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# Two Y Genes Can Replace the Entire Y Chromosome for Assisted Reproduction in the Mouse

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**The Y chromosome is thought to be important for male reproduction. We have previously shown that, with the use of assisted reproduction, live offspring can be obtained from mice lacking the entire Y chromosome long arm. Here, we demonstrate that live mouse progeny can also be generated by using germ cells from males with the Y chromosome contribution limited to only two genes, the testis determinant factor *Sry* and the spermatogonial proliferation factor *Eif2s3y*. *Sry* is believed to function primarily in sex determination during fetal life. *Eif2s3y* may be the only Y chromosome gene required to drive mouse spermatogenesis, allowing formation of haploid germ cells that are functional in assisted reproduction. Our findings are relevant, but not directly translatable, to human male**

The mammalian Y chromosome, once thought to be a genetic wasteland (*1*), is now known to encode a battery of genes, many of which are thought to be involved in male reproduction (*2*). A substantial amount of work has been done to define which genes are important for maintaining sperm function under normal, in vivo, conditions. In the era of assisted reproduction technologies (ART), it is now possible to bypass several steps of normal human fertilization using immotile, nonviable, or even immature sperm. We have shown that infertile male mice lacking the entire Y chromosome long arm can generate live offspring when their severely morphologically abnormal sperm are delivered into oocytes via intracytoplasmic sperm injection (ICSI) (*3*). In these mice, the Y chromosome is reduced from 78 Mb to ~2 Mb, and encodes only seven genes and three gene families (XY*$^{X}$*Sxr*$^{a}$ in fig. S1).

In most mammals, including humans and mice, testis determination is regulated by *Sry*, which directs developing gonads to male differentiation (*4*–*6*). Upon transgenic addition of *Sry*, mice with one X chromosome (XO*Sry*) develop testes that are populated with spermatogonia, male germ cells that have the potential to undergo differentiation and initiate spermatogenesis. In the absence of other Y chromosome genes, these spermatogonia undergo proliferation arrest, and the meiotic and postmeiotic stages of spermatogenesis are absent (*7*). Transgenic addition of individual missing Y genes led to the identification of *Eif2s3y* as the gene that restored normal spermatogonial proliferation (*7*). In XO*Sry* males transgenic for *Eif2s3y*, spermatogenesis was shown to complete meiotic prophase and the first meiotic division before the cells arrested as secondary spermatocytes, with the occasional production of spermatid-like cells (*7*, *8*). Here, we tested whether these spermatid-like cells were functional in assisted reproduction and what other components of the Y chromosome help to increase development of functional gametes.

We first examined mice with the Y gene complement limited to two transgenically derived genes, autosomally located *Sry* and X chromosome located *Eif2s3y* (X$^{E}$O*Sry* in fig. S1) (*9*). These mice had testes smaller than wild-type XY males (fig. S2) but populated with germ cells. Analysis of testicular sections confirmed that spermatogenesis was ongoing, which allowed development of germ cells with spermatid-like morphology (Fig. 1A, arrowheads), similar to those observed earlier (*7*, *10*). The occurrence of these spermatids was low, and their development was restricted to steps 5 to 7 of spermatid development, with the occasional presence of step 8 to 9 spermatids [Fig. 1A (inset) and fig. S3]. We also observed secondary changes to seminiferous tubules, with increased incidence of dying cells, multinucleate bodies, and vacuoles (Fig. 1B). These changes increased progressively as the males aged and ultimately led to Sertoli-cell-only (SCO) syndrome tubules (Fig. 1C).

We next tested the function of the spermatid-like cells from X$^{E}$O*Sry* in assisted reproduction. Round, spermatid-like cells could be found in testicular cell suspensions from all males used in ART trials, although these cells were rare and their morphology was often slightly abnormal (increased size, less pronounced nuclei, and rough rather than smooth surface) when compared with spermatids from control XY males (Fig. 1, D and E). Nevertheless, when we performed round spermatid injection (ROSI), the oocytes were successfully fertilized as evidenced by the development of two pronuclei and extrusion of the second polar body, as well as subsequent cleavage (fig. S4). When the developed two-cell embryos were transferred into the oviducts of recipient females, live offspring were obtained (Table 1 and Fig. 1F). Three out of four males that provided spermatids for injections successfully sired offspring. The efficiency of ROSI with X$^{E}$O*Sry* males was significantly lower than with XY controls (9% versus 26%) (Table 1). All the progeny had the genotypes as expected when derived from X$^{E}$O*Sry* fathers and were healthy; those bred were fertile (Fig. 1G, fig. S5, and supplementary text).

