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—Full Paper—

Sex-Reversed Somatic Cell Cloning in the Mouse

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Abstract. Somatic cell nuclear transfer has many potential applications in the fields of basic and applied sciences. However, it has a disadvantage that can never be overcome technically—the inflexibility of the sex of the offspring. Here, we report an accidental birth of a female mouse following nuclear transfer using an immature Sertoli cell. We produced a batch of 27 clones in a nuclear transfer experiment using Sertoli cells collected from neonatal male mice. Among them, one pup was female. This "male-derived female" clone grew into a normal adult and produced offspring by natural mating with a littermate. Chromosomal analysis revealed that the female clone had a 39,X karyotype, indicating that the Y chromosome had been deleted in the donor cell or at some early step during nuclear transfer. This finding suggests the possibility of resuming sexual reproduction after a single male is cloned, which should be especially useful for reviving extinct or endangered species.

Key words: Mouse, Nuclear transfer, Sertoli cell, Sex reversal

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Although somatic cell nuclear transfer (SCNT) has been successfully applied as a reproductive technique without gametes to a wide range of mammalian species, the rates of full-term development are still very low, irrespective of the species or the donor cell type used [1]. However, many researchers are intensively studying the epigenetics of genomic reprogramming, and indeed, some significant improvements have recently been achieved [2, 3]. In the near future, the low efficiency of SCNT should not be an insuperable problem [1]. In contrast, another problem associated with SCNT that seems very difficult to overcome is the inflexibility of the sex of the offspring. Cloning male cells inevitably results in the birth of males, not females, and vice versa. This sex irreversibility may become an important issue, especially when sexual breeding must be resumed for subsequent propagation, e.g., in projects for resurrection of extinct species or rescue of endangered species. Recently, two Japanese groups have reported the birth of normal clones following nuclear transfer cloning with somatic cells retrieved from bodies or organs frozen for many years [4, 5]. These studies are encouraging to those who want to bring back the woolly mammoths lying frozen under the Siberian permafrost. In such cases, all the anticipated clones would be the same sex as the donor, and they would never propagate by natural breeding.

In a recent cloning experiment, we obtained a female mouse from an immature Sertoli cell. This "male-derived female" clone grew into a normal adult and produced offspring by natural mating. Although this was an accidental phenomenon arising from a sex-chromosomal error, the result unequivocally suggests the possibility of producing females from male donor animals if the techniques of sex chromosome manipulation are sufficiently well developed. Here, we report a case of sex-reversed SCNT in the mouse.

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Materials and Methods

Animals

The nuclear donor strain, (B6x129)F1, was obtained by mating two strains, an $Xist^{1loxGFP}$ C57BL/6 female (BioResource Center [BRC] no. RBRC01260) and a 129/Sv-*ter* male (CLEA Japan, Tokyo, Japan). The $Xist^{1loxGFP}$ C57BL/6 line was originally generated by Sado *et al*. [6]. In our laboratory, these knockout mice have been used to examine the effects of *Xist* downregulation on the development of clones, and we believe that such genetic modification had no relation to the finding in this report. Eight- to 10-week-old (C57BL/6 × DBA/2)F1 (BDF1, Japan SLC, Shizuoka, Japan) and 8–12-week-old ICR (CLEA Japan) female mice were used for the collection of the recipient oocytes and as embryo transfer recipients, respectively. The mice were maintained under specific-pathogen-free conditions. They were provided with water and commercial laboratory mouse chow *ad libitum* and housed under controlled lighting conditions (daily light period of 0700–2100 h). All animals were maintained in accordance with the guidelines of the RIKEN BioResource Center.

Preparation of donor cells

Immature Sertoli cells were collected from day 7 male neonates. The cells were prepared as described previously [7]. Briefly, the collected testicular cells were treated with 0.1 mg/mL collagenase (Sigma Chemical, St Louis, MO, USA) and 0.01 mg/ml deoxyribonuclease (Sigma) for 30 min at 37 C followed by 0.2 mg/ml trypsin (Sigma) for 5 min at 37 C. The testicular cell suspension was washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) con-

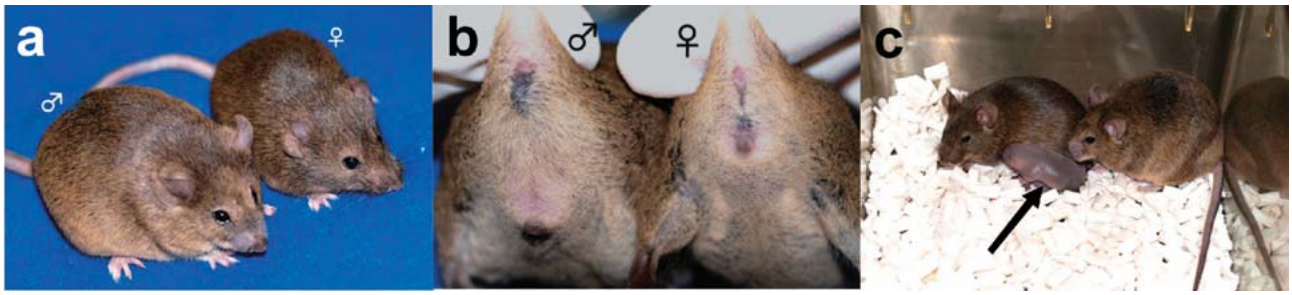


Fig. 1. a, b) Gross appearance (a) and external genitalia (b) of a female mouse and a male mouse cloned from Sertoli cells. c) Offspring (arrow) born from the female cloned mouse. The fertility of the female clone was confirmed by pregnancy and birth of normal offspring.

