arm of Y chromosome. The amplified products were electrophoresed in 2% agarose gel, dyed by ethidium bromide and visualized with a UV transilluminator.

Sry alleles: In addition to the primer pairs described above, a region of the *Sry* HMG box was amplified from genomic DNA using 5′-TGG GAC TGG TGA CAA TTG TC and 5′-GAG TAC AGG TGT GCA GCT CT as primers and the PCR product digested using *Mbo* I. The PCR conditions were 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec. The *Sry^{129}* allele contains an *Mbo* I recognition site, resulting in two fragments of 23 and 130 bp, while the *Sry^{POS}* allele lacks the *Mbo* I recognition site, resulting in an undigested 153 bp fragment.

Tissue preparation: Genital tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. The specimens were dehydrated with an ethanol series followed by xylene and then embedded in paraffin. Then, 4-μm-thick sections were cut by a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Germany) and placed on slide glasses (Platinum; Matsunami Glass Ind., Ltd., Kishiwada, Japan).

Histological and immunohistochemical analyses: For the general histological analyses, tissue sections were stained

with hematoxylin and eosin (HE; Merck Ltd., Japan, To-kyo, Japan) after their deparaffinization and hydration. To detect Nr5a1 (a generous gift from Dr. K. Morohashi, Kyushu University, Japan), Sox9 (Santa Cruz Biotechnol-ogy, Inc., Santa Cruz, CA, U.S.A.) and forkhead box L2 (Foxl2) (Affinity Bioreagents, Golden, CO, U.S.A.), we performed the following immunohistochemical protocol by using anti-Nr5a1 rabbit polyclonal antibody (1:16,000), anti-Sox9 rabbit polyclonal antibody (1:300) and anti-Foxl2 rabbit polyclonal antibody (1:500). The sections were im-mersed in absolute methanol and 0.5% H_2O_2 for 30 min at room temperature (RT) to quench the endogenous peroxi-dase activity. They were then incubated with Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) for 1 hr at RT for protein blocking and then incubated with each primary antibody in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST; pH 7.4) for 18 hr at 4°C. After being washed with PBST, the sections were reacted with Dako EnVision+ system-HRP (horseradish peroxidase) labeled polymer (EnVision+; Dako Japan, Tokyo, Japan) in Tris-HCl buffer (pH 7.6) for 1 hr at RT. Immunoreactivity was then detected by incubation with 3,3'-diaminobenzidine solution (EnVision+ kit/HRP[DAB]; Dako). The sections were counterstained lightly with hematoxylin solution for 1 min. Finally, the sections were placed in a graded series of ethanol, dehydrated with absolute ethanol, cleared by xylene and coverslipped with Eukitt (O. Kindler, GmbH, Freiburg, Germany).

Whole-mount immunohistochemistry: Gonad/mesoneph-ros complexes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. The specimens were dehydrated with 100% methanol for 8 hr at 4°C; then passed through three cycles of chilling in 1% H_2O_2 dissolved in absolute methanol to −80°C in a freezer and thawing to 4°C to break down the cell membranes and quench the endogenous peroxidase activity. Next, the specimens were rinsed with 50% methanol in 0.1 M PBS for 1 hr, followed by 0.1 M PBS alone for 1 hr, then incubated with 0.1% al-bumin serum in Blocking One Histo (Nacalai Tesque, Inc.) at 4°C for 18 hr and reacted with anti-Sry rabbit polyclonal antibody (a generous gift from Dr. D. Wilhelm, Monash University, Australia; 1:800) or anti-Sox9 rabbit polyclonal antibody (1:600) at 4°C for 24 hr. The tissues were washed with PBS three times for 2 hr each and incubated with a Dako EnVision+ system-HRP labeled polymer (Dako) at 4°C for 42 hr. After washing with PBS as described above, they were incubated with 0.17% DAB in 0.05 M Tris-HCl buffer (pH 7.6) at 4°C for 2 hr. Immunoreactivities were visualized by using 0.17% DAB and 0.3% H_2O_2 in the Tris-HCl (pH 7.6). Negative controls, in which the primary antibodies were re-placed with nonimmunized serum, did not show nonspecific staining.

Sequence analysis: The Sry coding sequence (CDS) was analyzed by direct sequencing of PCR products by the capil-lary electrophoresis systems. A total of 100–150 ng of ge-nomic DNA was extracted from liver tissue using a QIAamp DNA Microkit (Qiagen, Hilden, Germany). Genomic DNA was used as a template for the primers mSry-933: 5'-GCA

Table 1. Primers used for *Sry* sequencing

Name	Forward/ Reverse	Primer sequence (5' to 3' direction)
mSry-537	Forward	CGTTACAGGGCAAAAGAACTCA
mSry-328	Forward	GGAGGAGGGATAAATATTTTCTTACA
mSry-97	Forward	AGATCTTGATTTTTAGTGTTC
mSry+162	Forward	TTCCAGGAGGCACAGAGATT
mSry+320	Forward	CCCACATGCCATCACATACA
mSry+410	Forward	TATCCCCACTGGGCACCT
mSry+1083	Reverse	GTAAGCTGCTGGTCGTGGAA
mSry+1132	Forward	AGCATACACCATACCAGGAGCA
mSry+1195	Reverse	GTGTTGGCATAGGTAGGAGAAAAG

The first base of *Sry* nucleotide sequence, adenine, is set as position one. The sequences locate upstream of this adenine is shown with minus symbols. We used *Mus musculus* Y chromosome sequence from NCBI (Gene ID: 21674) as a reference.

