NOTE *Laboratory Animal Science*

Application of a New Convenience Gender Sorting Method for Mouse Spermatozoa to Mouse Reproductive Engineering Technology

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(Received 6 July 2012/Accepted 24 September 2012/Published online in J-STAGE 5 October 2012)

ABSTRACT. In this study, we attempted to apply new convenience gender sorting methods using sex-determining region Y (SRY) gene expression on Y spermatozoa to mice. Mouse spermatozoa labeled with Cy3-SRY antibody conjugate were used for intracytoplasmic sperm injection (ICSI). In addition, spermatozoa conjugated with SRY antibody were conjugated with magnetic beads (Mag) and were pulled to the bottom of the medium. The supernatant of the medium was used for *in vitro* fertilization (IVF). The rate of males reproduced by ICSI using the spermatozoa conjugated with Cy3-SRY antibody was 86.1%. The female proportion reproduced by IVF using the spermatozoa separated in the supernatant after Mag-SRY antibody conjugation was 67.3%. These gender sorting methods are effective for the reproduction of transgenic mice.

KEY WORDS: gender sorting, *in vitro* fertilization, Y spermatozoa.

doi: 10.1292/jvms.12-0303; *J. Vet. Med. Sci.* 75(2): 231–235, 2013

The goal of this research was to establish a new convenience gender sorting method for mammals, which has been difficult in the past. As a first step, a mouse reproductive engineering technology was established. The establishment of gender sorting technology is essential for the reproduction of laboratory animals [7], livestock [1, 3, 6] and humans [2, 5, 8, 15]. The reproduction of mice and rats by reproductive engineering technology included intracytoplasmic sperm injection (ICSI), *in vitro* fertilization (IVF) and embryo transfer (ET), which are important technologies in laboratory animal facilities. Therefore, we attempted gender sorting targeted at mice with an established reproductive technology. There is high demand for females for the production of mice. For example, the reproduction of mice carrying several mutant genes, such as IRS-1 deficient $Gck^{-/+}$ mice [12] and PPARγdeficient ob/ob mice [9], using reproductive engineering technology requires many females in the production process, although a male is required in the final stage. When no accidental bias occurs, the gender ratio after ET and Cesarean section is usually 50:50 (female:male). Modi *et al.* (2005) reported that SRY transcription occurs in spermatozoa in humans [10]. From their study, we assumed that the same phenomenon occurs in mouse spermatozoa. If this was true, then the sex-determining region Y (SRY) protein may also be produced by spermatozoa. Therefore, we examined the previous report indicating that SRY transcription occurs in spermatozoa in humans [10] and contrived a new technology for gender sorting using fluorescence or magnetic beads via SRY antibody, using mice as an example of mammals.

Because the SRY gene consists only of an exon, SRY gene expression was confirmed by two polymerase chain reaction (PCR) experiments using primers designed with a specific sequence of intra-exons to detect cDNA or a sequence ranging from the 5'- genomic region to the exon to detect genomic DNA (Fig. 1). To generate positive control vectors, RNA and genomic DNA were extracted from the sperm and testes of C57BL/6JJcl mice at the age of 14 weeks using TRizol reagent (Invitrogen, Gland Island, NY, U.S.A.) according to the manufacturer's instructions. As a negative control, RNA extracted from the pancreas of the same mice at the age of 14 weeks was used. RNA was then reverse-transcribed to cDNA using Super Script III RNaseH$^-$ reverse transcriptase (Invitrogen). The primers used to detect cDNA were 5'-GCAGGTGGAAAAGCCTTACA-3' (Forward) and 5'-AAGCTTTGCTGGTTTTTGGA-3' (Reverse). After an initial incubation at 95°C for 4 min, PCR amplification was performed for 35 cycles of denaturation at 94°C for 30 sec, annealing at 63.5°C for 20 sec and extension at 72°C for 25 sec. This was followed by a final extension at 72°C for 5 min. The primers used to detect genomic DNA were 5'-TGACCCGATGGCACTGTGT-3' (Forward) and 5'-AAGCTTTGCTGGTTTTTGGA-3' (Reverse). After an initial incubation at 95°C for 4 min, PCR amplification was performed for 35 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 50 sec in 20 μl of reaction mixture containing 1.5 mM Mg^{2+} and Ex-Taq (TaKaRa, Otsu, Japan). This was followed by a final extension at 72°C for 5 min in 20μl of reaction mixture containing 1.5 mM Mg^{2+} and Ex-Taq (TaKaRa). These PCR products were cloned into T-vectors and confirmed by sequence analysis. Cy3 (ANA SPEC, Campus Drive Fremont, CA, U.S.A.) labeling was performed according to the manufacturer's protocol as follows; 10 μl of DMSO was added to a vial containing Cy3 to produce a 2 mM dye solu-

