



Original

Generation of the Y-chromosome linked red fluorescent protein transgenic mouse model and sexing at the preimplantation stage

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Abstract: In mammals, sexual fate is determined by the chromosomes of the male and female gametes during fertilization. Males (XY) or females (XX) are produced when a sperm containing a Y or X-chromosome respectively fertilizes an X-chromosome-containing unfertilized egg. However, sexing of preimplantation stage embryos cannot be conducted visually. To address this, transgenic male mouse models with the ubiquitously expressed green fluorescent protein (GFP) transgene on X- (X-GFP) or Y-chromosomes (Y-GFP) have been established. However, when crossed with wild-type females, sexing of the preimplantation stage embryos by observing the GFP signal is problematic in some cases due to X-inactivation, loss of Y-chromosome (LOY), or loss of transgene fluorescence. In this study, a mouse model with the ubiquitously expressed red fluorescent protein (RFP) transgene on the Y-chromosome was generated since RFP is easily distinguishable from GFP signals. Unfortunately, the ubiquitously expressed tdTomato RFP transgene on the Y-chromosome (Y-RFP) mouse showed the lethal phenotype after birth. No lethal phenotypes were observed when the mitochondrial locating signal N-terminal of tdTomato (mtRFP) was included in the transgene construct. Almost half of the collected fertilized eggs from Y-mtRFP male mice crossed with wild-type females had an RFP signal at the preimplantation stage (E1.5). Therefore, XY eggs were recognized as RFP-positive embryos at the preimplantation stage. Furthermore, 100% sexing was observed at the preimplantation stage using the X-linked GFP/Y-linked RFP male mouse. The established Y-mtRFP mouse models may be used to study sex chromosome related research.

Key words: preimplantation embryo, sex chromosome, sexing, tdTomato

Introduction

The sexual phenotype in mammals depends on the combination of sex chromosomes and is controlled by the *SRY* (sex determining region Y) gene, which induces testicular development on the Y-chromosome [1, 2]. Fertilization of an egg with a Y- or X-chromosome sperm will result in the male (XY) and female (XX) phenotype, respectively, during development. Normal male and female function requires the genes from only one X-chromosome. Therefore, in females, one of its two X-chromosomes is inactivated during early development in a

process called X-chromosome inactivation (XCI).

However, sexing preimplantation stage embryos cannot be conducted visually, and visual phenotypes are only observable after testis formation.

The sex chromosome does not function only in sex determination. Sex chromosome abnormalities have been observed such as Klinefelter syndrome (XXY male) and Turner syndrome (XO female) [3, 4]. Furthermore, reports suggest that loss of the Y chromosome (LOY) in older men is associated with several diseases such as cancer and Alzheimer disease [5–8].

The sex chromosome-linked fluorescence protein (FP)

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Supplementary Table: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/expanim>



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transgenic animal models are helpful for these studies because the FP signal of the cell indicates the presence of the sex chromosome within the cell.

Initial mouse models were generated as having green fluorescent protein-linked X chromosomes (X-GFP) for sexing at the preimplantation stage [9–11]. When this X-GFP male mouse mated with wild-type females, fertilized eggs are classified as GFP-positive (XX^{GFP}) embryos (females) and GFP-negative (XY) embryos (males) until the blastocyst stage. This X-GFP male mouse enables sexing at the preimplantation stage. However, this system is unsuitable for some studies since the GFP signal in half of the cells and placental lineage cells disappears after implantation and cell differentiation by XCI [12–14]. A ubiquitously expressing Y-chromosome GFP transgene (Y-GFP) mouse was generated to improve this issue [15, 16], although GFP-negative cells via LOY remain following fertilization. The loss of the GFP signal in both the X-GFP and Y-GFP fertilized cells may or may not be caused by XCI in XX^{GFP} mice or LOY in XY^{GFP} mice, respectively. This is because the loss of GFP signal may be due to several reasons such as low pH, condition of the cell state, and promoter effect such as inserted locus efficiency, tissue specificity, or epigenetics.

Therefore, two marker proteins were combined to address the instability issue of GFP by initially generating the ubiquitously expressing Y-red fluorescent protein (RFP) mouse lines. Since the cell toxicity of RFP proteins are higher than GFP [17–19], tdTomato was used as the RFP; it is ubiquitously expressed in animal models [20, 21] and has an exceptionally bright signal that is clearly distinguishable from GFP. Furthermore, we generated X-linked GFP/Y-linked RFP male mice by crossing a Y-linked RFP male mouse with an X-linked GFP female mouse. The X-linked GFP/Y-linked RFP male mice facilitated 100% sexing at the preimplantation stage.