TGG TGA TGC TGT AAG GAA and mSry+1195: 5'-GTG TTG GCA TAG GTA GGA GAA AAG in a 50-μl reaction. PCR was performed by conventional techniques using Prime STAR® GXL DNA Polymerase (Takara Bio, Otsu, Japan) employing 35 cycles of 98°C for 10 sec, 60°C for 15 sec and 68°C for 100 sec. The PCR product was assayed for specificity on a 2% agarose gel (the expected PCR product size was 2,152 bp) and then purified for sequencing using a QIAquick Gel Extraction Kit (Qiagen). A total of 50 ng of purified Sry CDS PCR product was directly sequenced using the primers for Sry (Table 1). Sequencing of PCR products was outsourced and analyzed on an ABI3730XL DNA ana-lyzer (Applied Biosystems, Foster City, CA, U.S.A.) by Fas-mac Co., Ltd. (Atsugi, Japan). DNA sequence analysis and alignments were performed using the Gene Studio computer program with subsequent hand editing. Genomic DNA from at least three B6N-XY[POS] mice was used as a PCR template and for the sequence reactions.

Quantification of ovarian follicles: The number of folli-cles was counted in individual tissue sections. If one follicle was present in several continuous sections by using ocular micrometer under light microscopy, it was only counted in the first section in order to avoid repetition. The means were calculated, and the data are presented as the means ± stan-dard deviation (SD).

Fertility test: Each B6N-XY[POS] male or B6N-XY[POS] female offspring (5–8 weeks of age) was housed with a B6N-XX or B6N-XY[B6] mouse (6–10 weeks of age) that was known to be fertile. The presence or absence of copulation plugs was examined between 9:00 and 10:00 a.m. every morning up to 7 days. If copulation plugs were not found, mating was repeated two more times. The female mouse that had copulated was housed singly until the expected delivery day. If pregnancy was not confirmed after 12 days of deliv-ery, this process was repeated two more times.

Statistical analysis: Statistical analyses were performed with Excel Statistics 2012 (SSRI, Ltd., Tokyo, Japan). All data were analyzed by two-way analysis of variance (ANO-VA) followed by Tukey-Kramer's post hoc test. The results were considered significant when the *P*-value was <0.05.
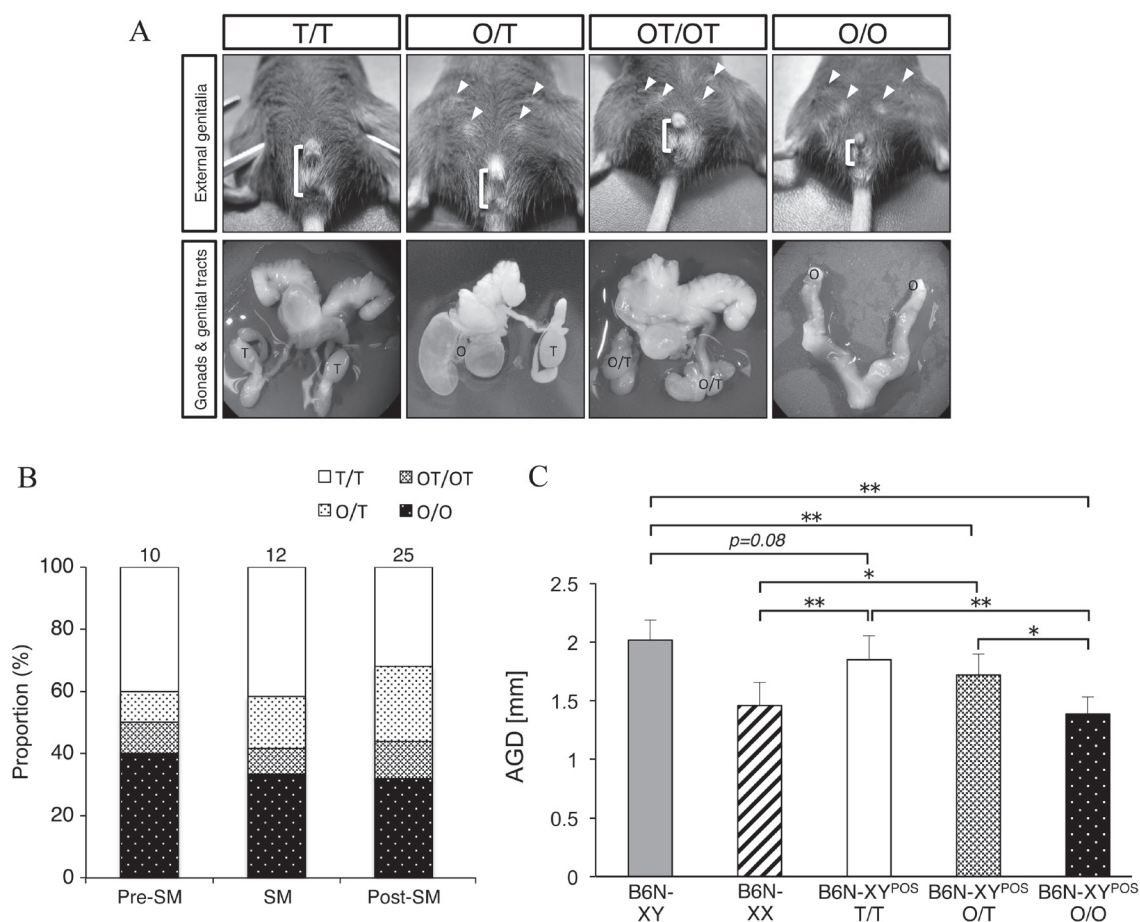
Fig. 1.   Sexual development of adult B6N-XY[POS] mice. (A) External genitalia of adult mice of the indicated genotypes. The distance between the anus and penis or vagina (AGD) is indicated. Arrowheads represent nipples. Gross anatomy of isolated internal genitalia and associated reproductive organs from adult B6N-XY[POS] mice. T: testis; O: ovary; OT: ovotestis (B) Frequency analysis of abnormal sex differentiation determined by examining the internal genitalia of adult mice. Numbers of examined animals are shown above the bars. (C) AGD of adult B6N-XY[POS] mice and control mice.

