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Substrain differences in Sry expression at the stage of sex determination in C57BL/6 mouse strains

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ABSTRACT. The expression of *sex determining region of the Y chromosome* (*Sry*) in the fetal gonads is important for male development. In a mouse model of disorders of sex development (C57BL/6 (B6)-XY^{POS}), the gonadal phenotype and the timing of *Sry* expression differ due to differences among B6 substrains as the genetic background. Since differences in *Sry* expression among B6 substrains have been speculated, the present study examined *Sry* expression in B6J, B6JmsSlc, and B6NCrl mice. These substrains differed in the number of *Sry*-expressing cells in the gonads of embryonic mice at each developmental stage, with B6NCrl having more than the other strains. The substrains differed also in the number of *Sry*-expressing cells between the left and right gonads, with B6J and B6NCrl, but not B6JmsSlc, showing left gonad-dominant *Sry* expression. Substrain differences existed also in the distribution of *Sry*-expressing cells in the medial and lateral directions of gonads. In addition, in the left gonad-dominant *Sry*-expressing substrains B6J and B6NCrl, the medial and central regions of the left gonad had more *Sry*-expressing cells than those of the right gonad. Substrains of B6 mice have not always been considered in sex differentiation studies. In the present study, however, we observed substrain differences in the number of *Sry*-expressing cells, left-right distribution, and medial/lateral distribution during the early stages of gonadal development in B6 mice. Therefore, future studies on sex differentiation in B6 mice should consider substrain differences.

KEYWORDS: C57BL/6, sexual differentiation, *Sry*, substrain differences in B6 mice

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The sex of mammals is determined by their chromosomes, with the XX sex chromosome being female and the XY being male. The reproductive system originates from the same gonadal primordium in both sexes and differentiates into testes in males and ovaries in females [38]. *Sex determining region of the Y chromosome* (*Sry*) plays an important role in male development [19, 20, 36]. *Sry* activates the transcription factor *SRY* (*sex determining region Y*-box 9 (*Sox9*)) and promotes testis formation by inducing the differentiation of supporting cell progenitors in the bipotential undifferentiated gonad into testis-specific Sertoli cells [35, 46]. *Sry* shows characteristic expression, spatially and temporally regulated, and expressed only in the supporting cell progenitors in the XY fetal gonad. *Sry* expression in mice begins in the central part of the gonad at the 11 ts (tail somite) stage and spreads throughout the gonad by 18 ts, reaching its peak expression level [15]. Thereafter, it fades from the middle to the cranial and caudal ends of the gonad and is almost undetectable by 30 ts [5, 6, 39, 45]. Sufficient *Sry* expression at 13 to 15 ts, corresponding to 11.0 to 11.25 dpc (days post-coitum), is required for Sertoli cell differentiation and testis cord formation to occur in the entire testis.

Previously we found that C57BL/6NCrSlc (B6NCrSlc) and Slc:ICR (ICR) mice have large individual differences in the early stages of *Sry* expression between the medial and lateral directions and that *Sry* expression in the left gonad is preceded in B6NCrSlc (Submitted). This left gonad-dominant *Sry* expression was not observed in the ICR mice, suggesting that it is a phenomenon unique to the B6 strain or to the B6NCrSlc substrain.

The B6 strain is an inbred mouse used in a variety of studies, including those on sex differentiation. The B6 strain was introduced into the Jackson Laboratory (Jackson Lab) in 1948 and distributed to the National Institutes of health (NIH) in 1951. Subsequently, the B6J substrains at Jackson Lab and the B6N substrains at NIH became the two major substrains, which branched into many substrains as they were maintained at various institutions [28]. Many phenotypic and genotypic differences among B6 substrains have been

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reported [29, 30, 49]. These include differences in behavior, alcohol tolerance, and glucose tolerance that cannot be ignored [4, 28], but many reports fail to identify the substrains of the B6 mice being studied [1, 7, 13]. B6 substrains are also not always considered in sex differentiation studies.

In fact, it has been reported that the timing of Sry expression and the gonadal phenotype are different in B6-XY^{POS} mice, a model of disorders of sex development (DSD) with a B6 strain background, due to the different genetic background among B6 substrain [10, 11, 42, 47]. XY^{POS} mice with the background of the Jackson Lab strain, which is the B6J substrain, have been reported to form ovaries or ovotestes [10, 11]. In addition, its Sry expression is reported to be delayed by 4 ts [6]. On the other hand, B6-XY^{POS} mice with the B6NCrSlc genetic background had delayed Sry expression by 2–3 ts, and some individuals formed testes in addition to ovaries and ovotestes [42]. In B6-XY^{POS} mice in which the genetic background was replaced by B6JJmsSlc, ovaries formed in all individuals [47]. Based on these differences in gonadal phenotype among substrains, we hypothesized that there are differences in Sry expression among substrains even in wild-type B6 mice. In the present study, we investigated Sry expression in B6J and B6JJmsSlc from the B6J substrains and B6NCrI from the B6N substrains by quantitative histology to determine whether there are differences in Sry expression among the B6 substrains.

