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CRISPR/Cas9-mediated knock-in of the murine Y chromosomal *Sry* gene

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Abstract. Mammalian zygote-mediated genome editing via the clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas9) system is widely used to generate genome-modified animals. This system allows for the production of loss-of-function mutations in various Y chromosome genes, including *Sry*, in mice. Here, we report the establishment of a CRISPR-Cas9-mediated knock-in line of Flag-tag sequences into the *Sry* locus at the C-terminal coding end of the Y chromosome ($Y^{Sry\text{-}flag}$). In the F1 and successive generations, all male pups carrying the $Y^{Sry\text{-}flag}$ chromosome had normal testis differentiation and proper spermatogenesis at maturity, enabling complete fertility and the production of viable offspring. To our knowledge, this study is the first to produce a stable *Sry* knock-in line at the C-terminal region, highlighting a novel approach for examining the significance of amino acid changes at the naive *Sry* locus in mammals.

Key words: CRISPR/Cas9, SRY, SRY-Flag, Testis, Y chromosome

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In mammals, the sex-determining region Y (*Sry*) gene on the Y chromosome is essential for the initiation of Sertoli cell differentiation from bipotential supporting cells, which induces testis formation (see review [1]). Without the action of *Sry,* the supporting cells differentiate into granulosa cells, resulting in ovarian development. In mouse embryogenesis, *Sry* is transiently expressed at ~11−12 days post coitum (dpc), following which it directly activates SRY-box 9 (*Sox9*), another testis-specific *Sry*-related transcription factor in fetal Sertoli cells [2, 3], resulting in testis formation in mouse embryonic gonads at 12.5 dpc.

Sry evolved rapidly in mammalian species, as its protein sequences, aside from the DNA-binding high mobility group (HMG) box domain, vary. Even within Muroidea, it is well known that the transfer of certain Y chromosomes, derived from *Mus domesticus*, together with *Sry*(DOM) alleles [e.g. Sry(POS) and Sry(AKR)], into the C57BL/6J mouse strain results in defective testis development (e.g. XY ovary and ovotestis), due to an insufficient interaction between the *Sry*(DOM) allele and the B6-derived autosomal genes [4]. SRY(DOM) sequences show a distinct repeat size of the C-terminal polyglutamine (polyQ) tract, suggesting the distinct functional activity and protein stability of each SRY protein among these species [5, 6]. The degraded C-terminal polyQ tract of SRY was also found on the Y chromosome of the African pygmy mouse *Mus minutoides*, in which individuals bearing a normal Y and a variant X (X*) develop as females, whereas XY individuals bearing a normal X chromosome develop as males [7]. Such functional variation of the SRY protein was demonstrated mainly by *in vitro* transfection experiments using cell lines [6, 7] and *in vivo* transgenic mouse approaches [8, 9]. To examine the significance and function of the SRY variation *in vivo*, novel approaches, such as direct genome editing of the *Sry* locus in naive Y chromosome locations, are required in the future.

Recently developed nuclease-based genome-editing technologies, such as transcription activator-like effector nucleases (TALENS) or clustered regularly interspaced short palindromic repeats/CRISPR-associated endonucleases (CRISPR/Cas), allow for the direct modification of various Y genes at their native Y chromosome locations [10–14]. Wang *et al.* and Kato *et al.* have reported the production of *Sry*-knockout mice using these techniques [11, 15]. Recently, Song *et al.* reported the direct mutation of rabbit *SRY* using the CRISPR/Cas9 system, revealing the importance of the Sp1-binding sites in the 5' flanking region of the *SRY* locus on the rabbit Y chromosome [16]. However, there have not been any reports of an *SRY* knock-in line using tagged sequences to modify the SRY-coding sequences of the Y chromosome.