RESULTS

Sexual development of B6N-XY^{POS} mice: When analyzing B6N-XY[POS] mice, which had been established by a cross between a male B6J-XY[POS] transgenic mouse with the 14.6 kb DNA fragment containing the *Sry^{129}* males and a B6N-XX females, we found a broad range of gonadal phenotypes, but typically four types of external and internal genitalia—*i.e.*, genitalia with bilateral testes (T/T), bilateral ovaries (O/O), bilateral ovotestes (OT/OT) and a testis on one side and an ovary on another (O/T, hermaphrodite)—with the same genotype (Fig. 1A). Hermaphrodites and individuals with unilateral testis or ambiguous gonads had both nipples and penises (Fig. 1A). Notably, O/T mice had a right ovary and left testis with extraordinary frequency at few years since assignation from the Jackson Laboratory (Fig. 1A). The frequency of these phenotypes in the growing stages (pre-SM: pre-sexual maturation at the ages of 2 weeks; SM: sexual maturation at the ages of 9 weeks; post-SM: post-sexual maturation at the

ages of 18 weeks) showed that over 36% of our B6N-XY[POS] mice displayed normal sex differentiation (T/T individuals) (Fig. 1B) and some of them were fertile. The boundary of these phenotypes seemed ill-defined, and some B6N-XY[POS] individuals had ambiguous gonads. Although the B6N-XY-[POS] testes and accessory reproductive glands looked similar to those of the B6N-XY[B6], some B6N-XY[POS] testes were smaller. Individuals with bilateral ovaries had nipples and vaginal openings, and no penises. The ovaries and accessory reproductive glands looked similar to those of the B6N-XX. The AGD of these animals was the same as that of the B6N-XX and shorter than that of the B6N-XY[B6] mice. They also had male accessory reproductive glands (Fig. 1C).

Gonadal descent was detected in T/T, OT/OT and O/T individuals, but not in O/O mice, even in the post-SM period. The body weights and AGDs in mice with gonadal descent were significantly higher than those in mice without gonadal descent (data not shown).

Histological and immunohistological findings: Histopa-

thology was evaluated using cross-sections of gonads stained with HE by light microscopy. To investigate the etiology of sex reversal, we examined the expression of Nr5a1, which is a critical regulator of sexual development and reproduction [33], the testicular Sertoli cell marker Sox9 [24] and the ovarian somatic cell marker Foxl2 [29]. Because the B6N-XY^POS T/T individual testes showed normal cell arrangements with well-developed seminiferous tubules consisting of spermatogonia, spermatocytes, spermatids and sperm according to each sexual development stage, the gonads contained no Foxl2-positive cells, but only Sox9-positive cells (Fig. 2A), indicating that the testes were normal and thus that there was no failure of the testis-determining pathway. While some seminiferous tubular structures in the testis of O/T individuals had abnormal vacuoles and no sperm (Fig. 2B), the pre-SM testis showed Foxl2-positive oocyte-like cells, but without a pellucid zone or follicular epithelium (Fig. 2B). The number of ovarian follicles was quite small in the ovaries of the O/T mice, and some follicles exhibited blood leakage or intrafollicular shedding of cells, while Sox9-positive cells were observed in some of the seminiferous tubule-like structures (Fig. 2C). Many secondary ovarian follicles as well as primary ones were observed in the pre-SM B6N-XY^POS females (O/O mouse), but no vesicular ovarian follicles were detected (Fig. 3A). These follicles were rare in the SM and post-SM sexual developing stages (Fig. 3A). The measurement of the number of ovarian follicles revealed a rapid decrease of germ cells at between 2 to 9 weeks of age (Fig. 3B).

The results of immunoreactive analysis in the genital ridges at 11.5 dpc are shown in Fig. 4. The immunoexpression of Nr5a1 in the genital ridges of B6N-XY^POS mice was compared to that in the B6N-XY^B6 males and females. Nr5a1 positivity was detected in the epithelium and internal somatic cells but not in the germ cells, and no difference was found in the staining properties (Fig. 4A). Although Sox9 was expressed in a diffusely-arranged manner in both the right and left gonads of the B6N-XY^B6, the level of Sox9 expression in the B6N-XY^POS gonads was much lower than that in the B6N-XY^B6 (Fig. 4B). In addition, a bilateral difference or very few Sox9-positive cells were demonstrated in B6N-XY^POS gonads (data not shown). By contrast, no immunopositive cells were detected in the B6N-XX (Fig. 4B). No Foxl2 immunoreactivity was detected in the genital ridges of both B6N-XY^B6 and B6N-XX, but extremely-weak reactivity was seen in the B6N-XY^POS (Fig. 4C).

Spatiotemporal expression of Sry: We found no expression at 11 ts in either the B6N-XY^B6 or B6N-XY^POS gonads (Fig. 5a and 5h). Weakly positive cells were observed chiefly in the central region of the 13 ts B6N-XY^B6 gonad (Fig. 5b). Getting to 15 ts, positive cells were expanded toward both the craniad and the caudal region, and the expression level in the central region was stronger than that in both acral regions (Fig. 5c). From 16 ts to 21 ts, strongly positive cells were seen throughout the gonad (Fig. 5d). Sry expression began to decrease from the central region after 23 ts, and then, it became limited by 25 ts (Fig. 5e). Few positive cells were observed in the thickening gonads at 26 ts (Fig. 5f), and there

were no positive cells at 27 ts (Fig. 5g). Briefly, Sry expression of B6N-XY^B6 gonads starts from the central region at 12–13 ts, rises toward a peak at 17–21 ts and then falls to a low level at 23–27 ts (Fig. 5a–5g).

