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Production of *Sry* knockout mouse using TALEN via oocyte injection

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Recently developed transcription activator-like effector nuclease (TALEN) technology has enabled the creation of knockout mice, even for genes on the Y chromosome. In this study, we generated a knockout mouse for *Sry*, a sex-determining gene on the Y chromosome, using microinjection of TALEN RNA into pronuclear stage oocytes. As expected, the knockout mouse had female external and internal genitalia, a female level of blood testosterone and a female sexually dimorphic nucleus in the brain. The knockout mouse exhibited an estrous cycle and performed copulatory behavior as females, although it was infertile or had reduced fertility. A histological analysis showed that the ovary of the knockout mouse displayed a reduced number of oocytes and luteinized unruptured follicles, implying that a reduced number of ovulated oocytes is a possible reason for infertility and/or reduced fertility in the KO mouse.

In most mammalian species, sex is determined by the presence or absence of the Y chromosome. In mice, the *Sry* gene locates to the minimum sex-determining region of the murine Y chromosome[1], is expressed in the male genital ridge at the time of sex determination[2] and has been proven to be a sex-determining gene based on gain-of-function experiments, i.e., the overexpression of *Sry* in XX mice achieved with transgenic mouse technology reveals a male phenotype[3]. Also in humans, the *SRY* gene has been shown to play a pivotal role in sex determination: point mutations or deletions of the *SRY* gene are found in approximately 15% of XY females, and translocated *SRY* is found in the autosomes of most XX males[4]. Although there are a number of suggestive observations, it is important to confirm the function of *Sry in vivo* using loss-of-function analyses with targeted mutagenesis in order to examine whether *Sry* is the one and only sex-determining gene on the Y chromosome and to finally confirm the *Sry* gene as the sex-determining gene and provide an animal model of XY female syndrome. However, it is difficult to create knockout (KO) mice of Y-linked genes using conventional homologous recombination-based methods with embryonic stem (ES) cells, as the process requires an adequate length of specific sequences of homologous arms to construct a KO vector, and the Y chromosome is rich in repeats.

In 2013, Sung *et al.*[5] first reported that KO mice can be produced using transcription activator-like effector nuclease (TALEN) technology without conventional homologous recombination-based methods. TALEN protein is an artificial sequence-specific endonuclease that contains *Xanthomonas* transcription activator-like effector (TALE) and a nuclease domain of FokI restriction endonuclease[6]. DNA binding domain of TALE consists of a tandem repeat of 33–35 amino acid motifs in which there are two critical adjacent amino acid pairs called a repeat variable diresidue (RVD) that determines the binding specificity for single nucleotide. There is a one-to-one relationship between the RVD and its recognition nucleotide[7,8]. Using this code, a TALEN can be constructed with a DNA binding motif recognizing the desired nucleotide sequence[6]. When two TALENs are expressed in a cell and bind to the genome at an appropriate distance, called a spacer, the nuclease domain of FokI dimerizes and generates a double-strand break (DSB) within the spacer. The lesion is frequently repaired via nonhomologous end joining (NHEJ), an error-prone mechanism that results in the introduction of small insertion or deletion (indel) mutations. It has been reported that TALENs are useful for creating KO animals, such as fruitflies[9], silkworms[10], zebrafish[11–14], *Xenopus*[15,16] and rats[17,18]. Recently, it has been shown that TALEN technology can be