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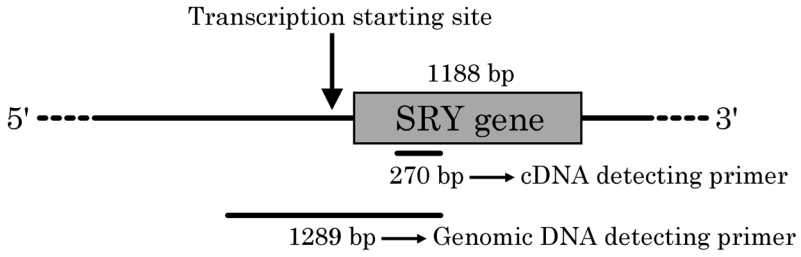

Fig. 1. Design of PCR primers for detecting SRY gene expression in mouse spermatozoa.

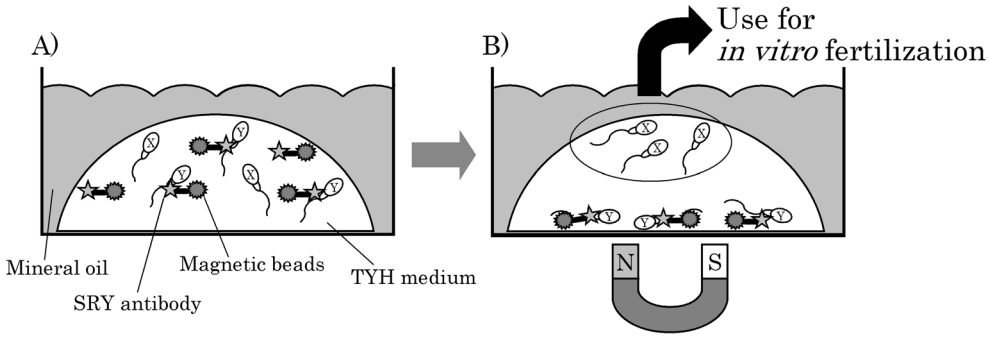

Fig. 2. Gender sorting method for Y spermatozoa. A) Addition of conjugates SRY antibody and magnetic beads to TYH medium containing mouse spermatozoa. B) Pulling of Y spermatozoa to the bottom of the TYH medium.

tion. The dye solution was added to an IgG or SRY antibody [Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A., SRY(M140): sc-33169; a rabbit polyclonal antibody raised against aminoacids 1–140 mapping at the N-terminus of SRY of mouse origin] solution at a dye-to protein molar ratio of 10:1. For the IgG solution, 6.7 μl of 2 mM Cy3 solution was added to 200 μg of IgG. The reaction mixture was kept away from light and shaken for 15 min at room temperature on a shaker. The gel to select Cy3-SRY antibody conjugate by size exclusion chromatography was resuspended in a spin column. The column was placed in a wash tube and centrifuged at 1,000 × g for 2 min. The gel-packing buffer was exchanged by adding 500 μl of elution buffer to the spin column, and the column was again centrifuged at 1,000 × g for 1 min. The spin column was then placed in a clean collection tube. The reaction mixture was applied to the center of the gel bed surface, and the column was centrifuged at 1,000 × g for 4 min. The Cy3-SRY antibody conjugate was added to the collection tube. Then, 10 μl of the Cy3-SRY antibody conjugate was added to 200 μl of TYH medium containing suspended mouse sperm.

To eliminate sodium azide, SRY antibody was filtered using Amicon Ultra-4 filters (Millipore, Billerica, MA, U.S.A.). For magnetic beads (Mag)-SRY antibody conjugation, 10 μl of SRY antibody was added to TYH medium [13, 14] containing mouse spermatozoa, and the reaction mixture was incubated at 37°C for 30 min. Then, 10 μl of Mag was added to TYH medium containing mouse spermatozoa and SRY antibody and incubated at 37°C for 1 hr (Fig. 2A). Y spermatozoa conjugated with SRY antibody and magnetic beads were magnetically separated to the bottom of the TYH medium. The supernatant was expected to include X spermatozoa and was used for IVF (experimental group) (Fig. 2B). Spermatozoa with magnetic beads only were used as control (control group). ICSI, IVF and ET were performed in C57BL/6JJcl mice (CLEA Japan, Inc., Tokyo, Japan) at 10–12 weeks of age as followed the past reports [4, 17].