On the other hand, Sry expression of B6N-XY^POS was not detected beyond 13 ts (Fig. 5h and 5i). Weakly positive cells were wholly observed in the 15 ts B6N-XY^POS gonad (Fig. 5j). At 17 ts, positive cells were seen throughout the gonad, though the expression level was weak around the caudal region (Fig. 5k). The expression level became strong at 19–26 ts (Fig. 5l and 5m), and it was reduced beyond 28 ts (Fig. 5n). Although there were few positive cells from the craniad to the central region at 30 ts, many strongly positive cells remained in the caudal region (Fig. 5o). In brief, Sry expression of B6N-XY^POS gonads starts from the central region at 14–15 ts, rises toward a peak at 19–26 ts and then falls to a low level after 28 ts (Fig. 5h–5o).

Spatiotemporal expression of Sox9: Weakly positive cells emerged chiefly in the central region of the 15 ts B6N-XY^B6 gonad (Fig. 6a). At 17 ts, positive cells were seen throughout the gonad, though the expression level was significantly low around the caudal region (Fig. 6b). Strongly positive cells were seen at 18–22 ts (Fig. 6c–6e). By the 20 ts stage, Sox9 expression was seen throughout the gonad (Fig. 6d–6g). An increase in positive cells and gonadal thickening were observed after 22 ts, and testis cords including positive cells were observed after 26 ts (Fig. 6e–6g). Briefly, Sox9 expression of B6N-XY^B6 gonads starts from the central region at 14–15 ts and expands to all regions at 18 ts, and then, the number of Sox9-positive cells increases, causing gonadal thickening and testis cords formation (Fig. 6a–6g).

By contrast, typically, Sox9 expression was not detected in B6N-XY^POS gonads at 16 ts (Fig. 6h), though expression in a small number of cells was sometimes seen in the central region (data not shown). Weakly positive cells started to expand toward both acral regions from 17 ts to 20 ts, but the number of positive cells in B6N-XY^POS gonads increased more moderately than that in the B6N-XY^B6 gonads (Fig. 6i–6k). The number of positive cells continued to rise in the central region, and no increase was observed at either acral region after 22 ts (Fig. 6l and 6m). Thickening was observed only in the center of the gonads at 26 ts (Fig. 6m). Although the gonads had developed to the same level as the B6N-XY^B6 gonads at 30 ts, they formed no testis cords and contained very few positive cells at either acral region. Moreover, the coelomic epithelium invaginated into the gonads at 30 ts, and weakly positive cells were eccentrically located at the mesonephric side (Fig. 6n). In brief, then, the Sox9 expression of B6N-XY^POS gonads starts from the central region at 16–17 ts, and then, it expands to all regions by 20 ts. The number of positive cells in B6N-XY^POS gonads increased more moderately than that in the B6N-XY^B6 gonads. Gonads at 30 ts have less positive cells at both acral regions, which results in no formation of testis cords (Fig. 6h–6n).

Fertility: All fertility-tested B6N-XY^POS female mice were infertile. Although all (four) B6N-XY^POS females that had been placed with B6N-XY^B6 mated as evidenced by the presence of copulation plugs, none of them were pregnant on