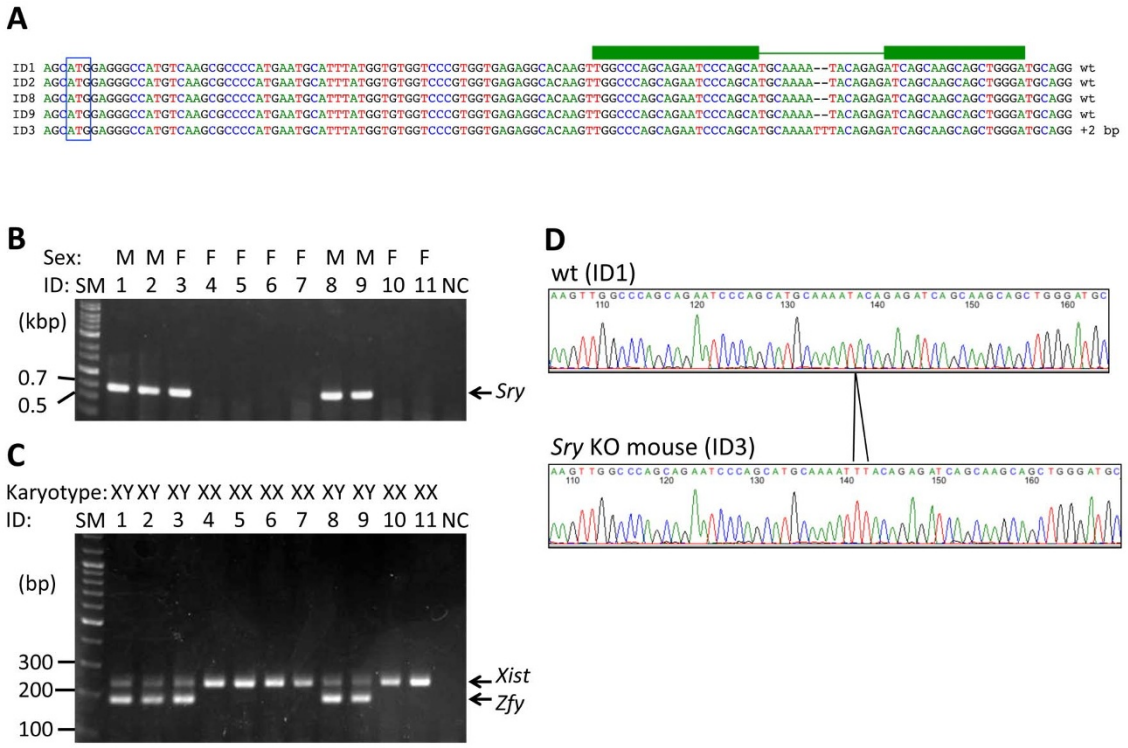

Figure 1 | Construction of the TALEN and generation of the *Sry* KO mouse. (A) Summary of the TALEN design and genotyping of the KO mouse. The positions of the TALEN targets and spacer are indicated by green boxes and a green line, respectively, above the nucleotide sequences. The blue box indicates the start codon of the *Sry* gene. ID of each mouse was shown at the left. Genotypes are indicated at the right. (B) Results of PCR amplification and agarose gel electrophoresis of *Sry*. M and F designate the phenotypic sex of male and female, respectively. DNA fragment lengths of the size markers are shown on the left. SM: size marker; NC: negative control. (C) Results of sexing PCR amplification and agarose gel electrophoresis. The karyotypes obtained from the experiment are shown at the top. DNA fragment lengths of the size markers are shown on the left. SM: size marker; NC: negative control. (D) Electropherograms around the TALEN spacer of the *Sry* KO (ID3) and wt male mice (ID1).

applied to generate autosomal gene KO mice via the microinjection of TALEN mRNA into fertilized oocytes[5,19,20]. TALEN can also be used to create Y-linked gene KO mice, as it recognizes specific sequences as short as approximately 45–65 nucleotides long.

In 2012, Wu et al.[21] reported the creation of a transgenic mouse in which *Sry* messenger RNA is knocked down *in vivo* using siRNA technology. In that report, the siRNA-treated developing gonads were feminized; however, it is difficult to knockdown target mRNA at 100% efficiency. Recently, the *Sry* gene was mutated using TALEN-mediated gene disruption in ES cells, and *Sry* KO mice were generated from the ES cells according to the tetraploid rescue method[22]. The authors reported that the *Sry* KO mice possessed sex reversed internal and external genitalia. In the current study, we generated *Sry* KO mouse using the microinjection of TALEN RNA into fertilized oocytes and present a detailed analysis of the KO mouse in regard to the hormone levels, histology of the gonads and brain, as well as gross morphology.

Results

Construction of TALEN and the production of *Sry* KO mouse. In order to generate *Sry* KO mouse, we adopted the TALEN-mediated method instead of the conventional homologous recombination-based ES cell modification strategy since *Sry* locates within 2.8 kb of a unique sequence at the center of a large inverted repeat structure[23]. The TALEN-mediated method is suitable for gene disruption of such repeat embedded genes and can be used to more quickly obtain KO mice since it can be applied to microinjection into oocytes, thereby bypassing gene targeting and chimera mouse generation using ES cells. To disrupt the *Sry* gene using TALEN, we set the TALEN recognition sequence at the 5′ part of the open reading frame (ORF) (Fig. 1A), so that almost the entire protein of SRY was lost due to a frameshift mutation once the TALEN caused an indel mutation. The TALEN RNAs were first microinjected into fertilized oocytes, then cultured at 37°C until transferred into pseudopregnant female mice (78% of the oocytes developed to the two-cell stage). PCR-sexing showed that 24 male pups were obtained out of 48 newborns, and PCR direct sequencing of *Sry* showed that no mutants were obtained (Table 1). It is possible that the optimal temperature for embryo culture, 37°C, is not suitable for TALEN. Therefore, we changed the temperature for the embryo culture to 30°C. This time, 129 oocytes were injected, 113 (88%) of which entered two-cell stage embryos (Table 1); therefore, the lower

Table 1 | Disruption of the *Sry* gene using TALEN technology

Temperature*	Injected	2-cell	Newborn	Males	Mutant
37°C	130	101 (78%)	48 (48%)	24 (50%)	0 (0%)
30°C	129	113 (88%)	23 (20%)	8 (35%)	1 (13%)

The percentages shown in parenthesis were derived from the number in the column as the numerator and the number in the column to the left as the denominator times 100.
*Temperature used for incubation after injection of the fertilized oocytes until transfer of the embryos into pseudopregnant females.

temperature of the embryo culture did not appear to hamper development. However, the ratio of delivered pups was reduced compared to that obtained when the injected oocytes were cultured at 37°C (Table 1). Based on visual inspection of sexual dimorphism of the external genitalia and the existence of nipples in the resulting pups obtained from the injected oocytes cultured at 30°C, we found that there were four males and seven females (Fig. 1B), whereas PCR genotyping in which the *Zfy* gene on the Y chromosome and the *Xist* gene on the X chromosome were amplified[24] showed that five mice had a Y chromosome (Fig. 1C), indicating that there was a sex reversal mouse (XY female, ID3). To investigate the effects of the TALEN, the flanking sequences of the TALEN target site on the *Sry* gene were amplified and sequenced (Fig. 1A, B,D). Four XY males had no mutations, while ID3 had two nucleotide insertions in the ORF of the *Sry* gene, causing a frameshift mutation (Fig. 1A, D). Our loss-of-function experiment with the *Sry* gene confirmed that *Sry* is the sex-determining gene on the Y chromosome, consistent with the findings of previous reports[3] utilizing gain-of-function experiments and the loss-of-function experiment of the *Sry* gene by Wang *et al.*[22] utilizing ES cells and the tetraploid rescue method.