In the present study, we designed a stable mouse line with a Flag-tagged sequence knock-in on the naive *SRY* locus on the Y chromosome ($Y^{Sry\text{-}flag}$). First, to knock-in the Flag-tag sequences, we prepared a single-stranded oligodeoxynucleotide (ssODN), corresponding to the Flag-tag sequences between nucleotides 57 and 61 of the homology arms of murine *Sry*, and then injected the ssODN, together with $Cas9^{D10A}$ mRNA and two guide RNAs (gRNAs), into mouse zygotes (Fig. 1A, B). Additionally, SRY-Flag-tag protein products were confirmed to be immunohistochemically detectable, albeit with weak signals, by anti-Flag-tag immunofluorescence staining

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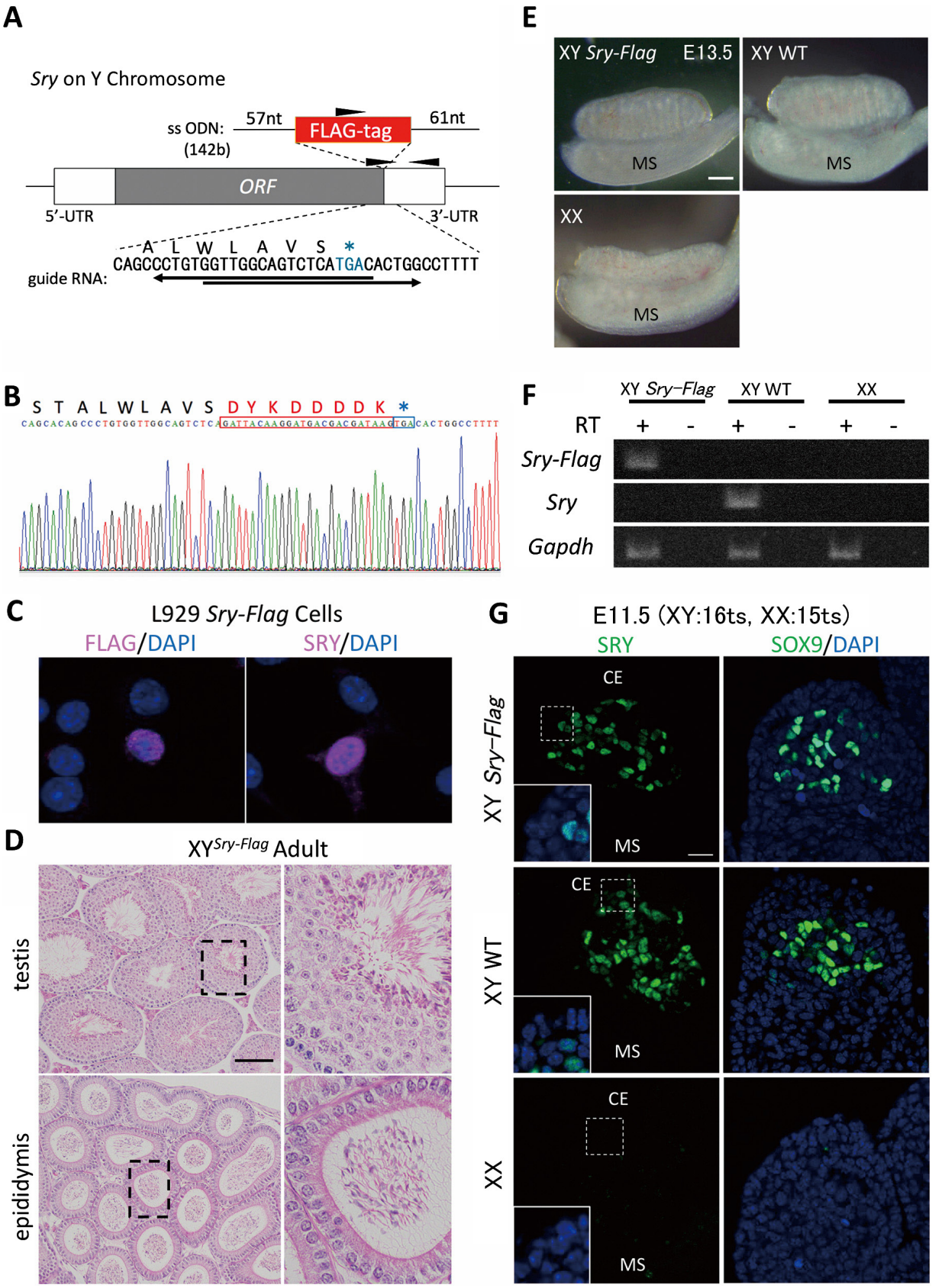
A
Sry on Y Chromosome
ss ODN:
(142b)
57nt
FLAG-tag
61nt
ORF
5'-UTR
3'-UTR
A L W L A V S *
CAGCCCTGTGGTTGGCAGTCTCATGACACTGGCCTTTT
guide RNA:
B
S T A L W L A V S D Y K D D D D K *
C
L929 Sry-Flag Cells
FLAG/DAPI
SRY/DAPI
D
XY Sry-Flag Adult
testis
epididymis
E
XY Sry-Flag E13.5
XY WT
XX
MS
F
XY Sry-Flag
XY WT
XX
RT + - + - + -
Sry-Flag
Sry
Gapdh
G
E11.5 (XY:16ts, XX:15ts)
SRY
SOX9/DAPI
XY Sry-Flag
XY WT
XX
CE
MS