taining 4 mg/ml bovine serum albumin and used for injection.

Nuclear transfer

Nuclear transfer was carried out as described previously, with slight modifications [2, 7, 8]. The BDF1 female mice were induced to superovulate by the injection of 7.5 IU of pregnant mare serum gonadotropin (Sankyo Yell, Tokyo, Japan) and 7.5 IU of human chorionic gonadotropin (hCG; Aska Pharmaceutical, Tokyo, Japan) with a 48-h interval between the injections. At 15 h after the hCG injection, cumulus-oocyte complexes were collected from the oviducts, and the cumulus cells were dispersed in KSOM medium containing 0.1% bovine testicular hyaluronidase (Calbiochem, San Diego, CA, USA). The oocytes were enucleated in Hepes-buffered KSOM containing 7.5 μg/ml cytochalasin B. The donor nuclei were injected into enucleated oocytes with a Piezo-driven micromanipulator. After culture in KSOM for 1 h, the injected oocytes were activated in Ca^{2+}-free KSOM containing 2.5 mM $SrCl_2$ and 5 nM trichostatin A (TSA; Sigma) for 1 h. The reconstructed embryos were cultured in KSOM containing 5 μg/ml cytochalasin B and 5 nM TSA for 5 h, and then in KSOM containing 5 nM TSA for 2 h.

Embryo transfer

The reconstructed embryos that had reached the four-cell stage after 48 h in culture were transferred into the oviducts of pseudopregnant ICR female mice on day 0.5 (the day following sterile mating). On day 19.5, the pregnant females underwent Caesarian section, and the live pups were nursed by lactating ICR females.

Chromosomal analysis

Tail-tip fibroblasts were subjected to chromosomal analysis. The fibroblast cells were treated with 25 ng/ml colcemide for 4–6 h in culture dishes. The cells were collected by trypsinization, treated with 0.075 M KCl for 15 min at 37 C, and fixed with methanol:acetic acid (3:1). The cell suspensions were spread onto clean glass slides and allowed to dry in air. The prepared slides were stained with 5% Giemsa solution (Merck, Darmstadt, Germany) and examined with a light microscope using a 100× objective lens (Nikon, Tokyo, Japan). For Q-banding staining, the cells were stained by a combined quinacrine-33258 Hoechst method [9]. Metaphase images were observed under a fluorescent microscope, and karyotype analysis was performed using an Ikaros karyotyping system (Carl Zeiss, Jena, Germany).

Results and Discussion

We reconstructed 347 embryos by nuclear transfer using immature Sertoli cells. After 48 h in culture, 196 embryos (56.5%) had developed to the four-cell stage and were transferred into the oviducts of recipient females. Twenty-seven (13.8% per transfer) cloned offspring were born by Caesarean section on day 19.5 and grew into adults. All offspring showed agouti coat color, as expected from the genotype of the donor cells. When they were examined for external genitalia by gross observation, one of the 27 clones seemed to be a female, with a vaginal opening and no penile structure (Fig. 1a and b). The remaining 26 clones were normal males in appearance. To determine whether this female-looking clone was behaviorally and functionally a female, we mated it with a male littermate. Within about five months, it gave birth to three litters (one, two, and five pups each; 4 females and 4 males). Thus, despite a low reproductive performance, it was a fertile female born after SCNT using a male mouse (Fig. 1c). We analyzed 10 chromosome spreads from tail-tip cells collected from the female clone. All spreads showed a 39,X karyotype, indicating that the clone was an XO female, resulting from the loss of the Y chromosome (Fig. 2).