External genitalia of the *Sry* KO mouse. The phenotype of the *Sry* KO mouse was examined in detail. Wild type females obtained at the same time as the KO mouse via microinjection were used as controls. The external genitalia of the *Sry* KO mouse at one and two months after birth was indistinguishable from that of the wt females (Fig. 2A). To compare the external genitalia of the *Sry* KO mouse and wt females in detail, we set out to observe the timing of vaginal opening and measure the anal-genital distance (AGD), an index of femaleness. If the sexual differentiation of the *Sry* KO mouse is not complete, it is possible that the vagina of the KO mouse would not be open, the timing of vaginal opening would be delayed and/or the AGD would not differ between males and females. We checked the timing of vaginal opening in three-week-old mice. There was little difference between the KO and wt female mice. The AGD was measured and normalized according to body weight at four and eight weeks of age, at which time, the AGD/body weight of the KO mouse was in the range of that of the wt females (Fig. 2B, C). These results suggest that the *Sry* KO mouse had a completely female type of external genitalia.

Internal genitalia of the *Sry* KO mouse. We next investigated the internal genitalia of the *Sry* KO mouse. The ovaries, oviducts and uterus in the *Sry* KO mouse were similar in size and morphology to those of the wt females (Fig. 3A–D). To compare the structure of the ovaries of the *Sry* KO mouse and wt females in detail, ovary sections were stained with hematoxylin and eosin (Fig. 3E–H). A few developing follicles, such as secondary and Graafian follicles, were observed (Fig. 3E, F) in both the wt and *Sry* KO ovaries; however, the number of follicles in the *Sry* KO ovaries was approximately half of that of the wt ovaries (Fig. 3J), and luteinizing had proceeded in the KO ovaries. In addition, it is noteworthy that luteinized unruptured follicles (LUFs), including oocytes retained in the corpus luteum, were observed in the *Sry* KO ovaries (Fig. 3I) but not in the wt ovaries. These results suggest that the *Sry* KO mouse had internal female genitalia; however, the ovaries were not completely the same as those of the wt females.

Hormone levels and the estrous cycle of the *Sry* KO mouse. In order to examine whether the *Sry* KO mouse had female type endocrinological features, the estrous cycle was investigated by smearing the vagina every morning at eight weeks of age. The *Sry* KO mouse exhibited a proestrus period for two days (Fig. 4A, B), followed by estrus for three days (Fig. 4C–E), metaestrus for one day (Fig. 4F) and diestrus for one day (Fig. 4G). Subsequently, the estrus cycle turned back to the proestrus period (Fig. 4H). The estrus period of the *Sry* KO mouse was a little longer than that of the wt females. Nonetheless, the estrus cycle of the *Sry* KO mouse was cycling. To confirm that the levels of sex hormones in the *Sry* KO mouse were in the range of females, the testosterone levels were measured. The blood plasma of four wt male mice obtained at the same time as the *Sry* KO mouse via microinjection, six wt females and the *Sry*

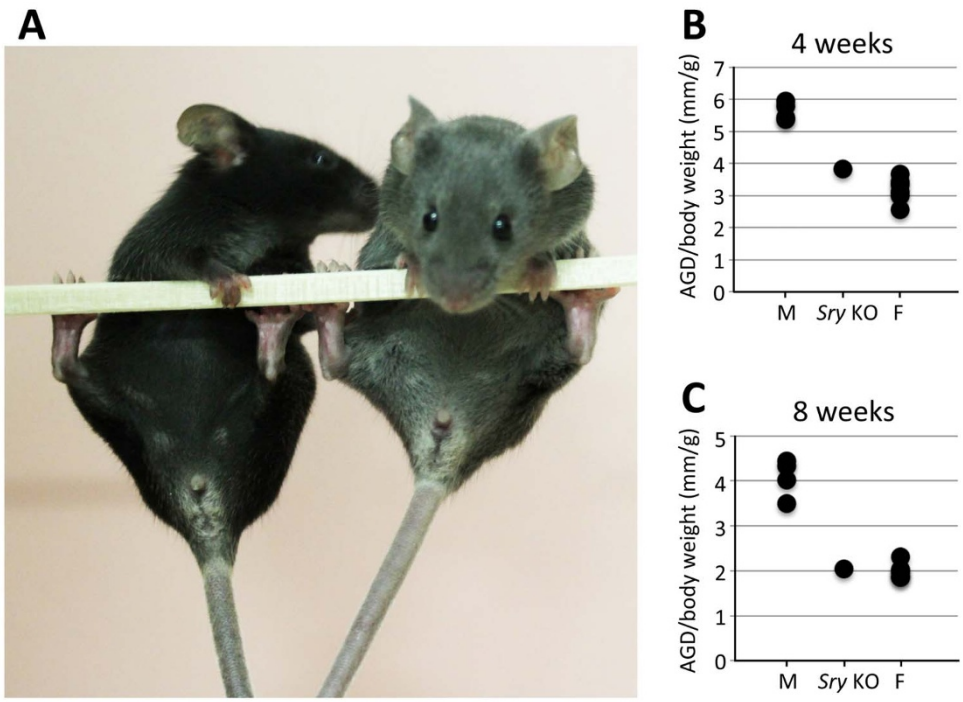

Figure 2 | External genitalia of the *Sry* KO mouse. (A) External genitalia of the *Sry* KO mouse. The photograph was taken in Animal Resources in National Research Institute for Child Health and Development by T.Kato and S.T. Left: *Sry* KO mouse; right: wt female mouse. (B) AGD normalized by body weight at four (top) and eight (bottom) weeks of age. Each dot shows the AGD/body weight of the individual mouse. M: male; F: female.