Fig. 1.

in transfected L929 cells (Fig. 1C). A total of 120 2-cell embryos were obtained from culturing of the injected zygotes and were then transferred into the oviductal ampullae of 8-week-old female ICR mice. We obtained 21 pups, including 15 male pups, and only one male F0 pup with mosaic sperm, carrying *Sry-flag* and *Sry*-11bp-del (the 11-base pair deletion corresponding to the C-terminal coding sequences, including the stop codon) at the *Sry* locus. The F0 male and all of the F1 males with the *Sry-flag* locus were fertile, which resulted in the establishment of the new $Y^{Sry\text{-}flag}$ allele in the C57BL/6 background (F5 generation at present).

We examined the histological phenotypes of the testis and epididymis in the $XY^{Sry\text{-}flag}$ males at 2 months of age and at later stages in the F1 and subsequent generations. In the adult testes, active spermatogenesis occurred in all of the seminiferous tubules throughout the sections examined (upper plate in Fig. 1D). Moreover, spermatozoa filled the lumens of both of the seminiferous tubules and epididymal ducts (lower plate in Fig. 1D), which confirmed the complete fertility and maintenance of offspring production in the $XY^{Sry\text{-}flag}$ male line, even for the F5 generation.

It was previously shown that some fetal testes, carrying *Sry*(MUS) in the C57BL/6 background, developed ovotestes with ovarian structures frequently exhibited at the posterior pole in the fetal stages (i.e. 12.5–14.5 dpc) [4, 17]. Such ovarian areas dissipated, consequently leading to fertile XY *Sry*(MUS) males, as was similarly shown in XX-XY chimeric ovotestes [18]. We then compared the morphology of the 13.5-dpc testes of the $XY^{Sry\text{-}flag}$ and wild-type (WT) XY lines to detect the phenotypes of fetal $XY^{Sry\text{-}flag}$ testis development (Fig. 1E). Dissecting microscopic observations confirmed well-defined, testis cord formation throughout the anteroposterior axis of the $XY^{Sry\text{-}flag}$ testes (n = 10), similar to the WT XY gonads.

Finally, we compared the expression profiles of SRY and SOX9, another testis factor immediately downstream of SRY [2], in the $XY^{Sry\text{-}flag}$ gonads at 11.5 dpc (17–18 tail somite stage [ts]; i.e., the stage with peak *Sry* expression in the WT XY line; see review [19]). Reverse transcription-polymerase chain reaction (RT-PCR) analyses confirmed the expression of the *Sry-flag* transcripts, but not the WT *Sry* transcript in the $XY^{Sry\text{-}flag}$ gonads, which was in sharp contrast to the expression of only the WT *Sry* transcripts in the WT XY gonads (Fig. 1F). Anti-SRY immunofluorescence of the serial sections revealed that in the $XY^{Sry\text{-}flag}$ gonads, presumptive SRY-Flag products were properly detected within the gonadal parenchyma, including in the gonadal cells, located just beneath the coelomic epithelium ("XY SRY-Flag" in Fig. 1G), similar to those in the WT testes ("XY WT" in Fig. 1G). Anti-SOX9 immunostaining also revealed proper nuclear staining of SOX9-positive signals in the $XY^{Sry\text{-}flag}$ gonads (Fig. 1G), suggesting the proper initiation of the *Sry-flag* gene during pre-Sertoli cell differentiation at 11.5 dpc. However, unfortunately, in these sections of the $XY^{Sry\text{-}flag}$ gonads, anti-Flag immunostaining did not reveal any significant positive signals in these tissues *in vivo* (figure not shown), despite detection by anti-SRY staining and proper SOX9 activation in them.

