Fertile offspring from sterile sex chromosome trisomic mice

Takayuki Hirota, Hiroshi Ohta, Benjamin E. Powell, Shantha K. Mahadevaiah, Obah A. Ojarikre, Mintinori Saitou, James M. A. Turner*

Having the correct number of chromosomes is vital for normal development and health. Sex chromosome trisomy affects 0.1% of the human population and is associated with infertility. We show that during reprogramming to induced pluripotent stem cells (iPSCs), fibroblasts from sterile trisomic XXY and XYY mice lose the extra sex chromosome through a phenomenon we term trisomy-biased chromosome loss (TCL). Resulting euploid XY iPSCs can be differentiated into the male germ cell lineage and functional sperm that can be used in intracytoplasmic sperm injection to produce chromosomally normal, fertile offspring. Sex differentiation can be differentiated into the male germ cell lineage and functional sperm that can be used in intracytoplasmic sperm injection to produce chromosomally normal, fertile offspring. Sex chromosome loss is comparatively infrequent during mouse XX and XY iPSC generation. TCL also applies to other chromosomes, generating euploid iPSCs from cells of a Down syndrome mouse model. It can also create euploid iPSCs from human trisomic patient fibroblasts. The findings have relevance to overcoming infertility and other trisomic phenotypes.

The mammalian sex chromosomes have specialized roles in male (XY) and female (XX) germ cell development (1). Sex chromosome abnormalities are the most common genetic cause of human infertility (2). In the sex chromosome trisomies (SCTs) Klinefelter (XXY) and double Y (XXY) syndrome, spermatogenesis is disrupted by excess X and Y genes, respectively (2). XXY men are commonly fertile due to spontaneous loss of the extra sex chromosome (mosaicism). In XXY men, mosaicism is less common. Testicular sperm retrieval has enabled reproduction from some young Klinefelter men but is less successful in older patients (3, 4). XXY and XYY individuals without XY germ cells are infertile.

To study SCT infertility, we generated adult XXY and XYY mice carrying the fluorescent reporter transgenes Blimp1-mVenus (BV) and Stella-ECFP (SC) (5) to monitor differentiation of pluripotent stem cells into primordial germ cell–like cells (PGCLCs) (6). XXY males were created by mating a wild-type female to a sex chromosome variant male that produces XY-containing sperm (fig. S1). Generation of XYY mice requires inheritance of a Y chromosome from both parents. We therefore used a paternally inherited wild-type Y chromosome and a maternally inherited Y chromosome that does not express the testis-determinant Sry (fig. S1) (7). As shown previously (8, 9), the spermatogenesis phenotype in both models recapitulated that in SCT men, with arrest at the spermatogonia stage in XYY mice and at pachynema in XYY mice (fig. S2). Spermatogenesis was normal in euploid XY BVSC transgenic siblings.

Next, we established fibroblasts from SCT and control XY and XX mice (Fig. 1A). DNA-fluorescence in situ hybridization (DNA-FISH) for the X gene Sla and Y gene Sly confirmed that passage 4 (P4) SCT and control fibroblasts had retained their original sex chromosome complements (Fig. 1B and fig. S3A). Fibroblasts were reprogrammed to induced pluripotent stem cells (iPSCs) (10) in a doxycycline (Dox)–inducible manner. DNA-FISH was performed in resulting P2 iPSCs (Fig. 1A).

A high proportion of SCT-derived iPSC lines exhibited sex chromosome loss. From XYY mice, we observed XY, XX, and XO iPSCs (Fig. 1, C and E). The incidence of loss was similar for the X and Y chromosomes (P = 0.062, Mann-Whitney test). From XYY mice, we observed XY and XO iPSCs (Fig. 1, D and E). Y chromosome loss in

Fig. 1. Chromosome loss through iPSC reprogramming of SCT fibroblasts. (A) Experimental scheme to generate XXY, XYY, XY, and XX iPSCs. 2i, inhibitors of GSK3β and Mek1/2; LIF, leukemia inhibitory factor. (B to D) Slx (green) and Sly (magenta) DNA-FISH of (B) fibroblasts and [(C) and (D)] P2 iPSCs from XXY and XYY mice (n = 50 cells). Scale bars, 5 μm. (E) P2 iPSC sex chromosome complements. Each bar represents an iPSC line and the percentage of cells exhibiting each complement (n = 50 cells per line). Numbers in brackets show number of iPSC lines examined. Data for two animals combined.
XY males occurred at a similar frequency to that observed for the X and Y chromosome combined in XXY males ($P = 0.08$, Mann-Whitney test). We then compared the incidence of sex chromosome loss between SCT-derived and euploid XY- and XX-derived iPSCs. Sex chromosome loss was more common in SCT- than euploid-derived iPSCs (Fig. 1E), irrespective of the cutoff used to define sex chromosome loss (Fig. S12D).