Although a more detailed systemic karyotype analysis is required, we tentatively infer that the Y chromosome was deleted in the donor cell or at some early step during nuclear transfer. It is known that the Y chromosome can be lost in populations of cultured mouse embryonic stem (ES) cells and that such XO ES cells can give rise to female mice via chimeric embryo formation [10] or nuclear transfer [11, 12]. However, because we used the donor cells shortly after their collection from autopsied mice, it is very unlikely that the *in vitro* manipulation of the donor cells caused the loss of the Y chromosome. As far as we know, there is no information available about how often XO cells are generated in a male body, but some may survive *in situ* because no essential genes are coded on the Y chromosome. Although the sex-reversed SCNT in our case was accidental, theoretically, we may be able to produce female clones from donor male animals if it becomes possible to

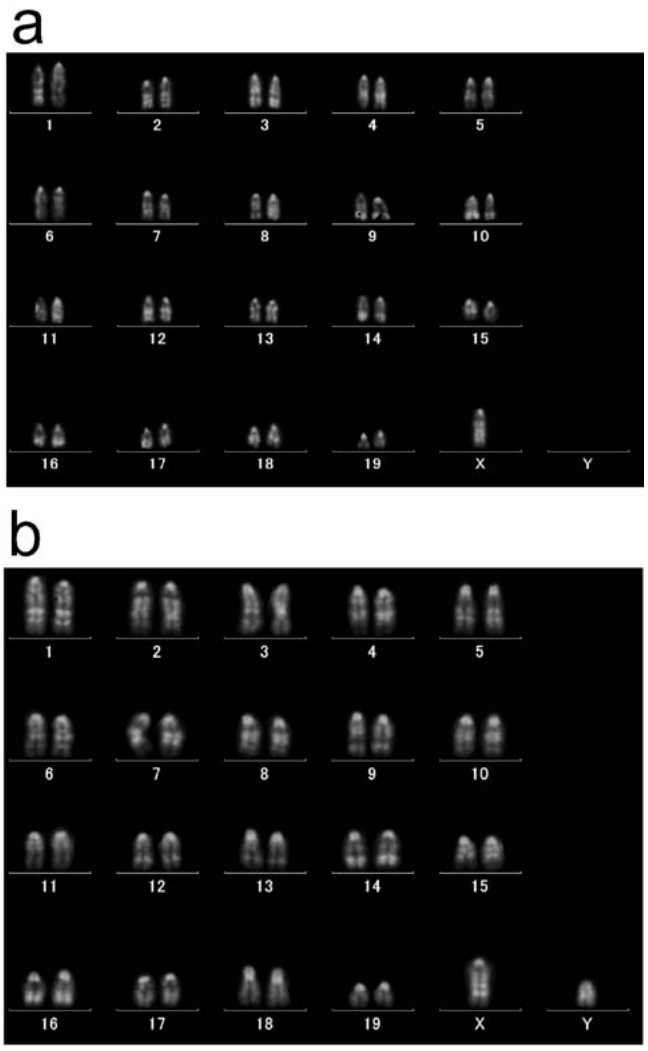


Fig. 2. Chromosome spreads from fibroblast cells from the female cloned from a Sertoli cell (a) and a male mouse (b). The 39 chromosome pairs and the absence of the Y chromosome in (a) indicate that it is an XO female.

sort XO cells from a suspension of male cells. Alternatively, establishment of ES cells from SCNT embryos may allow production of cloned XO females because ES cells facilitate *in vitro* genetic manipulation and increase SCNT efficiency. At present, nuclear-transferred ES cells can be generated efficiently only in mice [13], but recent significant improvements in controlling cell-autonomous pluripotency may allow the establishment of ES cells in other species [14, 15].

Another issue that should be considered in SCNT with XO cells is the normality of the resulting cloned females. In humans, the XO karyotype can arise from incomplete chromosomal segregation [16] and often results in the development of Turner syndrome, which is associated with various developmental, endocrine, cardiovascular, psychosocial and reproductive abnormalities [17]. Most (>95%) patients are infertile because of ovarian dysfunction. In contrast, XO female mice are anatomically normal and fertile [18], although they often show developmental retardation [19, 20]. This species-specific difference may arise from differences in the sensitivity of some X-linked genes to haploinsufficiency. The human *RPS4* (ribosomal protein S4) gene is responsible for the onset of Turner syndrome because it is biallelically expressed under normal conditions [21]. In contrast, its mouse homologue, *Rps4*, is a gene subject to X chromosome inactivation, and its function is assured by the presence of a single allele [22]. Little information about the occurrence of XO females is available for other species, but the laboratory mouse is not the only species known to produce normal XO females. A very few XO female shrews have been captured in their natural habitats [23].

If sex-reversed SCNT becomes available, it is expected to be most effectively used for production of offspring from very limited genetic resources. Recently, several lines of evidence have shown that the genetic integrity of somatic cells is more resistant to freezing processes than previously thought. Even after freezing without cryoprotectants [24], freeze-drying [25, 26] or freezing in intact bodies or organs for many years [4, 5], the nuclei of these dead cells retain their ability to support full-term development when transferred into enucleated oocytes. Therefore, we can speculate that, with this approach, we can rescue endangered species or resurrect animals that have already disappeared. However, SCNT is asexual reproduction and is expected to produce only animals of the donor sex. Although significant technical advances are required and inbreeding depression may become another issue, our finding clearly indicates the possibility that sexual reproduction may be resumed after the donor cells are cloned from one male animal. Our finding might provide a potential new reproductive technology for the rescue or maintenance of wild animals in the future.

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