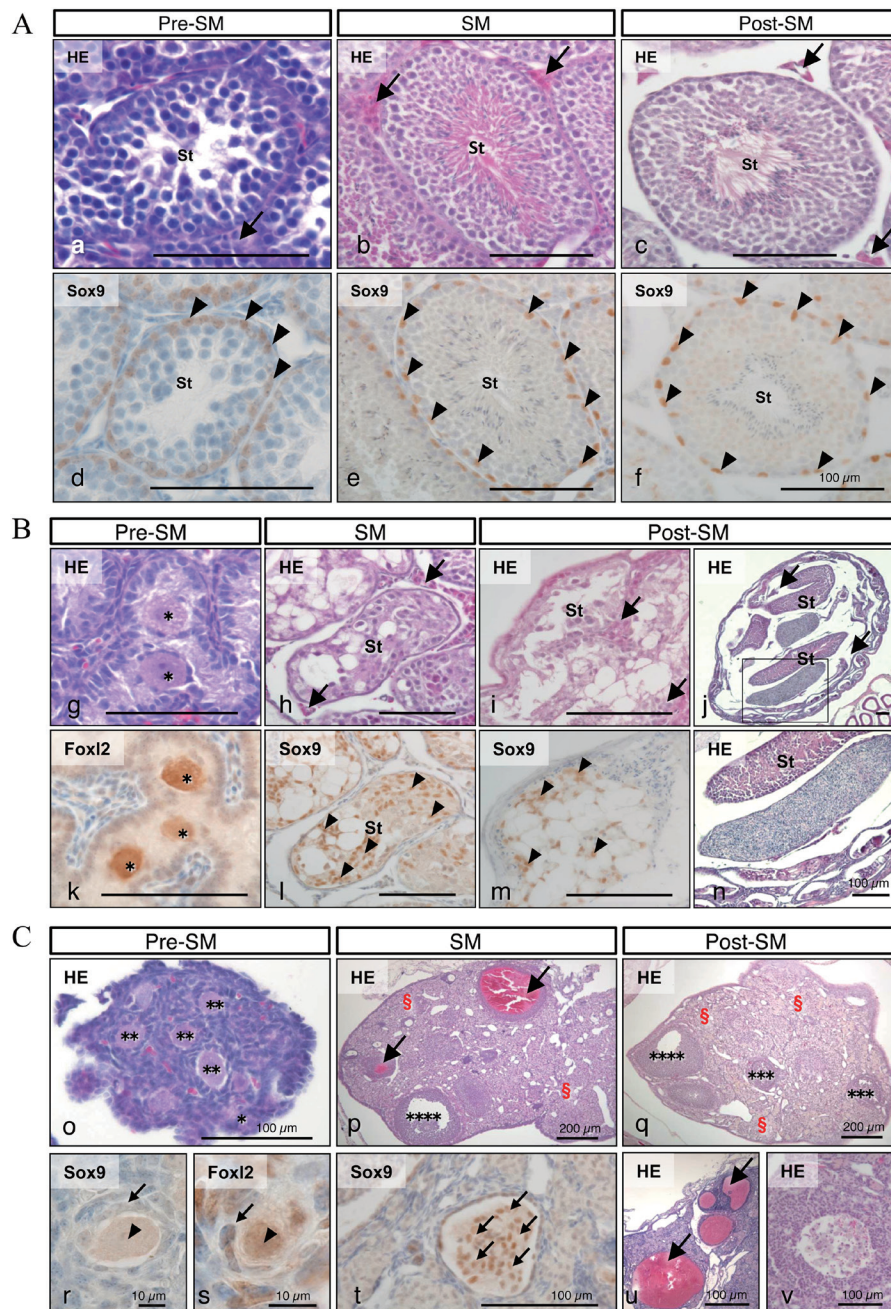Fig. 2. General histology and immunohistochemistry in the B6N-XY[POS] mice in each sexual developmental stage. (A) Hematoxylin and eosin staining (HE) and Sox9 immunostaining on transversal sections at pre-sexual maturation (pre-SM), SM and post-SM in T/T individuals. The testis shows a luminal structure and normal cell arrangements including elongated spermatids in the wall of seminiferous tubules in the SM and post-SM (a–c). Clusters of Leydig cells are present in the intertubular space (arrows, a–c). Sox9-positive Sertoli cells are observed (arrowheads, d–f). St: seminiferous tubule. (B) Abnormal structures and immunoreactivities of the testis in O/T individuals. Deformed seminiferous tubules and Foxl2-positive oocyte-like cells are seen in the lumen of the seminiferous tubules in the pre-SM (asterisk, g and k). Clusters of Leydig cells are present in the intertubular space (arrows, h–j). Detachment of Sox9-positive Sertoli cells (arrowheads) is also seen in the degenerated semi-niferous tubules in the SM and post-SM (l–m). Sperm masses are present in the seminiferous tubules without spermatogonia, spermatocytes, spermatids and Sertoli cells (j). The boxed areas are shown at higher magnification (n). (C) Degenerated structures and immunoreactivities of the ovary in O/T individuals. Slight primordial follicles and primary oocytes were observed in the pre-SM stage (o), vesicular follicles, corpus luteum and blood leakage (large arrows) in the SM stage (p), and secondary and vesicular follicles and corpus luteum in the post-SM stage (q). Moreover, blood leakage and intrafollicular shedding (large arrows) of cells were also seen (u, v). Oocyte (arrowhead) and follicular epithelium (arrow) slightly positive for Sox9 (r) and positive for Foxl2 (s) were seen in the pre-SM. Clusters of Sox9-positive cells (small arrows) were observed at SM (t). *primordial follicles; **primary follicles; ***secondary follicles; ****vesicular follicles; §corpus luteum.
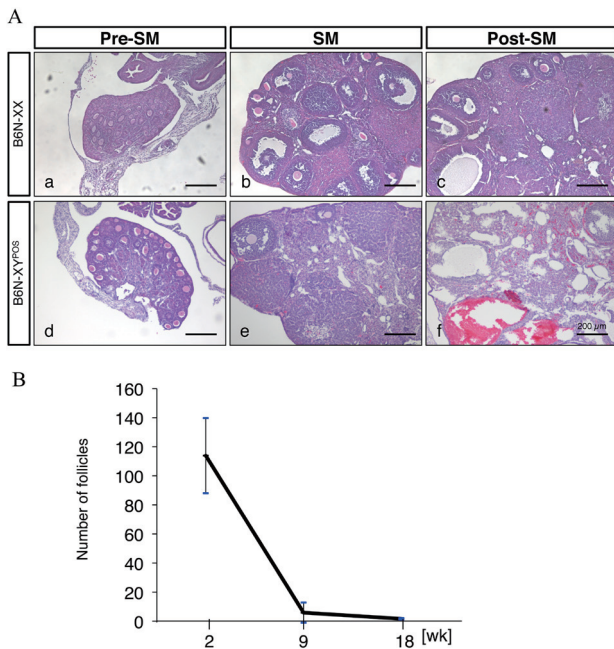
Fig. 3. Representative general histology and number of follicles related to sexual development in O/O individuals. (A) Growing follicles (primordial ~ secondary) are more abundant in the XY^{POS} ovary than in the B6N-XX at pre-SM (2 weeks age) (a and d). These follicles are rare in the SM and post-SM stages (b, c and e, f). Collapsing inner tissues are seen with bleeding (f). (B) The number of follicles at each stage at 2, 9 and 18 weeks of age. Total ovarian follicles in B6N-XY^{POS} rapidly decreased between 2 to 9 weeks of age.

Fig. 4. Immunoreactivity of Nr5a1, Sox9 and Foxl2 of the indicated genotypes in the genital ridges at 11.5 dpc. (A) Immunoexpression of Nr5a1 is detected in the epithelium and internal somatic cells, but not in the germ cells. Nr5a1 immunoexpression shows no difference between B6N-XY^{POS} and B6N-XY^{B6} and also no bilateral difference. (B) Sox9-positive cells (arrowheads) are observed scattered randomly in the B6N-XY^{B6} gonads, but not in the B6N-XX gonads. They are seen with extremely low frequency in the B6N-XY^{POS}. (C) In this stage, no Foxl2-positive cells are seen in both the B6N-XY^{B6} and B6N-XX gonads. However, faint staining is seen in each B6N-XY^{POS} gonad.

day 12 after copulation. In contrast, some of the B6N-XY^{POS} males that had functional seminiferous tubes mated with B6N-XX^{B6}, and most of them carried the pregnancy to full term. To date, no pups have been obtained from B6N-XY^{POS} (O/O individuals).

Sry CDS sequence: To determine whether our B6N-XY^{POS} mouse had a normal *Mus. domesticus*-derived *Sry* allele, we sequenced *Sry^{POS}* CDS and found the same sequence as previously reported for *Sry^{POS}* [1]. While SRY^{B6} is composed of 395 amino acids, SRY^{POS} is composed of 230 amino acids because of the point mutation creating a TAG stop codon in CAG repeats. In addition, the Q-rich domain of SRY^{B6} consists of 21 blocks of glutamine residues, and that of SRY^{POS} consists of 8 blocks.