An unpaired sex chromosome leads to meiotic arrest and apoptosis (*11*), so partial meiotic failure in X$^{E}$O*Sry* males was not unexpected. The few spermatids that could be found in the testes could be the cells that "leaked" through the meiotic arrest, i.e., finished meiosis and were haploid. Alternatively, these could be the cells that developed spermatid-like morphology without undergoing the second meiotic division (*8*, *10*). Spermatid nuclear DNA content (Fig. 2 and fig. S6) and zygotic chromosome analyses (Fig. 3) revealed that the great majority of spermatids from X$^{E}$O*Sry* males were diploid and yielded triploid zygotes, which would explain the poor ROSI success. In order to overcome the problem of meiotic block arising from X chromosome univalence, we used males in which a minute Y*$^{X}$ chromosome (fig. S1) was added to provide a second pairing region (PAR) for PAR-PAR chromosome synapsis (*12*). In these X$^{E}$Y*$^{X}$*Sry* males (fig. S1), successful pairing of Y*$^{X}$ and X was observed in 85% of pachytene spermatocytes (fig. S8). However, the testicular phenotype did not improve (figs. S2 and S7, A to C). The proportion of live offspring obtained after injection was similarly low as with X$^{E}$O*Sry* males (Table 1) and only five out of eight males that provided cells for ROSI sired offspring. We therefore tested for ploidy and demonstrated that most of the X$^{E}$Y*$^{X}$*Sry* spermatids were diploid (Fig. 2 and fig. S6) and that most zygotes after ROSI were triploid (Fig. 3). Thus, overcoming X chromosome univalence in X$^{E}$Y*$^{X}$*Sry* males did not allow overcoming meiotic arrest and increasing ROSI success (see supplementary text).

We next asked whether other Y chromosome genes may be beneficial for spermatid function in assisted reproduction. To address this, we

used males in which the *Sry* transgene driving sex determination was replaced with the sex reversal factor *Sxr*$^{b}$ ($X^{E}Sxr^{b}O$ and $X^{E}Sxr^{b}Y^{*X}$ in fig. S1). In these males, the X chromosome carries an *Eif2s3y* transgene necessary for spermatogonial proliferation (*7*), together with the *Sxr*$^{b}$ encoding for *Zfy2/1*, *Sry*, *H2al2y*, and the *Rbmy* gene cluster.

The testes from $X^{E}Sxr^{b}O$ and $X^{E}Sxr^{b}Y^{*X}$ males were larger than from $X^{E}OSry$ and $X^{E}Y^{*X}Sry$ but smaller than in wild-type XY males (fig. S2). The incidence of round spermatids increased only in $X^{E}Sxr^{b}Y^{*X}$ (fig. S3). In both male types, spermatid development was more advanced, with clear elongation up to step 10, occasional presence of step 11 to 12 spermatids, and even sporadic development to mature testicular sperm (fig. S7, D and G). There were secondary changes to the seminiferous epithelium (fig. S7, E, F, H, and I), albeit in $X^{E}Sxr^{b}Y^{*X}$ males less pronounced than in the other genotypes. Zygote chromosome analysis showed that most of the zygotes after ROSI were diploid (71 to 85%) (Fig. 3), and the ROSI outcome was significantly improved, with all tested males (three per genotype) yielding live offspring with rate now reaching 20 and 16% (Table 1). Nevertheless, the frequency of haploid spermatids in $X^{E}Sxr^{b}O$ males remained low (Fig. 2 and fig. S6). The discrepancy between the ploidy of the spermatids and zygotes raised the possibility that the second meiotic division took place in the oocytes. This suggests that *Sxr*$^{b}$ encodes a gene or genes that promote the second meiotic division when all chromosomes are paired and enables overcoming of meiotic arrest of the diploid spermatid in the oocyte when an X chromosome pairing partner is missing (see supplementary text).