PCR bands were detected in mouse spermatozoa, testes, mouse genomic DNA, and SRY cDNA cloned into T-vectors by specific cDNA primers (Fig. 3, Lanes 2–6) and in mouse genomic DNA and SRY genomic regions cloned into T-vectors only by genomic DNA primers (Fig. 3, Lanes 8–12). The PCR results revealed that the SRY gene was expressed in mouse spermatozoa and testes. Spermatozoa labeled with SRY antibody and Cy3 are shown in Fig 4. Observation by fluorescent (Fig. 4A) and light microscopy (Fig. 4B) revealed spermatozoa with Cy3 fluorescence as well as without Cy3 fluorescence. Because the antibody used was SRY antibody, the spermatozoa with Cy3 fluorescence displayed Y spermatozoa, and these spermatozoa with Cy3 fluorescence were selected from the medium and used for ICSI. The results of gender sorting and reproduction of mice produced by ICSI using spermatozoa with or without Cy3 fluorescence are shown in Table 1. ICSI using the spermatozoa conjugated with Cy3 and SRY antibody succeeded reproducing 31 males out of 36 progenies (86.1%). Conversely, ICSI using spermatozoa without Cy3 conjugation reproduced 20 males out of 44 progenies (45.5%). The results of magnetic gender sorting applied to IVF and ET are shown in Table 2. The number of oocytes in the experimental and control groups was 267 and 191, respectively. Their IVF rates of these groups were 66.3% (177 two-cell embryos) and 68.1%

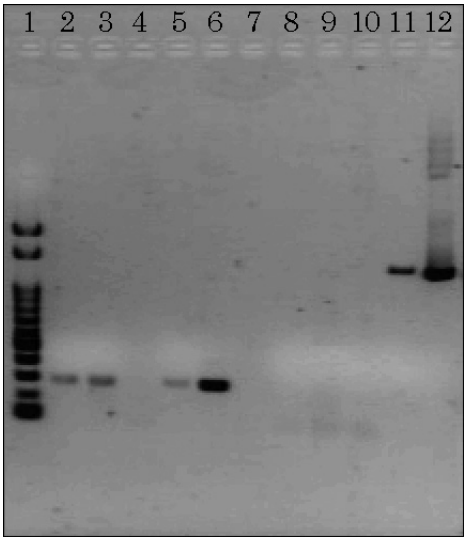

Fig. 3. Confirmation of SRY gene expression in mouse spermatozoa using PCR and electrophoretic separation. Lane 1: 100 bp Marker. Lane 2: Spermatozoa-cDNA detection primer. Lane 3: Testis-cDNA detection primer. Lane 4: Pancreas-cDNA detection primer. Lane 5: Genomic DNA-cDNA detection primer. Lane 6: SRY cDNA cloned into T-vector-cDNA detection primer. Lane 7: −. Lane 8: Spermatozoa-Genomic DNA detection primer. Lane 9: Testis-Genomic DNA detection primer. Lane 10: Pancreas-Genomic DNA detection primer. Lane 11: Genomic DNA-Genomic DNA detection primer. Lane 12: SRY cDNA cloned into T-vector-Genomic DNA detection primer.

(130 two-cell embryos), respectively, and the difference in rates was not significant between the groups. The mean female percentage among progenies was 50.3% in the control group, compared with 67.3% in the experimental group.

Modi *et al.* (2005) reported that SRY transcription occurs by spermatozoa in humans [10]. From this, we inferred that the same phenomenon may occur in mouse spermatozoa and SRY protein may also occur in spermatozoa. We investigated the expression of SRY cDNA in mouse spermatozoa. Our study confirmed that, the mouse spermatozoa expressed the SRY gene. Next, we examined whether Y spermatozoa could be differentiated from X spermatozoa by SRY antibody labeled with Cy3. We succeeded in conjugating Y spermatozoa with the Cy3-labeled SRY antibody and distinguishing Y spermatozoa from X spermatozoa. Based on these results, we thought that magnetic gender sorting would be possible after substituting Cy3 with magnetic beads. Gender sorting using flow cytometry and ICSI is effective for human [11] and livestock [3] studies that require gender certainty of spermatozoa. ICSI facilitated high-precision gender sorting in this study. In contrast, gender sorting with IVF using magnetic beads was not precise. ICSI is the technology which makes an immature spermatozoa succeeded in fertilization [16], so even if the spermatozoa activity is induced to be low by labeling for gender sorting, it is possible to obtain mouse infants sufficiently. On the other hand, the labeling to spermatozoa in IVF would influence negatively in mouse infant production. The labeling for gender sorting for IVF has a lot more steps compared with ICSI. In addition, there is a possibility that X spermatozoa is involved with magnetic beads by pulling to the bottom of the medium at a magnetic separa-