Recently, it was shown that tyrosine sulfation of the Flag-tag amino acid sequences alters epitope recognition by the anti-Flag antibody, leading to a drastic decrease in its signal intensity [20]. However, our preliminary experiments demonstrated that prior to immunostaining, the sulfatase enzyme and/or flash acid-base pre-treatments could not improve the signals of the SRY-Flag proteins from the anti-Flag antibody, despite considerable signal detection with an anti-SRY antibody under the same conditions (data not shown). As the present anti-Flag immunostaining could detect the SRY-Flag products in the transient transfection experiments with L929 cells (Fig. 1C), such a discrepancy might be due to a lower sensitivity of the anti-Flag antibody than the anti-SRY antibody in the detection of a small amount of SRY-Flag protein *in vivo*, although the possibility of C-terminal-specific protein degradation and modification in the $XY^{Sry\text{-}flag}$ pre-Sertoli cells remains. Further experiments are required to improve the sensitivity of anti-Flag-tag detection in the *in vivo* experiments and/or for the production of another knock-in line of 2 × or 3 × Flag sequences at the *Sry* locus in the near future.

To our knowledge, this study is the first to establish a stable *Sry* knock-in line at the C-terminal region of the naive Y chromosome. In this line, $XY^{Sry\text{-}flag}$ males underwent normal testis differentiation and subsequent spermatogenesis, producing completely fertile males. If the knock-in efficiency of the Y chromosome genes is improved in the near future, this technique will allow us to design a novel approach for the direct editing of the *Sry* coding sequences of *Sry* variants in various mammalian species to resolve their biological significance in the rapid sequence evolution of the SRY protein.

Methods

Animals

All of the animal experiments in this study were carried out in strict accordance with the Guidelines for Animal Use and Experimentation,

Fig. 1. Establishment of a stable knock-in mouse line of Flag-tag sequences into the C-terminal end of the *Sry* coding sequence on the naive Y chromosome ($Y^{Sry\text{-}flag}$). (A) Schematic representation of the murine *Sry* gene and the nucleotides constructed for the CRISPR-Cas9 system. The single-strand oligodeoxynucleotide (ssODN) was designed with the Flag-tag sequence between nucleotides 57 and 61 of the homology arm of *Sry*. The forward and reverse guide RNAs are indicated with arrows at the C-terminal end of the *Sry* open reading frame (ORF). Arrowheads show the positions of the forward and reverse primers for RT-PCR. (B) The genomic sequence image shows an example of the genotyping of the C-terminal sequence of the *Sry* ORF in the $XY^{Sry\text{-}flag}$ male line (F4 generation). Red letters, surrounded by the red rectangle, correspond to the insertion of the Flag-tag sequence. (C) Immunocytochemistry of L929 cells constructed to express SRY-Flag proteins using anti-Flag antibodies and anti-SRY antibodies. (D) Hematoxylin-eosin staining of the testis and epididymis of $XY^{Sry\text{-}flag}$ males at the adult stage (60 days old). Right insets are higher magnifications of the areas indicated by broken rectangles. (E) Dissecting microscopic images of the $XY^{Sry\text{-}flag}$ and wild-type (WT) XY testes and XX ovaries at 13.5 dpc showing testis cord formation throughout the anteroposterior axis of the $XY^{Sry\text{-}flag}$ testis. (F) RT-PCR analysis detected only the expression of *Sry-flag* transcripts, and not WT *Sry* transcripts, in $XY^{Sry\text{-}flag}$ gonads at 11.5 dpc (17–18 tail somite stage [ts]). Only the WT *Sry* transcripts, and not *Sry-flag,* were detectable in the WT XY gonads. *Gapdh* expression is shown as a positive control, in addition to the negative controls of using RNA samples without reverse transcriptase (RT). (G) Anti-SRY and anti-SOX9 immunostaining of serial sections of $XY^{Sry\text{-}flag}$, XY, and XX gonads at 11.3 dpc ($XY^{Sry\text{-}flag}$, XY:16 ts, XX:15 ts). Insets show SRY-positive signals in the gonadal cells just beneath the coelomic epithelia (ce). SRY and SOX9 expression in WT XY gonads is also shown. ce, coelomic epithelium; ms, mesonephros. Scale bars = 100 μm in D; 200 μm in E; 25 μm in G.