DISCUSSION

In recent years, not only the sex-determining gene *Sry*, but also some other genes have been identified as important factors involved in sex determination and sex differentiation. It is already known that knocking such genes out causes gonadal sex reversal in mice [45]. For example, XY gonadal sex reversal has been described in cases of homozygous deletion of a number of testis-determining genes, including *Sox9* [5, 8, 28], *Fgf9* [10], *Fgfr2* [4, 25], *Cbx2* (*M33*) [23]
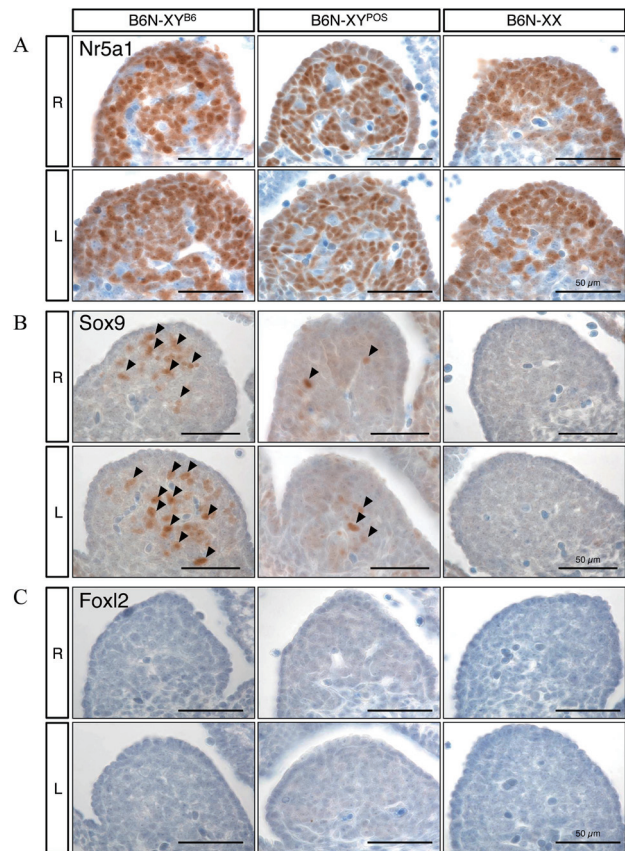
and *Wt1* (+KTS) [20]. A number of additional cases of XY sex reversal in the mouse depend on the presence of a *Mus m. domesticus* Y chromosome and the B6J genetic background: *i.e.*, so-called B6J-XY^{DOM} sex reversal [1, 16]. B6J-XY^{POS} mice carrying a *Mus m. domesticus poschiavinus*-derived Y chromosome show sex reversal, which was one of the B6J-XY^{DOM} sex reversal, and 75% of them develop as females (ovaries) while the remainder develop ovotestes; none develop a testis [14]. However, when we analyzed offspring of B6N-XY^{POS} mice in our laboratory, they had a broad range of gonadal phenotypes that could be divided into four types (T/T, O/O, OT/OT and O/T), and the frequency of the T/T animals, which displayed normal sex differentiation and were fertile, was over 30%. This implies that the B6N-XY^{POS} mice in our laboratory were somewhat different from those reported initially [16].
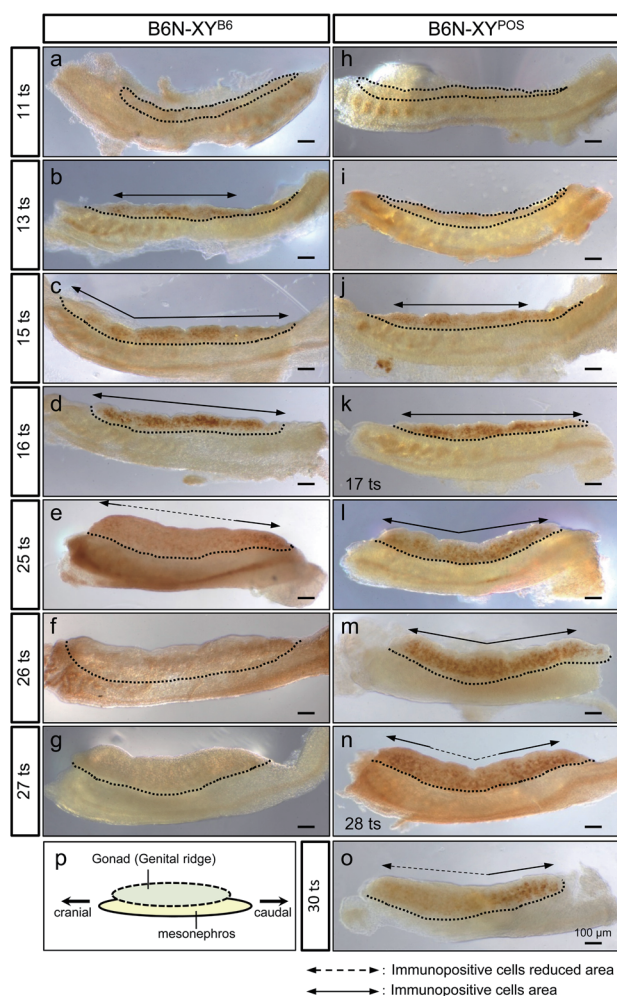
Fig. 5. Spatiotemporal expression of Sry in embryonic urogenital ridges from 11 ts to 30 ts in B6N-XY^{B6}(a–g) and B6N-XY^{POS}(h–o) mice. Dotted lines show outlines of the gonads (genital ridges) in each sample. The comparison of the expression pattern between different Sry alleles reveals an early and brief expression profile in B6N-XY^{B6} mice and a delayed and prolonged expression profile in B6N-XY^{POS} mice. The structures present in the gonad (urogenital ridge) explants are shown (p).