MATERIALS AND METHODS

Animals

B6J mice and B6NCrI mice were purchased from Jackson Lab Japan (Yokohama, Japan). B6JJmsSlc mice were purchased from SLC Japan (Hamamatsu, Japan). The mice were then maintained as described elsewhere [42]. Male fetuses at 11.0 dpc were collected immediately after euthanasia, which was accomplished under deep anesthesia with isoflurane. Noon of the day on which the mating plug was observed was designated as 0.5 dpc. Ts were counted as described previously to ensure the accurate estimation of developmental stages [15]. Embryos at 11.0 dpc were sexed by multiplex polymerase chain reaction (PCR) as described previously [42]. This study was approved by the Institutional Animal Care and Use Committee (Permission #29-05-02, #2022-07-01) and carried out according to the Kobe University Animal Experimental Regulations.

Histological analysis

The gonadal regions were fixed in 4% paraformaldehyde at 4°C for 24 hr. The specimens were dehydrated with an ethanol series followed by xylene and then embedded in paraffin. Then, 5- μ m-thick serial sections were cut by a sliding microtome and placed on slide glasses that had been precoated with 2% 3-aminopropyltriethoxysilan (Shin-Etsu Chemical, Tokyo, Japan) and stored at –18°C until use.

Immunohistochemical staining was carried out as described previously [42]. The primary antibody used was anti-Sry guinea pig polyclonal antibody (1:32,000; a generous gift from Prof. M. Tachibana, Osaka University, Japan). The secondary antibody was peroxidase-conjugated donkey anti-guinea pig IgG (1:200; 706-035-148, Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Analysis of Sry-expressing cells

Sry-expressing cells were counted every 10 μ m to prevent duplication of cell counts. The gonads were divided into three regions perpendicular to the midline, and the Sry-expressing cells were counted in each region (Fig. 1A). The Steel-Dwass test was used for statistical analysis to compare the numbers of Sry-expressing cells from substrain to substrain. The left and right gonads were considered to differ if the gonad with more Sry-expressing cells had at least 10 more than the other gonad and if the difference was more than 5% of the number of Sry-expressing cells in the dominant gonad. Statistical analysis was performed using the Wilcoxon signed-rank test to determine the tendency of left–right differences in each substrain. The caudal region of the gonads was curved, making it difficult to accurately divide the gonadal region. Therefore, the number of Sry-expressing cells in the 2/3 range from the head of the gonads was used to compare the numbers of Sry-expressing cells in the medial, lateral, and central regions of the gonads. The Wilcoxon signed-rank test was also used to compare the numbers of Sry-expressing cells in the medial, central, and lateral regions between the left and right gonads. In this case, *P* values were corrected using the Bonferroni method. When comparing left–right differences in the composition ratio of Sry-expressing cells in each region, the number of Sry-expressing cells in each individual was corrected to the mean of the gonads so that differences in cell numbers would not affect the statistical results. The values were used in a χ^2 test and in a residual analysis. A *P*-value of less than 0.05 (Steel-Dwass test, χ^2 test, and Wilcoxon signed-rank test) or less than 0.017 ($\approx 0.05/3$, Wilcoxon signed-rank test with the Bonferroni method) was considered statistically significant.

RESULTS

Differences in the total number of Sry-expressing cells in the gonads

Immunostaining for Sry revealed Sry-expressing cells scattered within the gonads (Fig. 1A). Immunoreactivity to Sry antibodies was observed in the nucleus and cytoplasm (Fig. 1A magnified view). The Sry-expressing cells in the entire gonad were counted and compared between 11 ts and 13 ts for each substrain. All substrains showed significantly greater variance in the number of Sry-expressing cells at 13 than at 11 ts (Supplementary Fig. 1). In addition, individuals not expressing Sry were observed in B6J and B6JJmsSlc at 11ts (Fig. 1A and 1B, Supplementary Fig. 1). Furthermore, in substrains other than B6NCrI, several individuals had fewer Sry-expressing cells at 13 ts than other individuals had at 11 ts.