as delineated by the University of Tokyo. The procedures were approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences of the University of Tokyo (approval ID: P13-764, P15-49, P15-017). The genotype was determined by direct sequencing of the *Sry* locus, in addition to PCR, using a *Sry*-specific forward primer 5′-TGG CAG TCT CAT GAC ACT GG-3′ [X67204: 9478 to 9497], a *Sry-flag*-specific forward primer 5′-TTG GCA GTC TCA GAT TAC AAG GAT GAC GAC-3′, and their common reverse primer 5′-AAA TGC CAC AAC AAT GCA AC-3′ [X67204: 9707 to 9726] (positions are indicated by arrowheads in Fig. 1A).

Production of the XY$^{Sry\text{-}flag}$ male line by CRISPR-Cas9

Production of the XY$^{Sry\text{-}flag}$ male line via CRISPR-Cas9 was conducted as described previously [21]. In brief, approximately 4 pL of an RNA solution containing Cas9^{D10A} mRNA (100 μg/ml), gRNA#1 (20 μg/ml), gRNA#2 (20 μg/ml), and the ssODN template encoding a Flag-tag (100 μg/ml) (see Fig. 1A) were injected into the cytoplasm of C57BL/6 zygotes (CLEA, Japan, Tokyo, Japan). The sequences of each gRNA and ssODN used in the present study were as follows: two guide RNAs, 5′-GTC ATG AGA CTG CCA ACC ACA GG-3′ and 5′-GGT TGG CAG TCT CAT GAC ACT GG-3′; and the ssODN template, including the Flag-tag sequence (indicated by lower case letters), 5′-GGT GAG CAT ACA CCA TAC CAG GAG CAC CTC AGC ACA GCC CTG TGG TTG GCA GTC TCA gat tac aag gat gac gac gat aag TGA CAC TGG CCT TTT CTC CTA CCT ATG CCA ACA CTC CCC CTT GCT ATG ATT TTT AAG TCT G-3′. After injection, zygotes were cultured in M16 medium, and just as the embryos reached the two-cell stage, they were collected and transferred into the oviductal papillae of ICR host female mice. After birth, genomic PCR and direct sequence analyses of the pups were conducted to confirm the mutation at the *Sry* locus.

In addition, no appreciable mutations in the four candidate off-target loci (Chr3: 66,768,523-66,768,544; Chr4: 46,674,865-46,674,886; Chr13: 24,855,189-24,855,211; Chr15: 9,452,138-9,452,159) were detected by direct sequence analyses of the genomic DNAs of the F1 generation.

Transfection of the Sry-flag plasmids in L929 cells

The pcDNA3-*Sry-flag* vector was prepared via PCR amplification using genomic C57BL/6 DNA. Either pcDNA3-*Sry-flag* or an empty control pcDNA3 vector was transfected into L929 cells using the FuGENE transfection reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. After 48 h of incubation at 37°C, the cells were used for an immunofluorescence analysis.

Histology and immunohistochemistry

The samples were fixed in 4% paraformaldehyde-phosphate buffered saline at 4°C for 12 h, and then dehydrated and embedded in paraffin. Serial sections (approximately 4 μm thick) were used for immunostaining and hematoxylin-eosin staining, as described previously [22, 23]. The deparaffinized sections were incubated with anti-SOX9 (1:1000 dilution; AB5535; Merck Millipore, Burlington, MA, USA), anti-SRY (1:100 dilution; [24]), or anti-Flag-tag (1:100 dilution; NBP1-06712; Novus Biologicals, LLC, Littleton, CO, USA) antibodies at 4°C for 12 h. The reactions were visualized with Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) or by a biotin-conjugated secondary antibody in combination with a tyramide kit (Thermo Fisher Scientific, Waltham, MA, USA).

RT-PCR analyses

Total RNA was extracted from the genital ridges using the RNeasy Micro kit (Qiagen, Hilden, Germany). Each RNA was reverse-transcribed using random primers with a Superscript III cDNA Synthesis System (Invitrogen). *Sry* and *Sry-flag* cDNAs were separately amplified by *Sry*- or *Sry-flag*-specific forward primers. *Gapdh* was used as an internal control, and a reverse transcriptase-free reaction was performed as a control.

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