Material and Methods

Animals

All animal experiments were conducted in accordance with the “Regulations and By-Laws of Animal Experimentation at the Nara Institute for Science and Technology,” and were approved by the Animal Experimental Committee at the Nara Institute of Science and Technology (approval no.1639). The *Mus musculus* strains: C57BL/6J and ICR were purchased from Japan SLC, Inc. B6.Cg-Tg (CAG/Acr-EGFP) CX-FM139Os and X-GFP mouse [10, 11] was kindly provided by Dr. Ikawa at Osaka University.

Construction of the knock-in vector and design of sgRNA

The targeting vector pCAG-tdTomato-2A-Puro^r ubiquitously expressing tdTomato was constructed by PCR amplification of the DNA sequence of 2A-Puro^r (Supplementary Table 1, Oligo-1 and Oligo-2) obtained from pSpCas9 (BB)-2A-Puro (pX459) V2.0 (Addgene #62988) and assembled using SLiCE (seamless ligation cloning extract) [22] after NotI digestion of pCAG-tdTomato (Addgene #83029).

A 1,091 bp fragment as the 5'-arm (Supplementary Table 1, Oligo-3 and Oligo-4) and a 1,014 bp fragment as the 3'-arm (Supplementary Table 1, Oligo-5 and Oligo-6) of the *DBY* locus were obtained by PCR amplification using genomic DNA derived from the mF1-05 embryonic stem cell (ESC) line [23], which was produced by the F1 embryo of 129X1/Sv and C57BL/6J genome as a template. The 3'-arm was inserted after SpeI digestion of pCAG-tdTomato-2A-Puro^r by SLiCE, then the 5'-arm was inserted after HindIII digestion and hereafter referred to as Y-RFP KI vector. Mitochondrion localizing signal was amplified by PCR from pCAG-Su9-DsRed2 to produce pCAG-Su9-tdTomato-2A-Puro^r (Supplementary Table 1, Oligo-7 and Oligo-8). Subsequently, the Su9 sequence was inserted after ApaI digestion of Y-RFP KI vector by SLiCE and is hereafter referred to as Y-mtRFP KI vector.

Each DNA fragment was amplified using KOD FX Neo (TOYOBO, Osaka, Japan) for 35 cycles with the following conditions: 94°C for 30 s, 60°C for 30 s, and 68°C for 60 s/kb.

Oligo DNAs of target guide RNA (gRNA) sequences (Supplementary Table 1, Oligo-9 and Oligo-10) were ligated at the BbsI site of pSpCas9 (BB)-2A-Puro (pX459) V2.0 plasmid (Addgene #62988). Target sites were designed using CRISPR direct [24].

Establishment of Y-RFP and Y-mtRFP ESC lines

Genome editing using ESCs was conducted according to a previous study [23, 25]. mF1-05 ESCs were seeded on mitomycin C-treated SNLP, which is an SNL cell line [26] with a puromycin-resistance gene, with the targeting vector and the designed pX459 plasmids transfected using Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transfected cells were selected by treatment with 1 µg/ml puromycin for one week. The resistant ESC colonies were screened by their positive RFP signal, then genotyped using PCR to confirm the fidelity. More than 70% of the normal karyotype clones progressed to tetraploid complementation. After puromycin treatment, ESCs were maintained on mouse embryonic fibroblast

(MEF) feeder cells with N2B27 medium supplemented with 3 μ M CHIR99021 (Axon1386), 1.5 μ M CGP77675 (Sigma SML0314), and mouse LIF (N2B27-a2i/L medium) [27]. RFP signals of the ESC colonies were observed using a fluorescent microscope (EVOS FL; ThermoFisher Scientific).

Tetraploid complementation

Tetraploid embryos were prepared as previously described [23]. Briefly, ICR two cell-stage embryos were placed in a fusion buffer, which consisted of 300 mM D-Mannitol, 0.2 mM MgSO₄, and 0.01% BSA; electrofusion was performed by applying 140 V for 50 ms after aligning the embryos between the electrodes; CFB16-HB and LF501PT1-10 electrodes (BEXCo., Ltd., Tokyo, Japan) were used. Tetraploid embryos were incubated in KSOM embryo medium at 37°C and 5% CO₂. Y-RFP or Y-mtRFP ESCs were injected into a tetraploid four-cell embryo at E2.5, cultured until the blastocyst stage at E3.5.