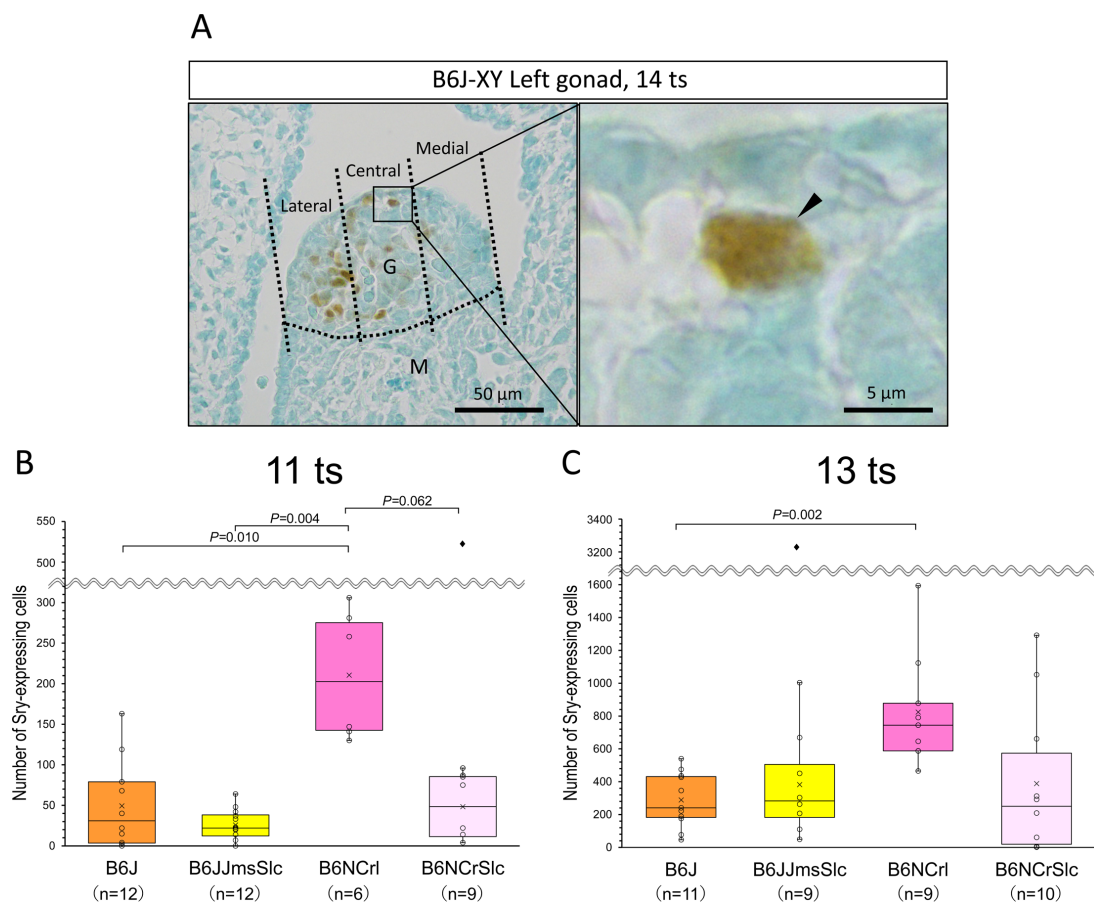


Fig. 1. Transverse section of the left gonad of a fetus (A). The gonad (G) was divided into medial, central, and lateral regions, and the positive cells (arrowheads) were counted (A). Comparison of the number of Sry-expressing cells in each substrain at 11 tail somite (ts) and 13 ts (B, C). At 11 ts, B6NCrI had significantly more Sry-expressing cells than B6J or B6JJmsSlc and tended to have more than B6NCrSlc (B). At 13 ts, B6NCrI had significantly more Sry-expressing cells than B6J (C). The data of B6NCrSlc are from our previous study (Submitted). Counterstain: toluidine blue. Rhombus: outlier, M: mesonephros, Steel-Dwass test; $P < 0.05$. ×: mean.

The numbers of Sry-expressing cells among the substrains were compared at 11 ts and at 13 ts. At 11 ts, B6NCrI had significantly more Sry-expressing cells than B6J and B6JJmsSlc and tended to have more Sry-expressing cells than B6NCrSlc (Fig. 1B). At 13 ts, B6NCrI had significantly more Sry-expressing cells than B6J (Fig. 1C). These results indicated that the substrains differed in the number of Sry-expressing cells at each developmental stage.

Left–right differences in the number of Sry-expressing cells

The effect of gonadal volume on left–right differences in the number of Sry-expressing cells was examined. As a result, the differences in both the number and density of Sry-expressing cells showed almost the same tendency (Supplementary Table 1). Therefore, only the numbers of Sry-expressing cells in the gonads were compared in this study.