Fig. 6. Spatiotemporal expression of Sox9 expression in embryonic urogenital ridges from 15 ts to 30 ts in B6N-XY^{B6}(a–g) and B6N-XY^{POS}(h–n) mice. Dotted lines show outlines of the gonads (genital ridges) in each sample. Sox9 expression was delayed in B6N-XY^{POS} mice; the staining observed in the B6N-XY^{POS} gonads at the 16 and 17 ts stages (h and i) shows a delay of 2 ts compared to the B6N-XY^{B6} mice. In addition, no increase of Sox9 positive cells was observed at either acral region even after 22 ts (l–n).

Accordingly, *Sry* CDS sequence analysis was carried out to investigate whether our B6N-XY^{POS} mouse has a different *Sry* allele from *Mus m. domesticus poschiavinus*. However, the results showed that our *Sry*^{POS} was identical to that reported previously [1], which means that they have the same published *Mus m. domesticus poschiavinus*-derived *Sry* allele. This suggests that the gonadal phenotypic divergence between our present data and the results published previously was not due to the *Sry* CDS.

Sry expression expands from the central region to the poles along the anteroposterior axis of the XY gonad [6, 40], and testis cord formation subsequently proceeds in a similar gradient manner [39]. The study of Bullejos and Koopman (2005) [7] clearly indicates that a delay of expression of
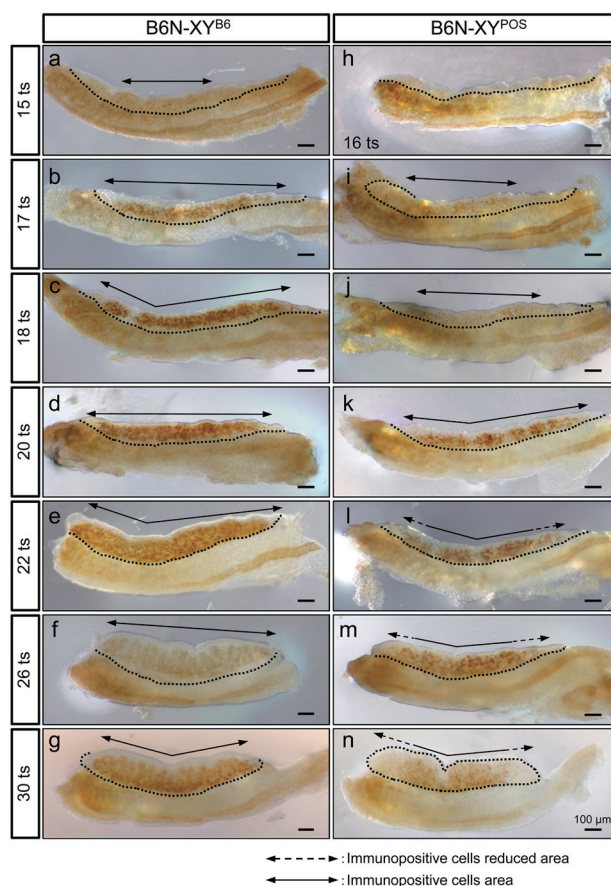
Sry^{DOM} relative to B6J-*Sry* underlies B6J-XY^{DOM} sex reversal, and *Sry* must act during a critical time window to appropriately activate *Sox9* and effect male sex determination before the onset of the ovarian-determining pathway. Their findings also showed that the expression of *Sry*^{POS} reached a peak level only after the expression of *Sry*^{B6} had all but ceased; this represents a delay of 4 ts (approximately 9.6 hr), and the onset of *Sox9* expression is delayed by a period corresponding to 6 ts (approximately 14.4 hr) compared with B6J-XY^{B6}. In contrast, the spatiotemporal expressions of Sry and Sox9 examined using whole-mount immunohistochemistry in the present study showed that the onsets of both Sry and Sox9 expressions in the B6N-XY^{POS} gonads were 2–3 ts later than those in B6N-XY^{B6} gonads. These results suggest that the onset of Sry^{POS} expression is earlier than that reported previously, that is, our Sry^{POS} expression seemed more similar to that in B6N-XY^{B6} gonads. Furthermore, it is possible that such a small difference in timing of

the Sry expression underlies testicular development in our B6N-XY^{POS} mice. At the same time, even at 30 ts B6N-XY^{POS} gonads, Sox9-positive cells were limited at the central region and were not seen at the cranial and caudal regions, which results in no formation of testis cords at both acral regions. *Sox9* expression normally persists throughout life in Sertoli cells, suggesting that Sox9 executes and maintains their differentiation and aspects of their function [39]. It suggests that the failure of expressing Sox9 at both acral regions of the gonad leads to the formation of ambiguous gonads seen in OT/OT individuals of B6N-XY^{POS}. Actually, it is mentioned that testicular tissue in ovotestis was invariably concentrated in the mid region of the gonad, with ovarian tissue distributed at the cranial and caudal poles [22].

Most studies on XY^{POS} mice have not focused on histological and immunohistological findings, so we provide indication of normality or abnormality of adult tissues (pre-SM, SM and post-SM) in detail. In T/T individuals, the testes showed normal cell arrangement and sperm formation, which is the histological evidence supporting fertility in our XY^{POS} male mice. On the other hand, the gonads in O/T individuals showed abnormal structures, especially in the testes, where the seminiferous tubular structures had abnormal vacuoles and no sperm. The pre-SM testes showed Foxl2-positive oocyte-like cells, but without a pellucid zone or follicular epithelium. Foxl2, a forkhead transcription factor, is expressed in fetal and adult ovarian follicular cells and involved in ovarian development and maintenance [9]. Testicular oocytes are also observed in MRL/MpJ XY mice with fertility, although they are surrounded by a pellucid zone and follicular epithelium unlike in the present case [36]. The ovaries in our O/T individuals had quite small follicles, and Sox9-positive cells were observed in the seminiferous tubule-like structures. Our studies are the first to histologically show the expression and localization of a female-related gene in the B6N-XY^{POS} testes and a male-related gene in the B6N-XY^{POS} ovaries. The ectopic expressions of such transcriptional factors as Foxl2 in the testes and Sox9 in the ovaries suggest that the normal differentiation system in B6N-XY^{POS} gonads is disturbed due to an imbalance between the male and female pathway since FOXL2 and SOX9 oppose each other's actions and thereby ensure the establishment and maintenance of the different female and male supporting cell types, respectively [43].