Embryo transfer

E3.5 embryos were transferred into the uterus of E2.5 pseudopregnant ICR mice. Offspring were recovered by natural delivery or caesarean section on E19.5.

The ESC derived offspring by the tetraploid complementation method were detected by their RFP signal and confirmed with genotyping using primers Oligo-11 and Oligo-12 for the detection of Y-RFP or Y-mtRFP (Supplementary Table 1). DNA fragments were amplified using GoTaq (Promega, Madison, WI, USA) for 40 cycles using the following conditions: 94°C for 30 s, 60°C for 30 s, and 68°C for 40 s.

Frozen spermatozoa from Y-mtRFP mouse (B6;129B6-Tg(CAG-Su9/tdTomato/T2A/Puro)1Gaitn, RBRC11535) will be available through RIKEN BRC (<https://web.brc.riken.jp/en/>).

Collection of eggs and observation

ICR females were treated by CARD HyperOva (Kyudo, Saga, Japan) and hCG (ASKA Animal Health, Tokyo, Japan) for superovulation, then mated with Y-mtRFP males or X-GFP/Y-mtRFP males. Zygotes at two-cell stage were collected from female oviducts after 42 h of hCG injection by the flush-out method. The collected two-cell stage embryos were incubated in KSOM at 37°C and 5% CO₂. RFP and GFP signals of eggs were observed using the fluorescent microscope (EVOS FL; ThermoFisher Scientific) after 48 h (E1.5), 96 h (E2.5), and 120 h (E3.5) following hCG injection. The expression of the RFP and GFP signals in the E1.5 embryos were analyzed using the Image J software (<https://imagej.nih.gov/ij/>).

RNA expression analysis by RT-PCR

Brains and thymi were collected from adult wild-type C57BL/6 males, wild-type C57BL/6 females, and Y-mtRFP males. Testes were collected from adult wild-type C57BL/6 males and Y-mtRFP males. Complementary DNA (cDNA) samples were prepared using SuperScript VI VILO Master Mix (Thermo Fisher Scientific) after purified RNA by Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA fragments were amplified using DreamTaq (Thermo Fisher Scientific) for 35 cycles under the following conditions: 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for *Ddx3y* (261 bp) and *Ddx3x* (341 bp), 94°C for 30 s, 57°C for 30 s and 72°C for 40 s for *tdTomato* (675 bp), 94°C for 30 s, 53°C for 30 s and 72°C for 30 s for *Gapdh* (153 bp). Primer sets were used Oligo-13 and Oligo-14 for *Ddx3y*, Oligo-15 and Oligo-16 for *Ddx3x*, Oligo-17 and Oligo-18 for *tdTomato*, and Oligo-19 and Oligo-20 for *Gapdh* in Supplementary Table 1.

Results and Discussion

Establishment of the red fluorescence protein-tagged on Y-chromosome mouse line

The RFP tdTomato was chosen to generate the Y-RFP mouse model since its signal is stronger compared with other RFPs, and the ubiquitously expressed mouse model has been reported [20, 21]. The *DDX3Y* locus on the Y-chromosome was selected as the targeted knock-in site since the *DDX3Y* knock-out mouse had a non-lethal and non-sterile phenotype [28]. It was predicted that no phenotypic changes would be observed even if the transgene affected *DDX3Y* gene expression. We established the Y-RFP ESC lines with a *CAG-tdTomato-T2A-Puro* transgene inserted in the intron between exon 1 and exon 2, and in the opposite direction to the *DDX3Y* gene two established Y-RFP ESC lines (Y-RFP #4 and Y-RFP #6). Subsequently, we generated Y-RFP mice implementing the tetraploid complementation technique using these two Y-RFP ESC lines (Figs. 1A and B; Table 1). Unexpectedly, all Y-RFP #4 mice died at 5-weeks old, and three of five mice from Y-RFP #6 died until 12-weeks old (Fig. 1D). The tdTomato is a DsRed variant which may be cytotoxic if it is highly expressed *in vivo* [29]. High expression of tdTomato may have caused the lethal phenotype of the Y-RFP mouse because it suppressed the growth of *E. coli* DH10B cells [29]; the cytotoxic components may have accumulated after birth.