In B6J mice (11–15 ts, $n=32$), 11 individuals had more Sry-expressing cells in the left gonad, 11 had equal numbers between left and right, and 8 had more in the right ($L > R: 11$, $L \approx R: 11$, $L < R: 8$; Fig. 2A). Sry expression was not observed in 2 of the individuals at 11 ts. Analysis of the relationship between the number of Sry-expressing cells in the gonads and the left–right difference revealed that in individuals with fewer than 100 total Sry-expressing cells, Sry was dominantly expressed in the right gonad ($L > R: 0$, $L \approx R: 6$, $L < R: 4$; $P=0.021$; Fig. 2A). On the other hand, individuals with more than 100 Sry-expressing cells tended to express Sry dominantly in the left gonad ($L > R: 11$, $L \approx R: 5$, $L < R: 4$; $P=0.059$; Fig. 2A). Moreover, Sry was expressed dominantly in the left gonad in individuals containing more than 200 Sry-expressing cells ($L > R: 9$, $L \approx R: 2$, $L < R: 2$; $P=0.071$; Fig. 2A). Thus, as the number of Sry-expressing cells increased, Sry tended to be expressed dominantly in the left gonad.

In B6JJmsSlc mice (11–14 ts, $n=25$), 7 individuals had more Sry-expressing cells in the left gonad, 11 had equal numbers between left and right, 5 had more in the right ($L > R: 7$, $L \approx R: 11$, $L < R: 5$; Fig. 2B). Two individuals did not express Sry at 11 ts. Analysis of the relationship between the number of Sry-expressing cells in the gonads and the difference in that number between the left and right gonads showed that individuals with fewer than 100 Sry-expressing cells had equal numbers between left and right ($L > R: 2$, $L \approx R: 9$, $L < R: 0$; Fig. 2B). Similarly, individuals with more than 100 cells showed no difference in the number of Sry-expressing cells between left and right ($L > R: 5$, $L \approx R: 2$, $L < R: 5$; Fig. 2B). Thus, B6JJmsSlc did not show a specific tendency in Sry expression between left and

right gonads.

In B6NCrl mice (11–14 ts, $n=23$), all individuals had more than 100 Sry-expressing cells. Of the 23 individuals, 22 had more Sry-expressing cells in the left gonad, with the remaining individual having more in the right ($L>R:22$, $L\approx R:0$, $L<R:1$; $P<0.001$; Fig. 2C). Thus, in B6NCrl, Sry was expressed dominantly in the left gonad.

Medial and lateral distributions of Sry-expressing cells

The gonads were divided into three regions in the medial and lateral directions (Fig. 1A), and the numbers of Sry-expressing cells in the medial and lateral regions were compared. Some of the individuals with fewer than 10 Sry-expressing cells contained more Sry-expressing cells in the medial region than in the lateral. However, most of the other individuals contained more Sry-expressing cells in the lateral region than in the medial (Fig. 3). This tendency was observed in B6J, B6JJmsSlc, and B6NCrl.

Regardless of the substrain, in the medial region the left gonad tended to have more Sry-expressing cells than the right. The number of Sry-expressing cells in the medial region relative to that in the lateral was also higher in the left gonad (Fig. 3).