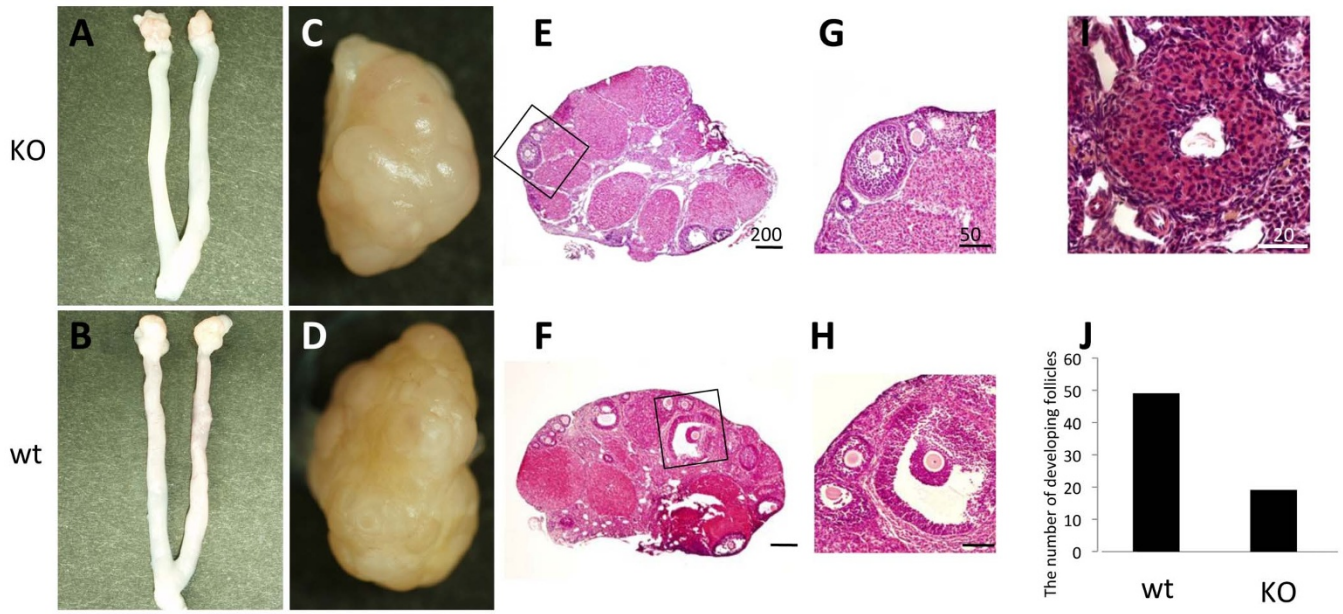

Figure 3 | Internal genitalia of the *Sry* KO mouse. (A), (B) Ovaries and uterus of the *Sry* KO mouse (A) and wt female (B). (C), (D) Ovaries of the *Sry* KO mouse (C) and wt female (D). (E–H) Ovarian cross-section of the *Sry* KO mouse (E), (G) and wt female (F, H). G and H are higher magnification images of areas within the rectangles in E and F, respectively. Scale bars in E and F = 200 μm. Scale bars in G and H = 50 μm. (I) LUFs in the *Sry* KO ovary. Scale bar = 20 μm. (J) Total numbers of counted developing follicles in the *Sry* KO mouse and wt female.

KO mouse were used. The testosterone levels were high in the wt males, whereas the testosterone levels in the females, including the *Sry* KO mouse, were below the confident limit (the levels in the four wt females were below the measurement limit) (Fig. 4I). This result shows that the testosterone level of the *Sry* KO mouse was in the range of those of the wt females.

Fertility of the *Sry* KO mouse. To examine the fertility of the *Sry* KO mouse, *Sry* KO mouse (nine weeks old) were mated with wt C57BL/6 male mouse, of which the fertility was confirmed before and after the experiment by mating with wt females, for four weeks. Although we confirmed the presence of the vaginal plug four times, pregnancy was not observed in the KO mouse, as we did not find any newborn pups