We have shown that only two mouse Y chromosome genes, *Sry* and *Eif2s3y*, are necessary for the development of male haploid germ cells that are sufficient for successful reproduction and yield live offspring. This minimal Y gene complement must be enough to ensure correct male-specific methylation and proper formation of any other epigenetic modifications that are required for embryogenesis in the paternal genome.

That *Sry* is one of the two genes is not surprising, as it drives testis determination (*4*–*6*). Mouse *Sry* expression and translation occur very briefly during fetal development. In adult testes, *Sry* transcripts are thought to be aberrant and not translatable (*13*, *14*), although their role as epigenetic regulator(s) cannot be excluded (*15*). Nevertheless, it is reasonable to conclude that *Sry* plays a role primarily during sex determination.

This suggests that the second Y gene, *Eif2s3y*, is the only gene necessary to drive spermatogenesis through the first meiotic division and with the occasional meiotic progression to form haploid round spermatids. *Eif2s3y* is a Y-encoded subunit 3 of the eukaryotic translation initiation factor 2. It is ubiquitously expressed and, in the testis, plays a role in spermatogonial proliferation (*7*). The *Eif2s3y* gene has been conserved on the Y chromosome during eutherian evolution, but there is no Y-linked copy of *Eif2s3* detected in any of the simian primates, including humans (*16*).

In men, spermatogonial proliferation arrest results most often from deletions of *AZFa* (azoospermia; SCO syndrome), and it has been related to a loss of *DDX3Y* (*17*–*19*). In both men and mice, the *Eif2s3* and *Ddx3* genes belong to the family of widely expressed genes encoding proteins involved in initiation of mRNA translation at the ribosome. These genes have X homologs that escape X-inactivation, and the Y and X copies are suspected to be, at least in part, interchangeable, with the Y copy conserved to provide two doses of gene product in both male and female. The loss of the mouse Y-encoded *Ddx3y* is not detrimental for spermatogonesis because of the compensation provided by an X copy retrotransposed on an autosome (*20*). Analogically, in men, the presence of a retrotransposed X copy of *EIF2S3X*, in addition to *EIF2S3X* itself, explains why the loss of the Y copy was still permissive for spermatogenesis. Although there is no human copy of *Eif2s3y*, men have a Y-encoded copy of the translation factor EIF1A (eukaryotic translation initiation factor 1A, Y-linked) (*21*), which likely acts as part of a multiprotein complex that includes EIF2S3X, as well as other EIF family members. Note that EIF1AY is found in the *AZFb* region, and its diminished expression sporadically contributes to azoospermia (*22*).

At present, our findings in mice do not translate directly to humans. ROSI is still considered experimental in human ART because of concerns regarding the safety of injecting immature germ cells and technical difficulties (*23*). In spite of this, some children have already been born (*24*, *25*), and those were healthy. As we learn more about the effects and improve technical aspects of ROSI, this method may become more acceptable. Indeed, studies on ROSI effects in mice have been encouraging (*26*). Thus, our study may bear importance for clinicians working in ART clinics by supporting the possibility that ROSI may be a viable option for overcoming infertility in men with nonobstructive azoospermia.

Considering that we have obtained live offspring using germ cells from males with only two Y chromosome genes, one could question the importance of the Y chromosome in male reproduction. We believe that the answer lies in defining the need. Human Y chromosome is not on the way to oblivion, as has been implied in the past (*27*), and its genetic information is undoubtedly important for many aspects of reproduction involving the development of mature sperm and its function in normal fertilization (*28*). Most of the mouse Y chromosome genes are involved in spermatogenesis and sperm function and, as such, are necessary for normal fertilization (*29*, *30*). However, when it comes to assisted reproduction, our mouse study proves that the Y chromosome contribution can be brought to a bare minimum consisting of *Sry* and *Eif2s3y*. Indeed, it may well be possible to eliminate mouse Y chromosome altogether if appropriate replacements are made for those two genes.