We have indicated that delayed Sry and Sox9 expressions disrupt normal testicular development, as described previously [7]. However, in the B6N-XY^{POS} gonads, Nr5a1 expression, which regulates Sry expression, was at the same level as in the B6N-XY^{B6} gonads. Several studies suggest that the C57BL/6J background is sensitized to disruptions to testis determination due to the relatively delayed expression of testis-determining genes and higher levels of expression of ovary-determining genes; in addition, in most sex-reversing mouse mutants examined, B6J increases the amount of ovarian tissue that forms in mutant XY embryos [11, 34, 44]. Therefore, there may be some interactions between autosomal factors specific to the B6J background and the *domesticus*-derived Y chromosome.

Let us consider these factors individually. First, there is the issue of the C57BL/6 background. Because testicular tissue develops in DBA/2J or 129S1/SvImJ (129) mice containing the *poschiavinus*-derived Y chromosome, sex reversal in B6J-XY^{POS} mice is attributable to the genetic background of B6J [15]. Previous studies of gene expression within B6J and another non-sex-reversing strain, 129S1, in developing testis showed that, compared to 129-XY^{129} gonads, B6J-XY^{B6} gonads had a higher average expression of "female"-related genes and a lower expression of "male"-related genes [34, 35]. Moreover, the B6 mouse, a common inbred strain of laboratory mouse, is classified into *Mus m. musculus*. However, commonly used classical inbred mouse strains have mosaic genomes with sequences from different subspecific origins, and with regard to B6, their genomes are derived predominantly from the Western European subspecies *Mus m. domesticus*, with the remaining sequences derived mostly from the Japanese subspecies *Mus m. molossinus* [42]. *Molossinus*-derived autosomal factors in B6 may provide sensitivity to sex reversal, because some other inbred strains, such as AKR, that are classified into *Mus m. domesticus* do not show sex reversal [7]. Furthermore, although they derive from the same B6 strains, B6J and B6N are genetically and phenotypically different because their breeding stocks have been maintained separately for a long period of time, allowing genetic differences to accumulate due to individual variety and genetic drift [30, 49]. At the beginning of our breeding, B6J-XX were mated to B6J-XY^{POS} Tg (129) mice over five generations, with the result that only individuals with ovaries or hermaphrodites were produced, which is the same result as reported in 1982. However, B6N-XX were mated to B6J-XY^{POS} Tg (129) mice subsequently, and the frequency of their progeny was the subject of this paper. Thus, it was suggested that the difference between B6J and B6N may be responsible for the phenotypes in our B6N-XY^{POS} mice.

Secondly, we would like to focus attention on the epigenetic factors upstream of *Sry*. Jumonji domain-containing 1a (Jmjd1a) (also called Tsga/Jhdm2a/Kdm3a) acts as a H3K9 demethylase, and *Jmjd1a*-deficient mice frequently show XY sex reversal [26]. The phenotypes of these animals have a broad range that is quite similar to that of our B6N-XY^{POS} mice. Jmjd1a directly and positively controls *Sry* expression by regulating H3K9me2 marks, which reveals that certain epigenetic factors, such as histone demethylation, have an important role in mammalian sex determination. Interestingly, the generation and comparison of XY *Jmjd1a*-deficient mice, which carried the Y chromosome from either CBA (Y^{CBA}) or B6 (Y^{B6}) on a B6N autosomal background, revealed that the sex-reversal phenotype was dependent on not only the loss of *Jmjd1a* but also the genetic origin of the Y chromosome combined with the B6 background [26]. In the case of the XY female in humans, without a functional mutation in *SRY*, pericentric inversion of the Y chromosome causes XY females: if *SRY* is inverted and relocated to the center of the heterochromatic region, this positional variegation will lead to *SRY* gene silencing, inducing XY female development [17, 31, 32]. These facts indicate that, even if

Sry is intact, the interaction between *Sry* and other genes will cause sex reversal because *Sry* expression is regulated in a strictly temporal and spatial manner.

Finally, a recent study on B6J-XY[POS] mice showed that the non-coding congenic region on chromosome 11 (110 region) that protects against B6J-XY[POS] sex reversal is located within the *Sox9* promoter and promotes the expression of *Sox9*, thereby driving testis development within the B6J-Y[POS] background. B6J-XY[POS] males which we originally believed to be "XY[POS]" were heterozygous for this 110 region derived from *Mus m. domesticus poschiavinus*, and heterozygosity for this region was sufficient to prevent the formation of complete embryonic B6J-Y[POS] ovaries. However, heterozygosity for the 110 region does not result in fully normal embryonic testis, with the majority of gonads showing some degree of ovarian formation [3]. This suggests that the *Sox9* promoter region of our B6N-XY[POS] mice has a similar congenic region that protects against B6J-XY[POS] sex reversal to a certain degree. By contrast, our B6N-XY[POS] progeny show a broad range of gonadal phenotypes even in littermates, and the frequency of T/T progeny is as high as that of O/O progeny. Our B6N-XY[POS] mice may have a similar autosomal non-coding region that prevents sex reversal in a dose-dependent manner. Therefore, further experiments will be needed to analyze the promoter regions of *Sry* and *Sox9* or the expression of sex-related genes affected by epigenetic regulations.

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