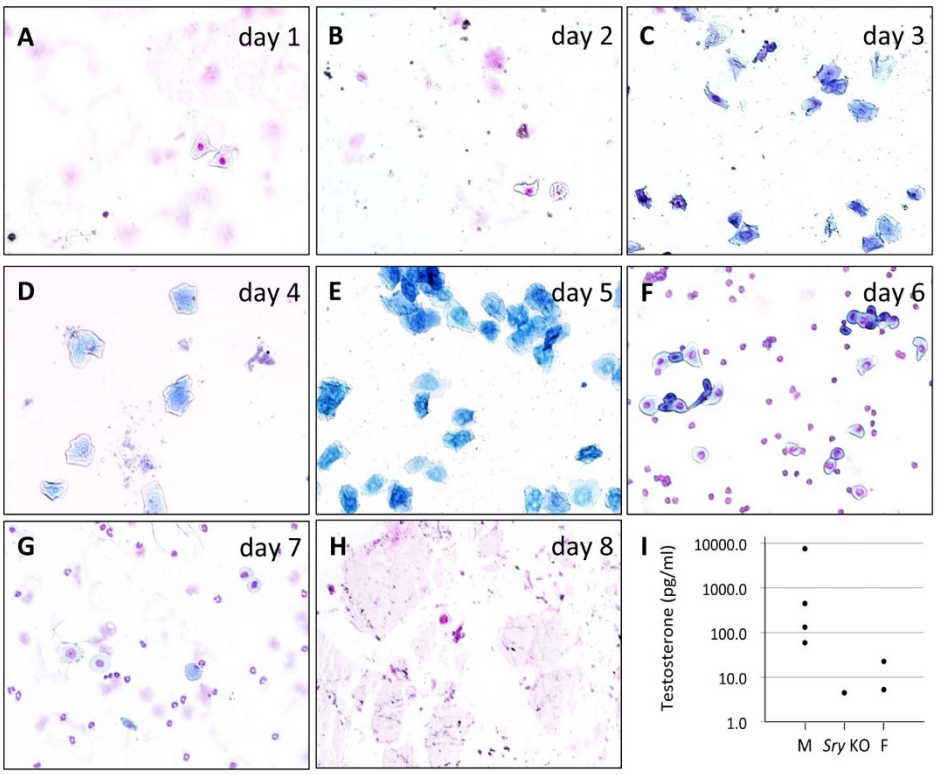

Figure 4 | Estrous cycle of the *Sry* KO mouse. Smear image of the *Sry* KO mouse. (A), (B), (H) Proestrus, (C–E) Estrus, (F) Metaestrus, (G) Diestrus. The day of the smear test is indicated on the top right in the image. (I) Testosterone levels of the *Sry* KO mouse. M: male; F: female.

or embryos at dissection at 14 weeks of age. This result implies that the *Sry* KO mouse was sterile or had reduced fertility, even though it had oocytes in their ovaries and performed copulatory behavior as females.

Brain of the *Sry* KO mouse. The *Sry* KO mouse demonstrated reproductive behavior when mated with the wt males, which prompted us to confirm whether the KO mouse had female type sexually dimorphic nuclei. The presence of a sexually dimorphic nucleus was determined in the medial preoptic area (MPOA) using staining with calbindin, with a higher number of immunoreactive cells observed in males[25]. We found that the number of calbindin expressing neurons in the anteroventral periventricular nucleus (AVPV) in the MPOA was higher in the wt male compared to that observed in the wt female (Fig. 5). In the *Sry* KO mouse, the number of calbindin expressing cells was equivalent to that observed in the wt female and lower than that observed in the wt male, especially in the medial dorsal portion. However, a small number of calbindin positive cells was detected in the ventromedial preoptic area of the AVPV, a finding that was relatively comparable to that observed in the wt male (Fig. 5).

Effect of *Sry* mutation on gene expression. In the end, we wanted to confirm whether functional SRY protein production was truly disrupted in our mutant mouse. However, since we obtained only one mutant mouse which did not produce an offspring, detection of the SRY protein in embryonic gonad, where it acts as sex determination factor, was technically impossible. Therefore we expressed wt and mutant *Sry* gene in cultured cell to confirm whether two nucleotide insertion that we observed in *Sry* KO mouse disrupts SRY protein production. Overexpression vector encoding ORF of *Sry* gene with or without the two nucleotide insertion were transfected to HEK-293T and protein products were detected by western blotting (Fig. 6B–D). Since *Sry* is an intronless gene, *Sry* ORF was obtained from PCR amplification using genomic DNA as a template. To facilitate the detection and to clarify which part of the gene is produced as protein, FLAG and S-tag were fused to N- and C- termini of *Sry*, in the reading frame of wt *Sry* ORF (i.e. C-terminal S-tag of mutant *Sry* construct is out of frame to the first methionine) (Fig. 6A). As shown in Fig. 6B and 6C, SRY protein containing FLAG and S-tag were detected from wt *Sry* expressed cells, whereas no signal was detected from mutant *Sry* expressed cells. This result suggested that *Sry* mutation we obtained actually disrupts SRY protein expression. We presume there could be two reasons why we did not detect any protein product from mutant *Sry* construct: first, mutated *Sry* transcript has premature stop codon and thus was degraded by nonsense-mediated mRNA decay. Alternatively, truncated SRY protein was indeed synthesized, but the expected protein is short and could be structurally unstable, and thus degraded by unfolded protein response, or too small to detect western blotting.

Discussion

We generated *Sry* KO mouse using TALEN RNA injection into fertilized oocytes. KO mice have been generated using ES cells via homologous recombination, which requires targeting vectors containing the homologous arm of several kilobases. This hampers the creation of KO mice of genes that are in or around repeat-rich regions, such as Y-linked genes. On the other hand, TALEN requires relatively short specific sequences for genome editing so that it can be used to create KO mice for genes within repeat-rich regions. Another advantage in using TALEN to make KO mice is that it enables KO mice to be obtained quickly when injected into fertilize oocytes because the time required to culture ES cells and produce chimera can be omitted.