Sex chromosome loss could occur during reprogramming of SCT cells or during iPSC propagation to P2, perhaps conferring a proliferative advantage to resulting euploid cells. Indeed, sex chromosome instability has been observed in pluripotent stem cells (II, 12). To test the latter hypothesis, we analyzed sex chromosome stability between P2 and P6 in iPSCs with highly parental (>$90\%$) complements (fig. S4A). We observed sex chromosome loss in XX and XXY iPSC lines ($P < 0.01$ and 0.05, respectively; Wilcoxon signed-rank test) but not in XY and XYY iPSC lines ($P = 0.21$ and 0.66, respectively; Wilcoxon signed-rank test). However, no iPSC line showed more than a 15% decrease in parental complement (fig. S4B). Furthermore, sex chromosome loss between P2 and P6 was not trisomy-biased (fig. S4B). SCT-derived euploid XY iPSCs also exhibited no proliferative advantage over XXY or XYY iPSCs (fig. S5). Because SCT fibroblasts were also karyotypically stable (fig. 1B and fig. S3A), chromosome loss is likely induced during iPSC reprogramming and is thus distinct from sex chromosome instability in pluripotent stem cells (II, 12). We refer to the phenomenon as trisomy-biased chromosome loss (TCL).

We next determined whether euploid XY iPSCs derived from SCT fibroblasts would form functional sperm. We selected highly euploid ($\approx 80\%$ of cells XY) P6 iPSCs adapted to Dox-free medium (fig. S6). For our XXY experiments, only XY iPSC lines that retained the wild-type Y rather than the Y$^{\text{mt}}$ chromosome were used for PGCLC experiments (fig. S7). Karyotyping confirmed that all SCT-derived XY iPSC lines and a control XY iPSC line were euploid (fig. S8). These iPSC lines were differentiated (6) through an epiblast-like state to create PGCLC aggregates positive for BV and SC (fig. 2A). BV-positive PGCLCs (table S1) were isolated by fluorescence-activated cell sorting (FACS) (fig. 2B) and transplanted into germ-cell deficient $W/W^{\text{v}}$ (Kit mutant) testes (12).

Spermatogenesis in recipients was evaluated 9 to 10 weeks after transplantation. Teratoma, which are observed after iPSC-derived PGCLC transplantation (6), were present in 29% of XXY-derived and 50% of XY-derived transplanted lines (fig. S9). Reconstruction of spermatogenesis, revealed by the presence of spermatogenic colonies (Fig. 2C) and by histology (Fig. 2D), was observed for all XXY- and XY-derived iPSC lines used (Table 1). Thus, SCT-derived XY iPSCs can differentiate to germ cells in vitro and complete spermatogenesis after transplantation.

We asked whether sperm created via transplantation could support reproduction. Intracytoplasmic sperm injection (ICSI) using sperm

**Table 1. Spermatogenesis from transplanted PGCLCs.**

<table>
<thead>
<tr>
<th>Sex chromosome complement</th>
<th>iPSC line name</th>
<th>No. of testes transplanted</th>
<th>No. of testes dissected*</th>
<th>No. of testes with spermatogenesis</th>
<th>No. of spermatogenic colonies in each testes</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXY from XXY</td>
<td>XY 7-6</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>10$^2$</td>
</tr>
<tr>
<td></td>
<td>XY 7-7-1</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>XX 7-7-2</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>XY 7-8-2</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>3.3,4.8</td>
</tr>
<tr>
<td></td>
<td>XY 7-15-2</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>7.10</td>
</tr>
<tr>
<td></td>
<td>XY 7-21-2</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>2,3,15</td>
</tr>
<tr>
<td></td>
<td>XY 7-29-1</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>2,6,8,16</td>
</tr>
<tr>
<td>XXY from XYY$^\dagger$</td>
<td>XY 5-4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>XY 5-5</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>2.10</td>
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<td>XY 5-8</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2,2.3</td>
</tr>
<tr>
<td></td>
<td>XY 7-3</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>5,11,14</td>
</tr>
<tr>
<td>Control XY</td>
<td>XY 6-6</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>1,15,20</td>
</tr>
</tbody>
</table>

*Some testes were not dissected and were used for histology analyses.