Meanwhile, mice with mitochondria-localized DsRed2 survive and exhibit a strong RFP signal [30]. Therefore, we generated a new Y-linked RFP transgenic mouse model with tdTomato localized in the mitochondria in

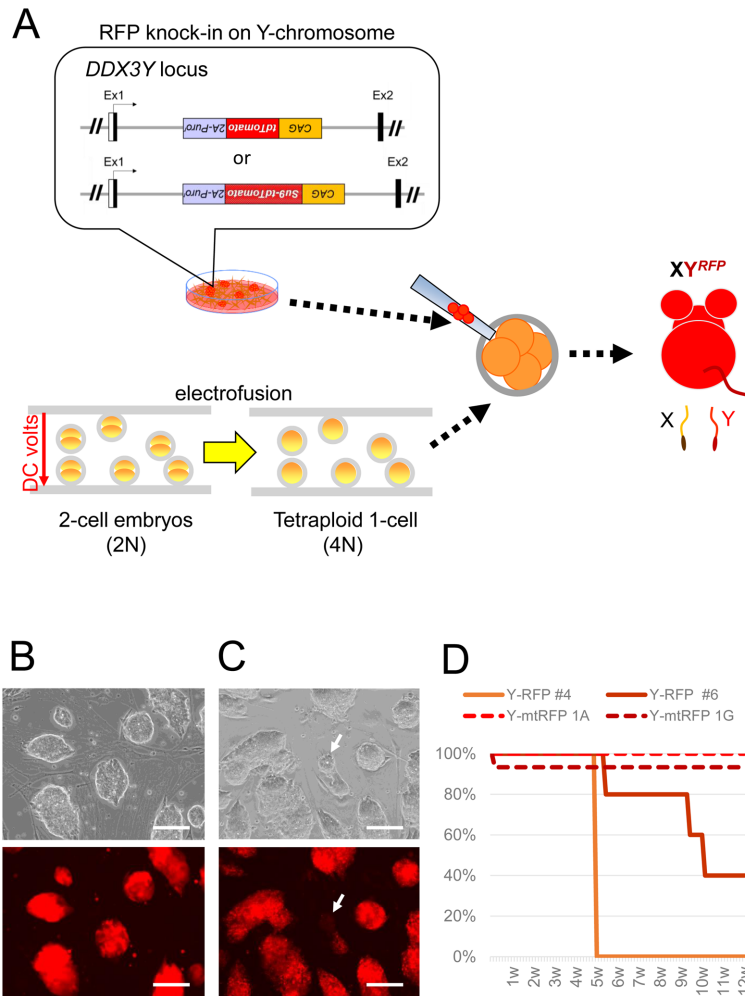


Fig. 1. Generation of Y-RFP mice. (A) Strategy for the generation of Y-RFP mice. (B) Y-RFP embryonic stem cell line (Y-RFP ESC #6). (C) Y-mtRFP embryonic stem cell line (Y-mtRFP ESC 1G). The arrow indicated a RFP-negative colony. The scale bars in B and C represent 100 μ m. (D) Survival rate of Y-RFP and Y-mtRFP offspring by the tetraploid complementation method.

Table 1. 4n complementation of Y-RFP ESC lines

ESC line	No. of transferred	No. of offspring	12-week survival
Y-RFP #4	10	4	40%
Y-RFP #6	20	5	25%
Y-mtRFP 1A	40	7	18%
Y-mtRFP 1G	40	15	38%

an attempt to reduce the cytotoxicity of RFP. Y-mtRFP ESC lines were established to generate the new Y-linked RFP transgenic (Y-mtRFP) mouse (Fig. 1C). Y-mtRFP_1A and Y-mtRFP_1G, offspring were obtained from two Y-mtRFP ESC lines by tetraploid complementation. All offspring grew at least until 12-weeks old except one mouse, which died one day after birth. This showed that the fluorescence protein's cytotoxicity was ameliorated by its localization to the mitochondria.

The Y-mtRFP male mice were mated with wild-type female mice to confirm their germline transmission po-

tency. F1 generation pups were born from both lines, and all males had RFP signals (Fig 2A). These F1 generation of Y-mtRFP male mice grew normally, had fertility, and had no other clear phenotypes as same as funders.