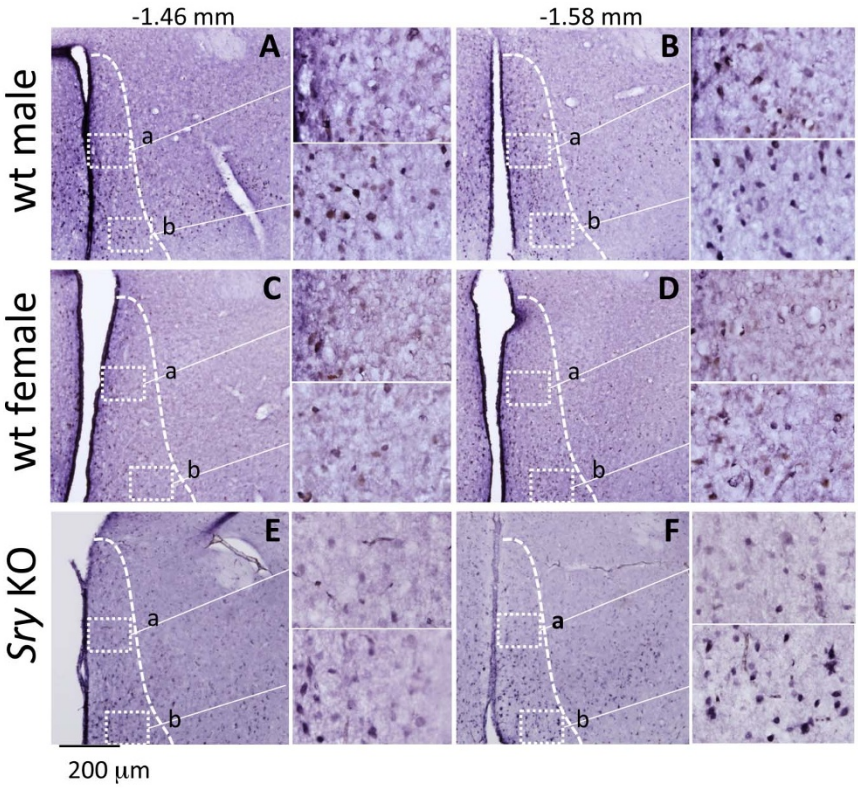

Figure 5 | Brain of the *Sry* KO mouse. Calbindin immunoreactive neurons were observed in the AVPV (dotted line) in the wt male (A, B), but not wt female (C), (D). The *Sry* KO mouse (E), (F) exhibited a lower expression level of calbindin in the medial dorsal portion of the AVPV compared to that observed in the wt male (square a); however, some positive cells were detected in the ventromedial preoptic area of the AVPV (square b). Scale bar = 200 μm.

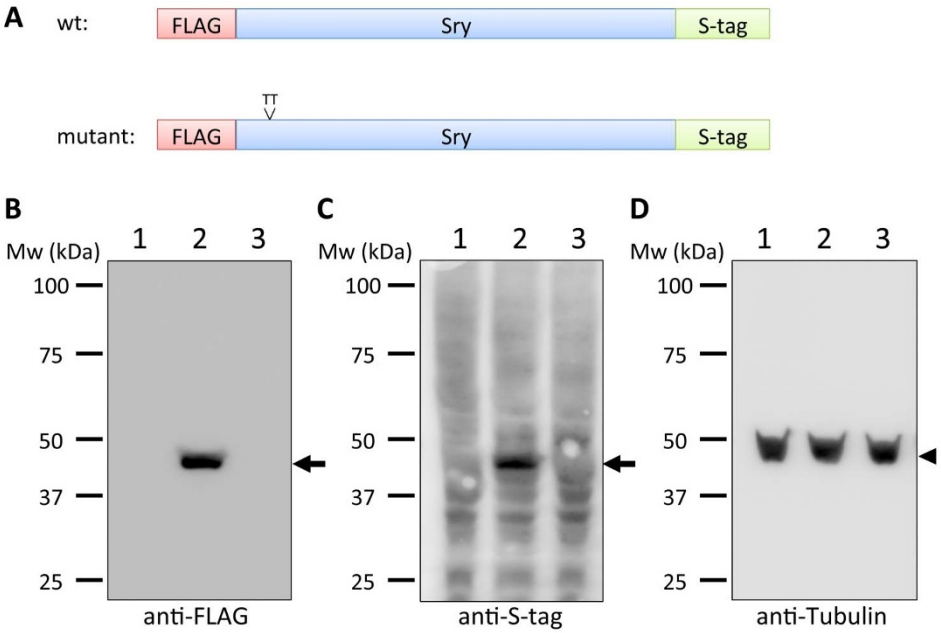

Figure 6 | Wild type and mutant *Sry* gene expression detected by western blotting. (A) Schematic representation of constructed gene structures in the expression vector. Position of the two nucleotide insertion (TT) is shown above the mutant gene structure. (B) Detection of wt and mutant SRY protein containing FLAG and S-tag at N- and C-termini, respectively, by western blotting using anti-FLAG antibody (arrow). Lane 1: empty vector transfected cell lysate; lane 2: wt *Sry* with FLAG and S-tag; lane3: mutant *Sry* with FLAG and S-tag. Size marker is shown at left of image. (C) Detection of wt and mutant SRY protein containing FLAG and S-tag at N- and C-termini, respectively, by western blotting using anti-S-tag antibody (arrow). Lanes and markers are same as those of (B). (D) Detection of tubulin by western blotting (arrowhead) as a control for amount of protein loading. Lanes and markers are same as those of (B).

We could not obtain *Sry* KO mouse by incubation of TALEN RNA injected oocytes incubated at 37°C. Since successful mutagenesis using TALEN was reported at the temperature lower than 37°C in heterothermic animals such as fruit flies[9], silkworms[10], zebrafish[11–14] and *Xenopus*[15,16], we thought lowering the incubation temperature could ameliorate the mutagenesis rate. Thus we incubate TALEN RNA injected oocytes at 30°C and could obtained the *Sry* KO mouse. We cannot conclude which temperature is better for making knockout mouse using TALEN from our data, but incubating injected oocyte at 30°C could be an option for whom cannot obtain knockout mouse when injected oocytes are incubated at 37°C.

To evaluate sex reversal observed in *Sry* KO mouse is caused by *Sry* gene mutation, the best way is rescue of the phenotypes of the *Sry* KO mouse by crossing with *Sry* transgenic mouse. However this experiment could not be performed because the *Sry* KO mouse did not produce an offspring. We introduced a dinucleotide insertion at the 5′ part of the ORF of the *Sry* gene, so that the protein product contains 32 intact amino acid residues (intact deduced SRY (RefSeq: NM_011564): 395 amino acid residues) with 30 new amino acids or mRNA is degraded by nonsense-mediated mRNA decay. In the former case the expected mutant SRY lacks functionally important motifs, such as a glutamine-rich domain, one of two nuclear localization signals (NLSs) and more than half of the HMG domain. Previous studies have reported that *Sry* transgenic constructs without the glutamine-rich domain fail to cause sex reversal in mice with the XX karyotype[26], mutations in either NLS cause XY sex reversal in humans[27–30] and most cases of human XY sex reversal involve mutations in HMG box (review in Harley and Goodfellow[31]). Considering these reports, it is reasonable that the *Sry* KO allele in our KO mouse does not produce functional SRY protein because 92% of the ORF was not translated and functionally important motifs were located on the missing residues.