$^\dagger$XY iPSC lines with wild-type Y chromosome were used for transplantation.

$^\ddagger$Sperm were very few.

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from two XXY- and two XYY-derived XY iPSC lines (Fig. 2E and fig. S10A) generated zygotes that developed into two-cell embryos in vitro (efficiency 76.7% to 87.3%) (Fig. 2F, fig. S10B, and table S2) and grossly normal offspring when transplanted into recipients (efficiency 46.9% to 59.4%) (Fig. 2G, fig. S10C, and table S2). Polymerase chain reaction genotyping confirmed that the offspring were derived from the transplanted PGCLCs (fig. S10D). Pups from XXY- and XYY-derived iPSC lines showed comparable survival (efficiency 76.7 to 87.3%) (Fig. 2F, fig. S10B, and table S2). Each bar represents an iPSC line and the percentage of cells exhibiting each complement (n = 50 cells per line). Numbers in brackets show number of iPSC lines examined. Data for two animals combined.

We addressed whether TCL is specific to trisomic iPSC lines that were generated (62.5%) showed loss of the hChr.21 in >90% of cells (fig. 3A). DNA-FISH showed that the vast majority (>96%) of Tc1 fibroblasts retained hChr.21 (Fig. 3B and fig. S3B). These Tc1 fibroblasts were reprogrammed without G418 selection, and the resulted iPSCs were analyzed at P2. Ten of the 16 iPSC lines that were generated (62.5%) showed loss of the hChr.21 in >80% of cells (Fig. 3, C and D, and fig. S12D). In contrast, after G418 removal, hChr.21 was retained in Tc1 fibroblasts cultured for the same period as that used for iPSC reprogramming (18 days) and in P6 iPSC lines that had highly parental (>90% hChr.21 positive) complements at P2 (fig. S12, A and B). We conclude that the loss of hChr.21 in Tc1 cells is promoted by reprogramming rather than G418 removal and therefore that TCL also affects an accessory chromosome.

We next asked whether TCL occurs in human cells. Instances of chromosome loss have been observed during human trisomic cell culture (16, 17), but its prevalence and relationship to reprogramming has not been systematically analyzed. We selected human Klinefelter syndrome, Down syndrome, and euploid XY and XX fibroblast lines exhibiting minimal mosaicism (fig. S13, A and B), reprogrammed them, and determined the chromosome complements of resulting iPSC lines. We observed XY and XX iPSCs from Klinefelter syndrome fibroblasts and euploid iPSCs from Down syndrome fibroblasts (fig. S13, C and D). Chromosome loss was more common in trisomic than in disomic cells, demonstrating that TCL also occurs during human reprogramming. However, the frequency of highly euploid iPSC lines was lower than that observed in trisomy-derived mouse iPSCs (fig. S13, E to H).

We have shown that TCL produces euploid iPSCs from SCT and autosomal trisomic mice and patients (fig. S12E). In mice, the resulting “corrected” iPSCs can form functional sperm, enabling production of chromosomally euploid offspring from infertile SCT individuals. TCL complements existing iPSC therapies for chromosome abnormalities (17–20). The mechanisms that cause TCL are unknown. Cellular stresses associated with reprogramming may select against trisomic cells, permitting emergence of euploid cells. We observed less frequent TCL in human than in mouse cells (figs. S12D and S13H). Even if rare, TCL could offer treatments for infertile SCT patients for whom alternative approaches are unsuccessful. However, the clinical use of human germ cells made in vitro should be carefully considered ethnically and legally (22–24). Furthermore, complete in vitro spermatogenesis will have to be developed to avoid the risk of teratoma formation arising through germ-cell transplantation.

TCL also permits production of female iPSCs from males, offering potential for the genetic dissection of sexual dimorphisms (25). By creating isogenic iPSC lines that differ only with respect to their sex chromosomes, sex differences identified during iPSC disease modeling could be attributed to X or Y chromosomal effects.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS

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Trisomic animals lose third chromosome

Generally, when a third sex chromosome is added to the normal two in mammals (XX for female and XY for male), developmental defects result. Mice that are trisomic for the sex chromosomes are infertile. Hirota et al. demonstrate that reprogramming cells from sterile mice with chromosome trisomies XXY or XYY generates XY stem cells. Sperm generated from these XY stem cells could give rise to healthy, fertile offspring. Reprogramming also promoted loss of the extra chromosome in cells from patients with Klinefelter (XXY) or Down (trisomy 21) syndrome. Science, this issue p. 932