Some sequences in the intron are necessary for RNA splicing [31]. Therefore, RT-PCR was used to ascertain whether the insertion of the *CAG-tdTomato-T2A-Puro*' transgene, which is located in the intron between exon 1 and exon 2 of the *DDX3Y* locus on the Y-chromosome in the Y-mtRFP male, affected the expression of *Ddx3y*. *Ddx3y* was expressed in the wild-type male brain,

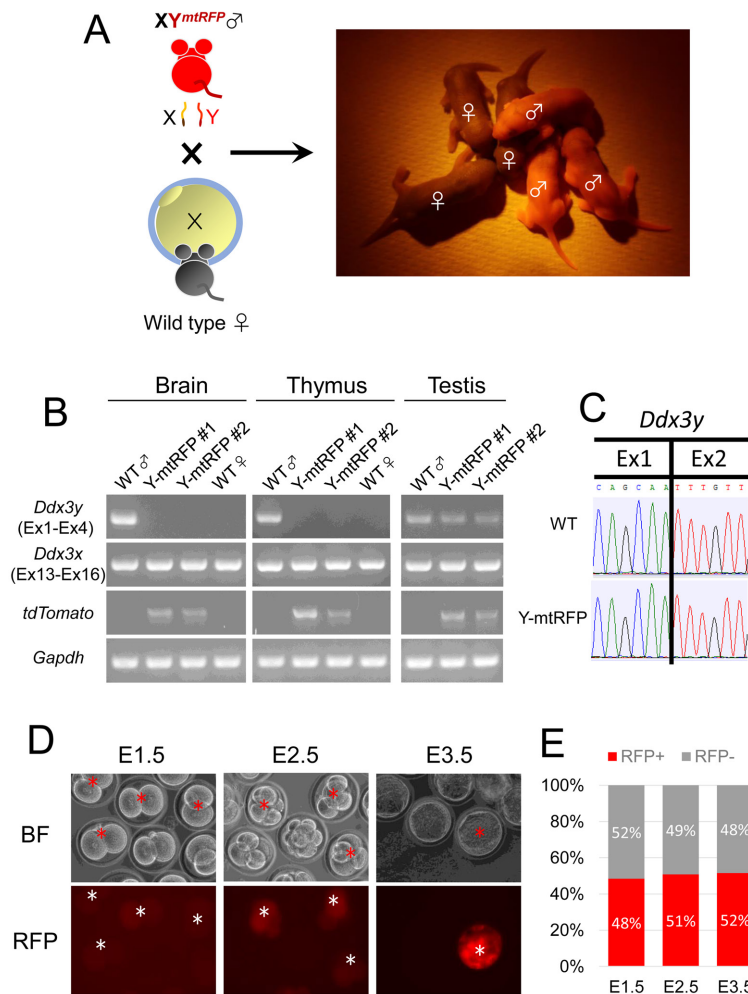


Fig. 2. Generation of a Y-mtRFP mouse line and sexing of embryos at the preimplantation stage. (A) F1 pups from mated experiments using a wild-type female mouse and a F0 Y-mtRFP male mouse. All RFP-positive pups were males. (B) Gene expression of the *DDX3Y* locus in the adult brain, thymus and testis. WT indicates wild-type mice, and Y-mtRFP indicates Y-mtRFP_1G male mice. (C) Sequences of a part of exon 1 and exon 2 in the expressed *Ddx3y* in the testes. (D) Identification of RFP-positive embryos at the preimplantation stages. Asterisks indicate RFP-positive embryos. (E) The ratio of RFP-positive (RFP+) and non-fluorescent (RFP-) embryos at the preimplantation stages. The numbers of counted embryos are showed in Table 2.

Table 2. Sexing in preimplantation stage embryos

Female	Male	No. of pairs	Total eggs	E1.5		E2.5		E3.5	
				RFP+	GFP+	RFP+	GFP+	RFP+	GFP+
Wild-type	XY ^{mtRFP}	3	126	61	N/A	64	N/A	65	N/A
Wild-type	X ^{GFP} Y ^{mtRFP}	5	217	95	0	112	82	113	104

“N/A” means “not applicable”.

thymus, and testis and the Y-mtRFP male testis but not in the Y-mtRFP male brain and thymus. Meanwhile the expression of *Ddx3x*, which is a homolog of *Ddx3y* on the X-chromosome, was detected in the brains and thymi of wild-type male, wild-type female, and Y-mtRFP males (Fig 2B). The RNA splicing of *Ddx3y* in the Y-mtRFP testis was executed with accuracy (Fig 2C).