In the present study, we analyzed the effects of *Sry* gene depletion in XY mouse. Recently Wang *et al*[22] reported *Sry* KO mouse created through TALEN mediated gene disruption in ES cells. They showed the KO mouse had female type genitalia and reduced fertility. Our KO mouse, which had different mutant allele to previously reported mice, recapitulated the sex reversal phenotype which confirmed the fundamental role of *Sry* gene for male sex determination. Moreover, in this study we provided the histological and physiological phenotypes of *Sry* KO mouse. The external and internal genitalia were, as expected, female, the testosterone levels were within the female range, the estrous cycle was the same as that of the wt females, the copulatory behavior was the same as that of females and the dimorphic nucleus in the AVPV was of the female type. These results indicate that *Sry* KO mouse is sex reversed and healthy female, although the structure of the ovaries was different from that of normal females and the number of oocytes was reduced. We were unable to distinguish whether the *Sry* KO mouse had reduced fertility or was sterile, although we confirmed the presence of a vaginal plug four times, and no embryos or newborns were identified. Considering that Wang *et al.*[22] reported that *Sry* KO mice created using ES cell KO and tetraploid rescue have reduced fertility, it is possible that our KO mouse also had reduced fertility. If so, it is possible that the number of ovulated mature oocytes in the *Sry* KO mouse was reduced. Indeed, the ovaries of the *Sry* KO mouse contained fewer developing follicles and were more luteinized than those of the wt females. In addition, a few LUFs, including oocytes retained in the corpus luteum, were identified in the *Sry* KO ovaries only. In humans, LUFs are observed in 5–10% of menstrual cycles of normal fertile females[32], while a higher incidence of LUFs has been reported in infertile females[33]. In the latter cases, an estrous cycle is normal[33–35]. This situation is similar to that of the *Sry* KO mouse. This symptom of anovulation is considered to be a cause of human female infertility or reduced fertility[35]. However, we do not know whether the LUFs observed in the *Sry* KO mouse were caused by the existence of the Y chromosome or dysfunction of the ovaries. If the latter case is true, then it is possible that *Sry* KO mouse could be used as a model for infertility or reduced fertility containing LUFs.

We found that the *Sry* KO mouse demonstrated female-like calbindin positive cells in the MPOA, especially in the medial dorsal portion of the AVPV. The distribution patterns of the calbindin expressing neurons in the wt males were different from the findings of a previous report of C57/BL6 males[25]. This is likely due to the difference in mouse strains[36]. In fact, clear sex differences in the number of calbindin expressing neurons were observed in the MPOA in this B6CBAF1/J strain in the medial dorsal portion of the AVPV, in which many Kisspeptin containing neurons exist. Kisspeptin neurons in the AVPV stimulate GnRH secretion[37], which results in an increase in female copulatory behavior[38]. Interestingly, the *Sry* KO mouse exhibited female-like calbindin expressing neurons in the AVPV, suggesting that this female-like AVPV structure is related to the normal female-type copulatory behavior observed in the *Sry* KO mouse. However, a small number of calbindin positive cells was detected in the ventromedial preoptic area of the AVPV in the *Sry* KO mouse, which was relatively comparable to that observed in the wt male (Fig. 6). The neurons in the ventromedial preoptic area of the AVPV are related to the onset of anestrous in ewes[39] and project to and stimulate hypothalamus dopaminergic neurons[40]. A higher level of dopaminergic activity blocks pregnancy by decreasing the release of prolactin from the pituitary[41]. Therefore, the male-like structure of the ventromedial preoptic area of the AVPV may be related to the prolonged estrous cycle and reduced fertility observed in the *Sry* KO mouse. It is unclear why MPOA was not fully reversed from a male- to female-type structure in the *Sry* KO mouse. One possibility is a parent-of-origin imprinting gene expression in the hypothalamus. In the hypothalamus, sex-specific imprinted genes are found in females, which suggests a parental allelic influence over the hypothalamic function in XX females[42]. Therefore, *Sry* KO mouse lacked some female functions in the hypothalamus.

Methods

TALEN. The TALEN plasmids were designed using the online TAL Effector Nucleotide Targeter 2.0 software program (https://tale-nt.cac.cornell.edu/node/add/talen-old) with the following parameters: Minimum Spacer Length at 12 bp, Maximum Spacer Length at 16 bp, Minimum Repeat Array Length at 16 and Maximum Repeat Array Length at 20. The query sequence used was the reverse complementary sequence of nucleotide number 1919368–1919568 on chromosome Y (Assembly by July 2007 (NCBI37/mm9)). Position 1 of the query sequence corresponds to the initiation codon of the *Sry* gene. One of the possible TALEN target sequences identified by the program was selected empirically. The TALENs were assembled in pcDNA-TAL-NC vector plasmids using a previously described protocol[43]. A Golden Gate TALEN and TAL Effector Kit was obtained from Addgene[44]. The target sequences of the *Sry* TALENs were as follows: left 5′-TGG CCC AGC AGA ATC CCA GCA-3′ and right 5′-TCC CAG CTG CTT GCT GAT-3′.