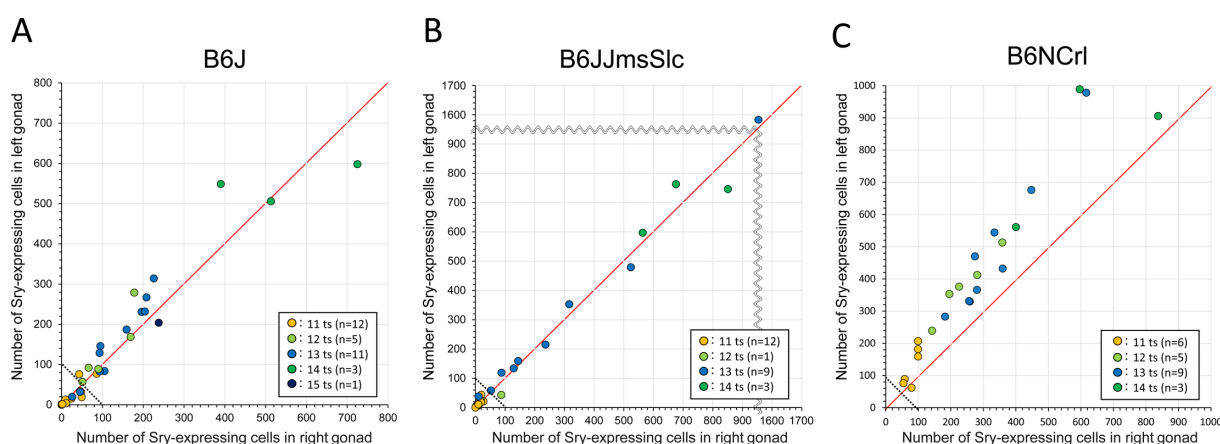


Fig. 2. Scatterplots of left–right differences in the number of Sry-expressing cells of individuals in each substrain (A–C). The numbers of Sry-expressing cells in the right and left gonads are plotted on the horizontal and vertical axes, respectively. The red line indicates that the left and right gonads have equal numbers of Sry-expressing cells. The dotted line indicates that the total number of Sry-expressing cells is 100. In B6J, when the total number was less than 100, the right gonad tended to have more Sry-expressing cells. As the total number of Sry-expressing cells increased, the number of individuals with more positive cells in the left gonad increased (A). In B6JJmsSlc, regardless of the total number of Sry-expressing cells, no specific trend was observed between the left and right gonads (B). In B6NCrl, all individuals had more than 100 Sry-expressing cells, and all but one had more Sry-expressing cells in the left gonad (C).

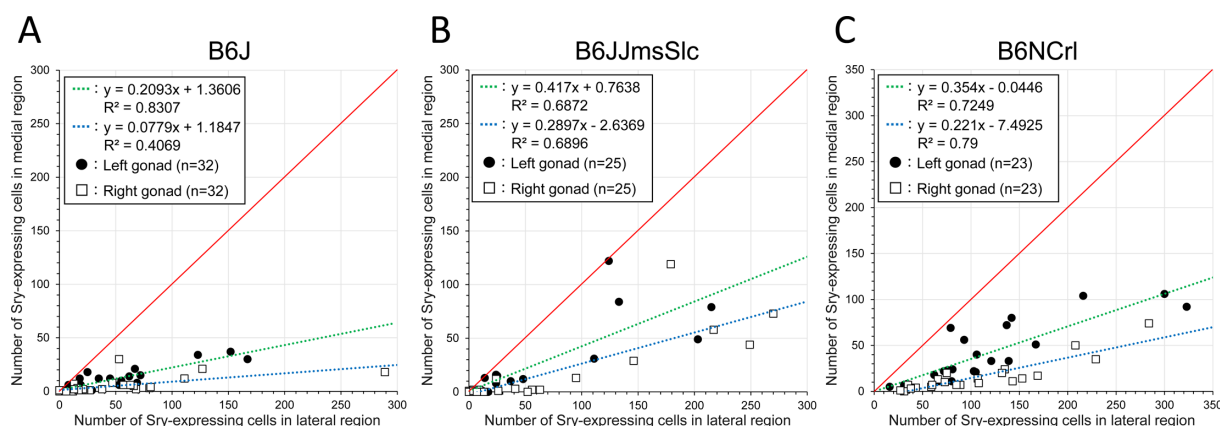


Fig. 3. Scatterplots of the numbers of Sry-expressing cells in the medial and lateral regions of the gonads in each substrain (A–C). The numbers of Sry-expressing cells in the lateral and medial gonads are plotted on the horizontal and vertical axes, respectively (B6J: 11–15 tail somite (ts), B6JJmsSlc: 11–14 ts, B6NCrl: 11–14 ts). In all three substrains, the lateral region contained more Sry-expressing cells than the medial in most individuals. The slope of the approximate line for the left gonad was greater than that for the right gonad. Green dotted line: approximate straight line of left gonad. Blue dotted line: approximate straight line of right gonad.

Left–right differences in the medial and lateral distributions of Sry-expressing cells

In each substrain, left–right differences in both the number and composition ratio of Sry-expressing cells in the medial, central, and lateral regions of the gonads were compared in individuals containing at least 100 Sry-expressing cells. In B6J, there was no difference in the number of Sry-expressing cells in the lateral region between the left and right gonads. In the central and medial regions, on the other hand, the left gonad had more Sry-expressing cells (Fig. 4A). There was no significant difference between the left and right gonads in the composition ratio of Sry-expressing cells in each region (Fig. 4D).

In the lateral region of B6JJmsSlc, the right gonad had more Sry-expressing cells than the left, whereas the reverse was true in the medial region. In the central region, B6JJmsSlc showed no significant difference in the number of Sry-expressing cells, unlike the case with B6J (Fig. 4B). The composition ratio of Sry-expressing cells in the lateral region was significantly higher in the right gonad, while the ratios in the medial and central regions were significantly higher in the left (Fig. 4E).

