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 (YSry-flag). In the F1 and successive generations, all male pups carrying the YSry-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

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 ovarotestis), 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 S' 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 (YSry-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 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.
Fig. 1.

A

**Sry on Y Chromosome**

- ss ODN: (142b)
- 57nt
- FLAG-tag
- 61nt
- 5' UTR
- ORF
- 3' UTR
- guide RNA:
  - CAGCCCTGTGTTGCGAAGTCTCATGACGAGGCTTTT

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

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**

- CE
- MS

**XY WT**

- CE
- MS

**XX**

- CE
- MS
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-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 the Sry gene and the nucleotides constructed for the CRISPR-Cas9 system. The single-strand oligodeoxynucleotide was used as a guide RNA to the Y chromosome Sry gene during pre-Sertoli cell differentiation at 11.5 dpc. However, unfortunately, these sections of the XY-Sry-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-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-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,
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 GTG TAC TCA AAG GAT GAC 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-flag male line by CRISPR-Cas9

Production of the XY Sry-flag male line via CRISPR-Cas9 was conducted as described previously [21]. In brief, approximately 4 μg of an RNA solution containing Cas9D10A mRNA (100 μg/ml), gRNAA1 (20 μg/ml), gRNAA2 (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 AGG-3′ and 5′-GGT GAG CAT ACA CCA TAC CAG GAG 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).

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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