Microinjection. TALEN plasmids were digested by PvuII restriction endonuclease. One microgram of the digested plasmids was used as a template for the *in vitro* transcription reaction using the mMESSAGE mMACHINE T7 Kit (Life Technologies) according to the manufacturer's instructions. The synthesized RNAs were purified using the MegaClear kit (Life Technologies) according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop 1000 spectrophotometer and diluted with injection buffer (10 mM Tris-HCl/0.1 mM EDTA (pH 7.4)) at 600 ng/μl in a total of two TALEN mRNAs (1 : 1 ratio, i.e. 300 ng/μl each). The microinjection of the two TALEN mRNAs mix into cytoplasm of pronuclear stage oocytes was carried out under standard procedures using oocytes obtained from superovulated (C57BL/6 × DBA2) F1 mice mated with male mice of the same strain (Sankyo Labo Service Corporation). The injected oocytes were cultured in M16 medium at 30 or 37°C. The following day, embryos developed into the two-cell stage were transferred into pseudopregnant ICR female mice. All animal care protocols and experiments were approved by the Animal Care and Use Committee at the National Research Institute for Child Health and Development.

PCR-based genotyping and sexing. Genomic DNA was extracted from tail tips using amputation. For genotyping of *Sry*, PCR was carried out using the following primers: SryF475 (5′- CTG TCC CAC TGC AGA AGG TT -3′) and SryR412 (5′- GGG CTG GAC TAG GGA GGT CCT G -3′). The PCR products were treated with ExoSAP-IT (Affymetrix) and used as templates for sequencing. The sequencing primer was SrySeq476 (5′- CAG CCC TAC AGC CAC ATG AT -3′). For PCR-based sexing, the Y chromosome-specific gene, *Zfy*, and the X chromosome-specific gene, *Xist*, were amplified using the primer set described by Obata *et al.*[24].

Vaginal smear test. The estrous cycle was analyzed using a vaginal smear test. *Sry* KO mouse and wt females (eight weeks of age) were used for eight successive days. Every morning, PBS was applied to the vagina and pipetted well. The liquid was collected and uniformly smeared on a slide glass. The samples were air dried, fixed in methanol, stained with Giemsa solution and washed in tap water. The specimens were micrographed using an optical microscope.

Fertility check. Female mice nine weeks of age were mated with wt C57BL/6 male mice, of which fertility was confirmed by crossing with C57BL/6 females before and after crossing with the *Sry* KO mouse. The presence of the vaginal plug and parturition were checked every morning for four weeks.

Hormone assay. The mice were anesthetized, and blood was collected from the heart before euthanasia. The blood plasma was separated immediately after collection and stored at −80°C. The hormones levels were analyzed using a radioimmunoassay which was performed by Asuka Pharmaceutical Co., Ltd.

Histology. The mice were perfusion fixed through the heart using 4% paraformaldehyde. The brains and gonads were collected and embedded in paraffin. The embedded ovary paraffin block was sectioned at 6 μm. Deparaffinized sections were stained with hematoxylin and eosin. Six representative sections with more than 75 μm of distance between each other were chosen, and the numbers of developing follicles, including secondary follicles and Graafian follicles, per ovarian cross section were counted. As to calbindin immunohistochemistry, sections containing the MPOA (−0.5 to −1.8 mm from bregma) were sectioned at 30 μm. One of two sequential sections was subjected to immunohistochemistry. The staining methods have been previously described[25]. Briefly, the sections were incubated with the first monoclonal antibody to calbindin (C9848-2ML; Sigma-Aldrich, 1 : 12000) for 48 hours. After rinsing, the sections were incubated with biotinylated horse anti-mouse IgG antibodies (BA-2000, Vector Lab, 1 : 500), then reacted with the ABC kit (VECTASTAIN Elite ABC kit, Vector Labs).

Transfection and western blotting. ORF of *Sry* was PCR amplified using genomic DNA purified from wt male and *Sry* KO mouse using primers SryORF XhoI-FLAG F (5′- gcc tcg agA TGG ACT ACA AGG ACG ATG ATG ACA AGG GCa tgg agg gcc atg tca agc g-3′), where XhoI recognition site and FLAG tag indicated with underline and capital letters, respectively, and SryORF EcoRI StagR (5′- gcg aat tct caG CTG TCC ATG TGC TGT CTC TCG AAC TTG GCA GCG GCG GTC TCC TTt gag act gcc aac cac agg g-3′), where EcoRI recognition site and FLAG tag indicated with underline and capital letters, respectively. PCR products were cloned in pIRES2-EGFP (Clontech) using EcoRI/XhoI. Plasmids were transfected to HEK-293T cell using Fugene HD (Promega) according to the manufacturer's instruction. After 48 hours incubation at 37°C, transfection efficiency was confirmed by checking EGFP emission and cells were lysed with cell lysis buffer (50 mM HEPES [pH 7.8], 200 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol, freshly complemented with 1 mM DTT, protease inhibitor cocktail (Roche)). The cell lysate was analyzed by western blotting using 10% SDS-PAGE, Immobilon PVDF memmbrane (Millipore), monoclonal anti-FLAG M2 antibody (SIGMA), anti-S-tag antibody (Abcam), anti-mouse IgG HRP conjugated (SIGMA), anti-rabbit IgG HRP conjugated (SIGMA) and ECL (GE).

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Author contributions

K.Miyata, S.Y., S.M. and S.T. constructed the TALEN plasmids, T.S. and T.Y. provided instructions regarding the construction of the TALEN plasmids, M.T. performed the microinjection experiments, M.S. and T. Kikusi carried out the detection of the dimorphisms in the brain, S.Y., M.I., K.Miura. and Y.K. performed detection of SRY protein, T. Kato and S.T. conducted the remainder of the experiments, M.I., H.A. and S.T. designed the project and S.T. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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