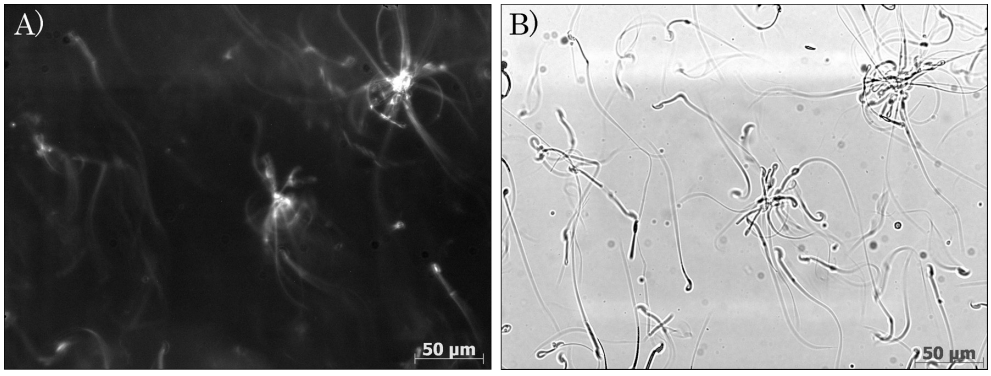

Fig. 4. Confirmation of SRY protein expression in mouse spermatozoa using a Cy3-SRY antibody conjugate. A) Y spermatozoa were detected using the Cy3-SRY antibody conjugate. B) Observation by light microscopy.

Table 1. Gender sorting and reproduction of mice produced by ICSI with Cy3 fluorescence labeled spermatozoa

	No. of embryo	No. of 2-cells (%)	No. of infants	No. of Males (%)	No. of Females (%)
With Cy3 fluorescence	81	70 (86.4 %)	36	31 (86.1 %)*	5 (13.9 %)
Without Cy3 fluorescence	74	71 (95.9 %)	44	20 (45.5%)	24 (54.5 %)

*(P<0.05); vs No. of females (Fisher's exact probability test).

Table 2. Results of magnetic gender sorting applied to *in vitro* fertilization and embryo transfer

A) Experimental group

Number of pseudo-pregnant mice	Number of transferred embryos	Number (%) of deliveries	Number of female infants	Number of male infants	Rate (%) of female infants
1	20	14 (70.0)	9	5	64.3
2	20	15 (75.0)	10	5	66.7
3	20	12 (60.0)	8	4	66.7
4	20	13 (65.0)	8	5	61.5
5	20	13 (65.0)	9	4	69.2
6	20	14 (70.0)	9	3	75.0
7	18	11 (61.1)	7	5	58.3
8	19	12 (63.1)	9	3	75.0
9	20	13 (65.0)	9	4	69.2
Sum or Mean	177 [1)]	13.0 [2)] (66.0)[2)]	81 [1),**]	35 [1)]	67.3 [2),**]

B) Control group

Number of pseudo-pregnant mice	Number of transferred embryos	Number (%) of deliveries	Number of female infants	Number of male infants	Rate (%) of female infants
10	19	10 (52.6)	5	5	50.0
11	19	9 (47.3)	4	5	44.4
12	16	11 (68.7)	6	5	54.5
13	16	10 (62.5)	4	6	40.0
14	20	16 (80.0)	11	5	68.8
15	20	9 (45.0)	3	7	30.0
16	20	14 (70.0)	9	5	64.3
Sum or Mean	130	11.2 [2)] (60.9) [2)]	42	38	50.3

1) Sum of data. 2) Mean of data. *(P<0.05), **(P<0.01); vs control group (Student's t-test).

tion. We think that these possibilities influenced the results of the gender sorting for ICSI and IVF. Therefore, the gender sorting method using magnetic beads needs improvement. However, these gender sorting methods appear sufficient for mouse IVF and ET when mass reproduction is desired because these methods are simple and only conjugates are added to a culture medium. The reproduction of transgenic mice with more than 2 mutant genes requires several rounds of IVF and ET. Substantial female reproduction is possible with these processes, because the homozygous or heterozygous females obtained in the first round of IVF and ET will be available for the second round of IVF and ET.

Future studies will attempt to improve the precision and accuracy of gender sorting with IVF using SRY antibody and magnetic bead conjugates.

ACKNOWLEDGMENTS. The authors are grateful to Ryoji ITO, Tsutomu KAMISAKO, Keiko ENDO, and Gen ITAI for important advice on immunoreactions and reproductive technology. This study was supported by a Grant-in-Aid for Challenging Exploratory Research (No. 22650095) and a Grant-in Aid for Scientific Research (C) (No. 24500500) to Haruo HASHIMOTO from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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