In B6NCrl, there was no difference between the left and right gonads in the number of Sry-expressing cells within the lateral region. On the other hand, in the central and medial regions the left gonad had more Sry-expressing cells (Fig. 4C). This tendency was similar to, but more pronounced than, that in B6J. The composition ratio of Sry-expressing cells in the lateral region was significantly higher in the right gonad, whereas that in the medial region was significantly higher in the left (Fig. 4F).

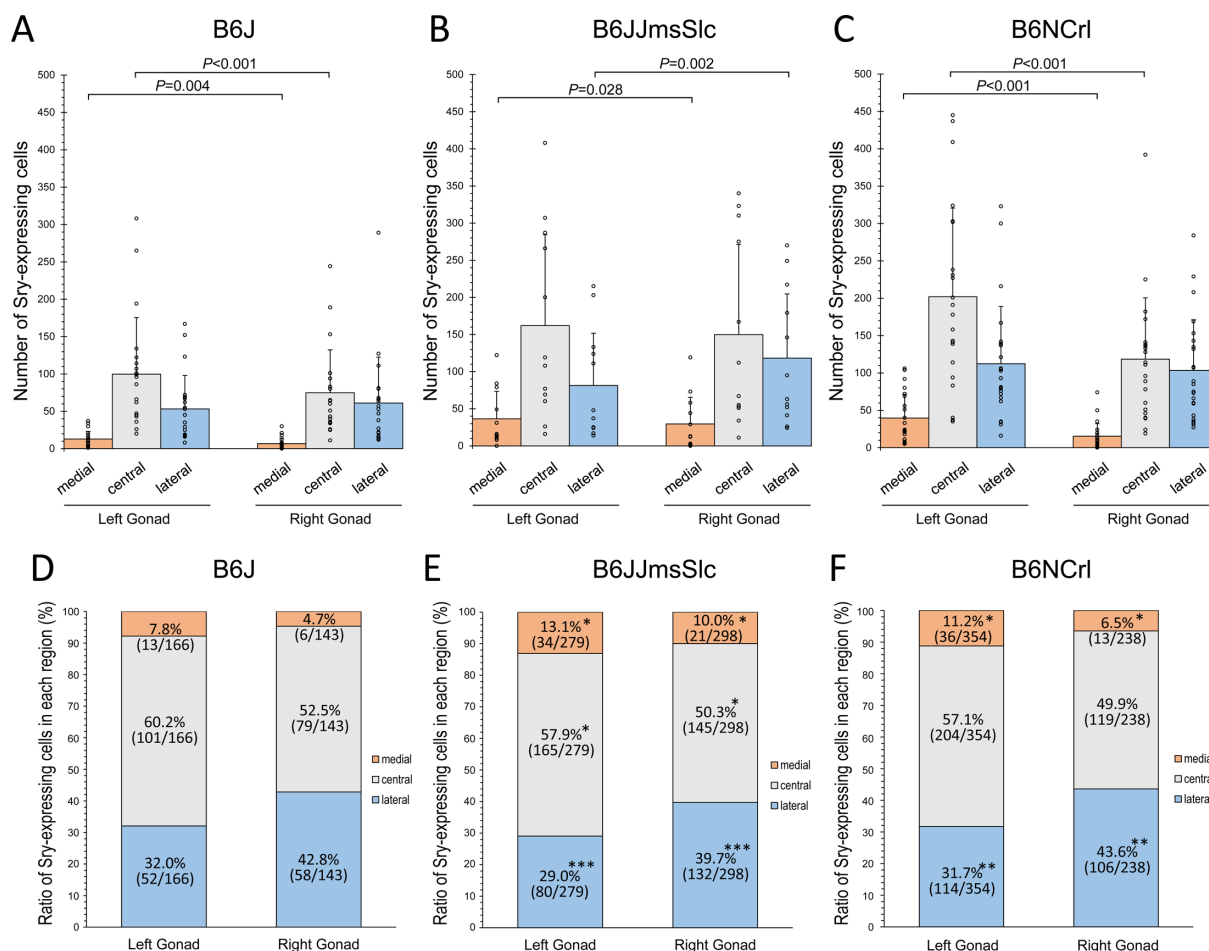


Fig. 4. The numbers of Sry-expressing cells in the medial, central, and lateral regions of the left and right gonads (A–C) and the composition ratio of Sry-expressing cells in each region in the gonads (D–F). Individuals containing more than 100 Sry-expressing cells were analyzed (B6J: 11–15 tail somite (ts), n=20; B6JJmsSlc: 12–14 ts, n=12; B6NCrl: 11–14 ts, n=23). In B6J, the left gonad contained more Sry-expressing cells in the central and medial regions (A). There was no significant difference between left and right gonads in the composition ratios of Sry-expressing cells in each region (D). In B6JJmsSlc, there were more Sry-expressing cells in the lateral region in the right gonad, whereas in the medial region there were more in the left gonad (B). The composition ratio of Sry-expressing cells in each region differed significantly between the left and right gonads (E). In the central and medial regions of B6NCrl, the left gonad had more Sry-expressing cells (C). The composition ratio of Sry-expressing cells in the medial and lateral regions differed significantly between the left and right gonads (F). A P-value of less than 0.017 ($\approx 0.05/3$, Wilcoxon signed-rank test, A–C) or less than 0.05 (χ^2 test and residual analysis, D–F) was considered statistically significant. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Values are means + SD.

DISCUSSION

All B6 substrains showed a significant increase in variance in the number of Sry-expressing cells in each individual at 13 ts compared to 11 ts (Fig. 1B and 1C, Supplementary Fig. 1). Sry expression in the fetal gonads begins in the central part of the gonads at 11 ts, which is around a fetal age of 11.0 dpc, and rapidly spreads to the entire gonads by around 18 ts, around 11.5 dpc [15]. The rapid increase in Sry-expressing cells may have amplified small differences in the degree of development between individuals, resulting in a large variance in the number of Sry-expressing cells at 13 ts.

The method utilizing tail somites proposed by Hacker *et al.* (1995) is widely used in sex differentiation studies to determine the developmental stage around 10.5–12.5 dpc. It is more accurate than the traditional Theiler stages because tail somites increase by one every 2–3 hr [40]. However, several individuals of B6J and B6JJmsSlc had no Sry-expressing cells at 11 ts. In addition, some individuals had fewer Sry-expressing cells at 13 ts than other individuals had at 11 ts (Fig. 1B and 1C, Supplementary Fig. 1). These results demonstrated large individual differences in the onset time of Sry expression. Therefore, it should be noted that the