**References and Notes**


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**Supplementary Materials**
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**Table 1. The results of round spermatid injection (ROSI) with spermatids from males with limited Y gene complement.** For Y gene contribution, see fig. S1. Percentages of live offspring and implants were calculated from embryos transferred. Male ages ranged from 63 to 229 days. Statistical significance (Fisher's exact test): [a]*P* < 0.01 and [b]*P* < 0.001 versus $XY^{RIII}$ control. *Sxr*[b]: *Sry, Zfy2/1*, *H2al2y*, *Rbmy cluster* (reduced).

| Male genotype | Y gene contribution | Live offspring % (no.) | Implants % (no.) |
|---|---|---|---|
| $X^{E}OSry$ | Eif2s3y and Sry | 9.1 (12/132)[a] | 29.5 (39/132)[b] |
| $X^{E}Y^{*X}Sry$ | Eif2s3y and Sry | 5.7 (13/227)[b] | 27.3 (62/227)[b] |
| $X^{E}Sxr^{b}O$ | Eif2s3y and Sxr[b] | 20.0 (24/120) | 45.8 (55/120)[a] |
| $X^{E}Sxr^{b}Y^{*X}$ | Eif2s3y and Sxr[b] | 16.0 (24/150) | 37.3 (56/150)[b] |
| $XY^{RIII}$ control | Intact Y | 26.0 (19/73) | 69.9 (51/73) |

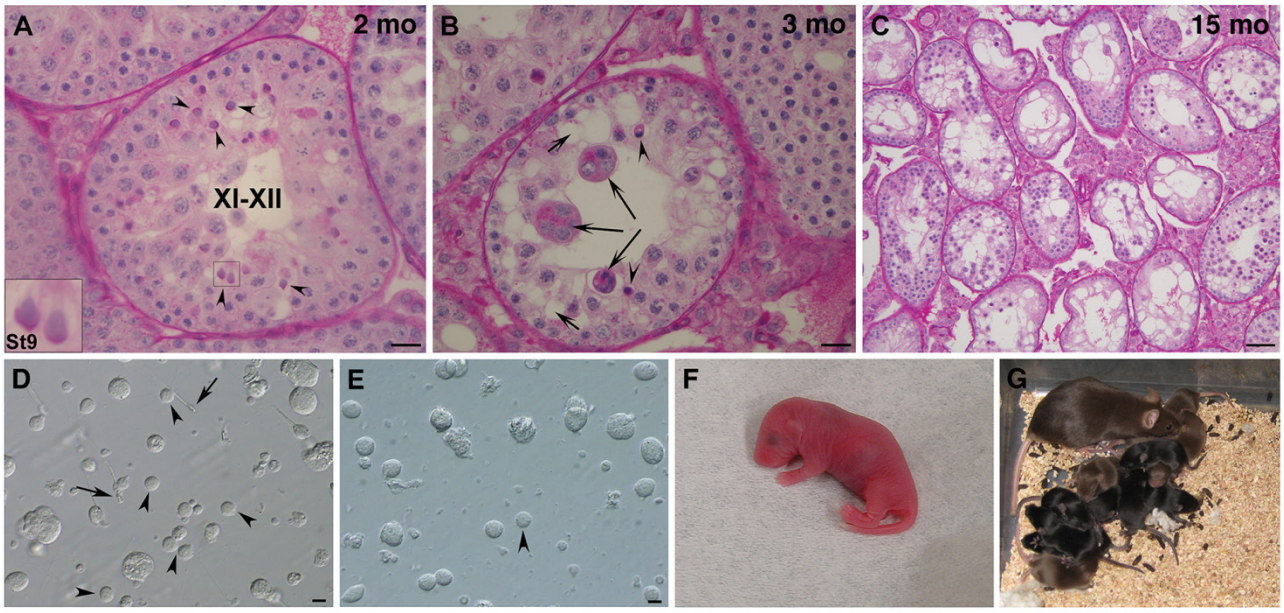



**Fig. 1. Testis histology, round spermatids, and live offspring.** (**A**) Males with only two Y genes, *Eif2s3y* and *Sry*, have meiotic and postmeiotic arrests that only occasionally allow formation of spermatids (arrowheads) that are frequently delayed and do not develop beyond St8/9. (**B**) Tubule degeneration with formation of "multinucleate bodies" (long arrows), vacuoles (short arrows), and dying and/or apoptotic germ cells (arrowheads) is frequently observed. (**C**) SCO syndrome–like phenotype in an old male. (**D**) Testicular suspension from wild-type males contains testicular sperm (arrows) and many round spermatids (arrowheads) with clear morphological features. (**E**) Males with two Y genes have substantially fewer germ cells in testicular cell suspension, with no sperm and very few round spermatids (arrowhead). These spermatids are functional in ROSI. (**F**) ROSI pup obtained after transfer of embryos generated with spermatids from a male with only two Y genes. (**G**) An adult female developed from the pup shown in (F) with her own litter. Roman and arabic numerals in (A) are tubule stages and steps of spermatid development (St), respectively (see fig. S3). Scale bars, 50 μm (A to C) and 10 μm (D and E); insets, ×3 magnification; mo, months of age.