The mRNA expression of *Ddx3y* in the Y-mtRFP mouse only in the testis, but not in the brain and thymus, suggested that the effect of the transgene inserted in the intron was differed according the kind of tissues, and it may have been induced by tissue-specific transcription mechanisms such as splicing and RNA stability.

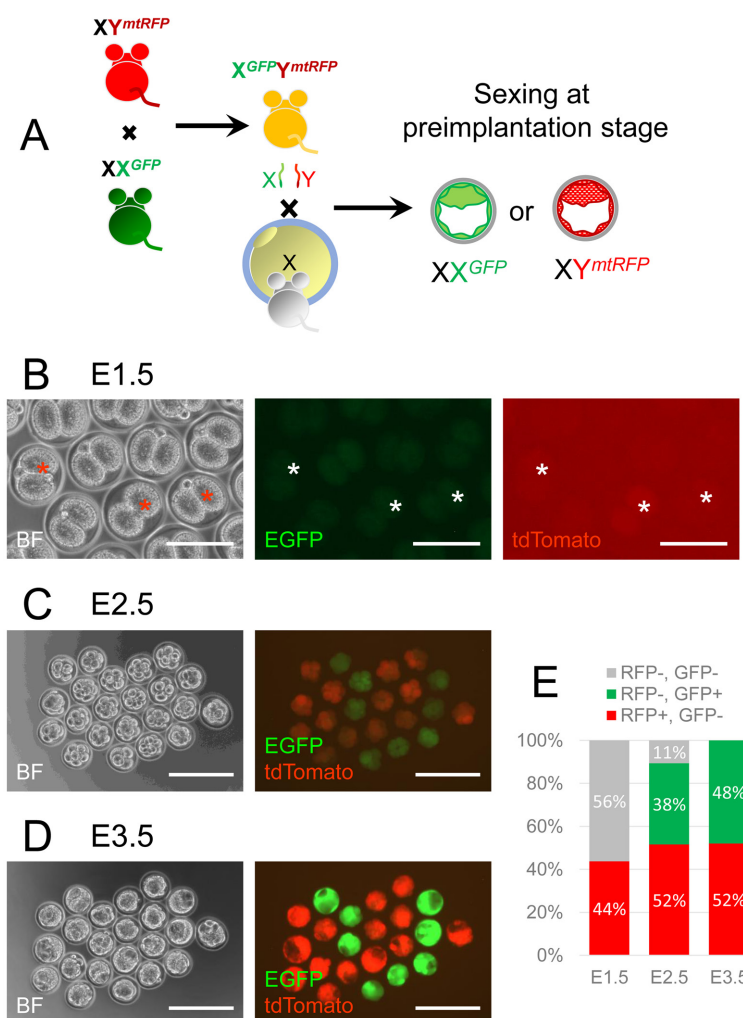


Fig. 3. Generations of X-GFP/Y-RFP mice and sexing of embryos at the preimplantation stage. (A) Strategy for the generation of X-GFP/Y-mtRFP mice. (B–D) Identification of RFP-positive or GFP-positive embryos at the preimplantation stages. Asterisks in B indicate RFP-positive embryos. Scale bars represent 100 μm in B, and 200 μm in C and D. (E) The ratio of non-fluorescent (RFP-, GFP-), GFP-positive (RFP-, GFP+), and RFP-positive (RFP+, GFP-) embryos at the preimplantation stages. The numbers of counted embryos are showed in Table 2. BF refers to bright-field microscopy.

Sexing at the preimplantation stage using Y-mtRFP mouse

Non-invasive sexing in the preimplantation stage was observed by cellular differentiation from the two-cell stage embryos at E1.5 (weak RFP signal) to the blastocyst stage embryos at E3.5 (strong RFP signal) (Figs. 2D and E, and Table 2). This study showed that it is possible to observe sexing of male embryos at the two-cell stage which is earlier than that stated in previous reports, which were at the eight-cell stage [9, 11].

We concluded that non-invasive sexing at the preimplantation stage embryo was possible by the Y-mtRFP mouse. However, we could not eliminate the possibility that the RFP-negative XO-type embryo may be caused by LOY. LOY occurs at a frequency of 1–3% in ES cells

[15, 32], and we also observed some RFP-negative colonies in the Y-mtRFP ES cell line (Fig. 1C).