developmental stage estimated from the number of tail somites may not always correspond to the degree of testis differentiation in the undifferentiated gonads. Because the B6 strain is inbred, mice within the same substrain are considered genetically homogeneous. On the other hand, it has been reported that the B6 strain is more unstable than other strains in sexual differentiation under certain conditions [9, 34]. For example, different gonadal phenotypes have appeared from the same genetic background when *Mus. musculus Domesticus*-type Sry, which has low stability, was replaced, such as B6-XY^{POS} and B6-XY^{TIR} [2, 3, 8, 24, 41, 47]. In contrast, other strains, such as DBA/2J (D2) and 129S1/SvJ (129S), are resistant to the disturbance of sexual differentiation by *M. m. Domesticus*-type Sry [12, 26]. In addition, B6 mice showed different gonadal phenotypes when the epigenetic status was disturbed by *Jumonji domain-containing 1a* (*Jmjd1a*, *Kdm3*) knockout [22]. Taking these previous and present results together, we speculate that B6 mice are prone to individual differences in the process of sexual differentiation.

When we examined the substrain differences in the number of Sry-expressing cells, we found that B6NCrI had more than the other substrains at 11 ts and, at 13 ts, significantly more than in B6J (Fig. 1B and 1C). These results suggest that either the onset of Sry expression or the rate of increase of Sry-expressing cells, or both, differ among the substrains. In B6J, B6JJmsSlc, and B6NCrSlc, a few individuals did not express Sry at 11 ts, while in B6NCrI, all individuals had more than 100 Sry-expressing cells. This suggests that Sry expression in B6NCrI started earlier and was elevated more than in the other three substrains.

Moreover, the substrains differed in their Sry expression tendencies between left and right gonads (Fig. 2A–C). The tendency of Sry expression in B6NCrI was similar to that in B6NCrSlc (Submitted) but different from those of B6J and B6JJmsSlc. Various phenotypic and genotypic differences between the B6J and B6N substrains have been reported [28]. For example, some B6J substrains have abnormal glucose tolerance and insulin secretion levels due to a defect in the *Nnt* gene [14, 17, 41]. In addition, nonsense mutations in the *Crb1* gene have been observed only in B6N substrains [25, 27]. Many other phenotypic differences between the B6J and B6N substrains have also been reported [28]. These results suggest that the B6J and B6N substrains may differ distinctly in their Sry expression tendencies between left and right gonads. Moreover, in addition to defects in the *Nnt* gene, mutations and defects in other genes and many SNPs have been reported within the B6J substrains [28, 37]. The differences in Sry expression between B6J and B6JJmsSlc in this study suggest that differences in gene sequences between the two substrains may have affected Sry expression [29]. Furthermore, many SNPs correlating with branching time have been reported in the B6N substrain [30]. The number of Sry-expressing cells was higher in B6NCrI than in B6NCrSlc (Fig. 1B and 1C), presumably due to differences in gene sequence.