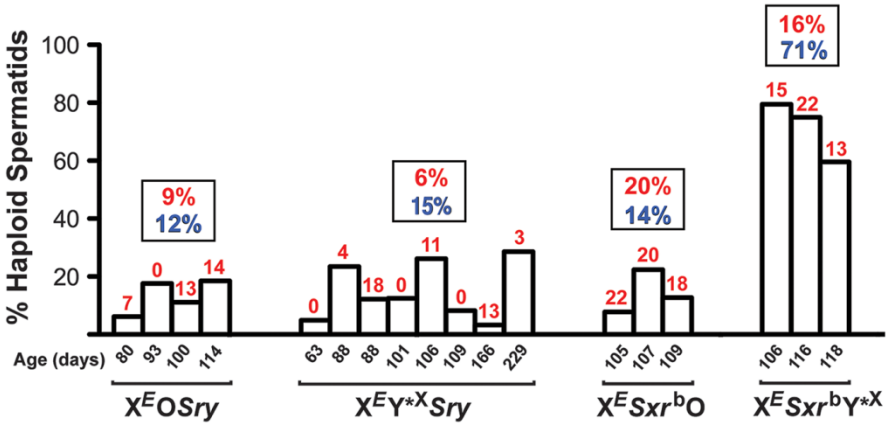
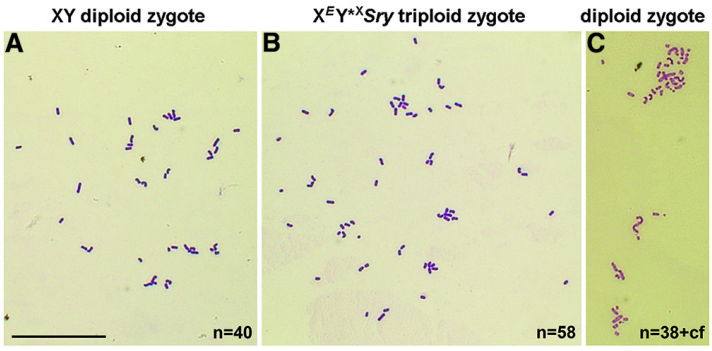



**Fig. 2. Incidence of haploid spermatids.** Each graph bar represents an individual male providing testicular cells for analysis; the numerals above show the percentage of pups obtained after ROSI. The data in rectangular boxes represent average percentage of haploid spermatids (blue) and ROSI offspring (red) for each genotype. Control male with intact Y chromosome had 97% (67 out of 69) of spermatids haploid. $X^{E}Sxr^{b}Y^{*X}$ males had higher average incidence of haploid spermatids than other genotypes ($P < 0.01$).




D

| Genotype | Diploid zygote % (No) |
|---|---|
| $X^{E}OSry$ | 23 (8/35)[b] |
| $X^{E}Y^{*X}Sry$ | 25 (13/53)[b] |
| $X^{E}Sxr^{b}O$ | 71 (5/7)[a] |
| $X^{E}Sxr^{b}Y^{*X}$ | 85 (47/55)[a] |
| XY | 100 (36/36) |

**Fig. 3. Zygote chromosome analysis after ROSI.** (**A**) Normal diploid mouse zygote generated after ROSI with XY male contains 40 chromosomes. (**B**) Triploid zygote obtained after ROSI with spermatids from a male with two Y genes (~60 chromosomes). (**C**) Diploid zygote after ROSI with spermatids from male of the same genotype (~40 chromosomes). (**D**) Incidences of diploid zygotes generated after ROSI. cf, chromosome fragments. *n* = chromosome number. [a]$P < 0.05$, [b]$P < 0.001$ versus XY control. Scale bar, 50 μm.