To discover the probability of the appearance of the RFP-negative embryo at the preimplantation stage, we generated the X-GFP/Y-mtRFP mouse by mating the Y-mtRFP male with the X-GFP female (Fig. 3A). Theoretically, when a wild-type female mouse mated with the X-GFP/Y-mtRFP male mouse, the obtained female embryos were RFP-negative and GFP-positive, while male embryos were RFP-positive and GFP-negative. If RFP and GFP-negative embryos were observed, it was predicted that LOY was induced before starting zygotic gene activation in the embryo or during spermatogenesis. The RFP signals in the male embryos were detected in some of the E1.5 embryos similar to Fig. 2D. The GFP signal

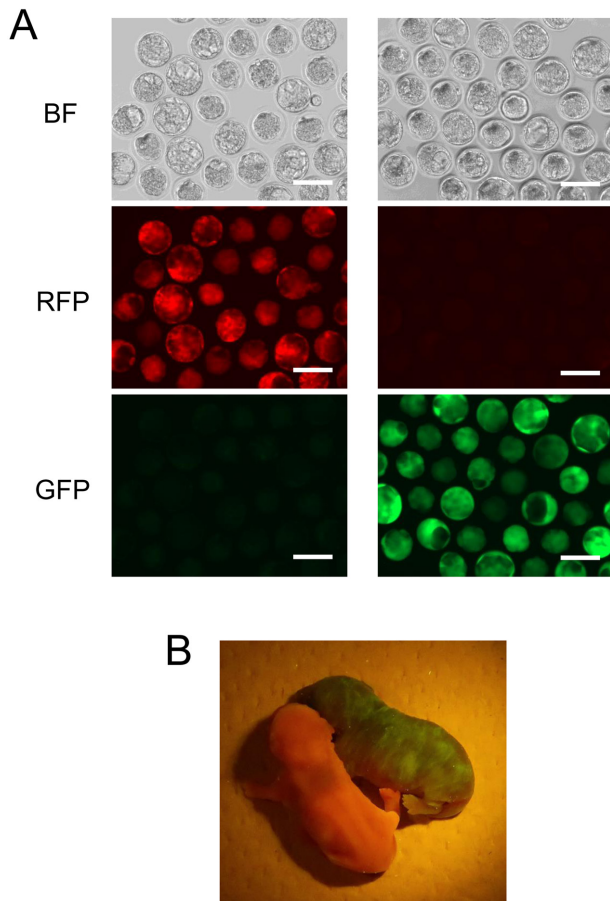


Fig. 4. Offspring after sexing in preimplantation stages. (A) After embryos were observed for sexing from E1.5 to E3.5, they were separated into RFP-positive (RFP+, GFP-) embryos and GFP-positive (RFP-, GFP+) embryos at E3.5. The embryos were transferred into E2.5 uteri of pseudopregnant females. Scale bars represent 100 μm . (B) The RFP-positive male pup (left) from an RFP-positive embryo and the GFP-positive female pup (right) from a GFP-positive embryo were born.

in female embryos were observed after approximately E2.5 (Figs. 3B–E, and Table 2). No RFP- and GFP-negative embryos were found in this study.

Furthermore, embryos after sexing were transferred into the uteri of pseudopregnant mice. Forty-three male offspring were born from RFP-positive embryos ($n=55$, 78%) and twenty-eight female offspring were born from GFP-positive embryos ($n=54$, 52%) (Fig. 4). All offspring were breathing, and significant abnormalities were not found. Since embryos after sexing were transferred into five pseudopregnant mice (two mice were transferred RFP-positive embryos only, two mice were transferred GFP-positive embryos only, and one mouse was transferred RFP-positive embryos into the right uterus and GFP-positive embryos into the left uterus), their condition may have affected the difference in the birth rate.

In summary, we established non-lethal, RFP-tagged Y-chromosome mouse lines with RFP signals distinguishable from the GFP signal. These established Y-mtRFP mouse models allow for sex determination before the preimplantation stage, and may be useful for further research into LOY and sex chromosomal aneuploidy.

Author Contributions

W.H., and A.I. performed most experiments, assisted by T. T. who performed establishment of Y-mtRFP ESC lines, and S.Y., performed construction of plasmids. W.H., and A.I. analyzed the data. A.I. wrote the manuscript and all authors discussed the results and commented on the manuscript.

Conflict of Interests

The authors declare no competing financial interests.

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