In the three substrains in the present study, the lateral region of the gonads contained large numbers of Sry-expressing cells (Figs. 3 and 4). From this result, it is speculated that dominant Sry expression in the lateral region of the gonads may be common in B6 strains and even in many mice. On the other hand, the difference in the distribution of Sry-expressing cells between the left and right gonads (Fig. 4) suggested that the left gonad-dominant Sry expression observed in B6J and B6NCrI is caused by the difference in the number of Sry-expressing cells in the medial and central regions of the gonads. This tendency was especially more pronounced in B6NCrI than in B6J (Fig. 4). Even in B6JJmsSlc, which did not show any tendency in Sry expression between the left and right gonads, the lateral region had more Sry-expressing cells than the medial region. However, the number of Sry-expressing cells within the lateral region and the composition ratio of each region in B6JJmsSlc showed different trends from the other two substrains. In B6JJmsSlc, the right gonad had more Sry-expressing cells in the lateral region or the left one had fewer. As a result, the number of Sry-expressing cells in the gonads was almost equal between the sides. The mechanisms underlying these differences are unknown but may be due to differences in gene sequences among the B6 substrains.

Sry expression is directly regulated by transcription factors such as WT1 transcription factor (Wt1); GATA binding protein 4 (Gata4); nuclear receptor subfamily 5, group A, member 1 (Nr5a1/Sf1/Ad4bp) [23, 31]; and other transcription factors. Other MAPK signals, consisting of mitogen-activated protein kinase kinase kinase 4 (Map3k4), p38 α , and p38 β , also activate Gata4 [43]. It has also been reported that Gadd45g, which is involved in the regulation of Gata4, shows wave-like expression in the cranial–caudal axis of the gonad, similar to that of Sry, and is required for the proper initiation of Sry expression [43, 44]. Based on the substrain differences in Sry expression observed in this study, we assume that there are substrain differences in the expression of factors involved in Sry expression. On the other hand, the detailed mechanisms underlying the heterogeneous expression of Sry, such as the left-dominant Sry expression observed in B6J and B6NCrI and the change in the left–right trend observed in B6J, remain unclear. Factors such as *Nodal*, *Lefty*, and *Pitx2* are involved in the formation of the left–right axis during embryogenesis [16, 33]. *Pitx2* is reported to be involved in asymmetric gonad formation in birds [18, 48]. However, no such differential expression has been reported in mammalian gonads. The present study showed the existence of both a substrain with strong left–right differences in Sry expression in the gonads (B6NCrI) and a substrain without such differences (B6JJmsSlc). Substrains with distinct left–right differences, such as B6NCrI, may

be useful for studying the mechanisms that generate left–right differences in sexual differentiation.

B6 mice have been used in many studies, and various non-negligible phenotypic differences among B6 substrains have been reported, as mentioned above [21, 28, 32]. However, according to Åhlgren and Voikar (2019), 39.5% of researchers at a Finnish research institute did not consider the importance of substrain differences, and 26% did not know which B6 substrain they had been using [1]. Moreover, among reports published in *Diabetes* between 2010 and 2014, 58% of those that used genetically modified mice had incomplete descriptions of the mouse strains as the genetic background [13]. Even among sex differentiation studies, not all of them considered differences among the B6 substrains, and different countries and institutions use different B6 substrains and sometimes do not describe them in detail. Furthermore, the present results suggest that differences among the background B6 substrains may change the phenotypes of mutant and DSD model mice. Therefore, comparisons with results from other mouse strains or crossbreeds, including the B6 substrains, may lead to misinterpretation and an inability to replicate experiments. Therefore, in studies of sex differentiation, it is also important to pay attention to the genetic background of the mice used, including substrains of B6 mice.

In the present study, substrain differences were observed in the number of Sry-expressing cells, their distribution in the left and right gonads, and in the medial and lateral directions during the early stages of gonadal development in B6 mice. From these results, it is assumed that the expression of sex differentiation-related factors also differed between the left and right gonads. Therefore, sex differentiation studies should account for the possibility that the expression of sex differentiation-related factors may differ between the left and right gonads, depending on the B6 substrain used.

CONFLICT OF INTEREST. The authors have no conflicts of interest.

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