Engineering a genetic system for female-biased, genetically unmodified progeny in mice without affecting litter size

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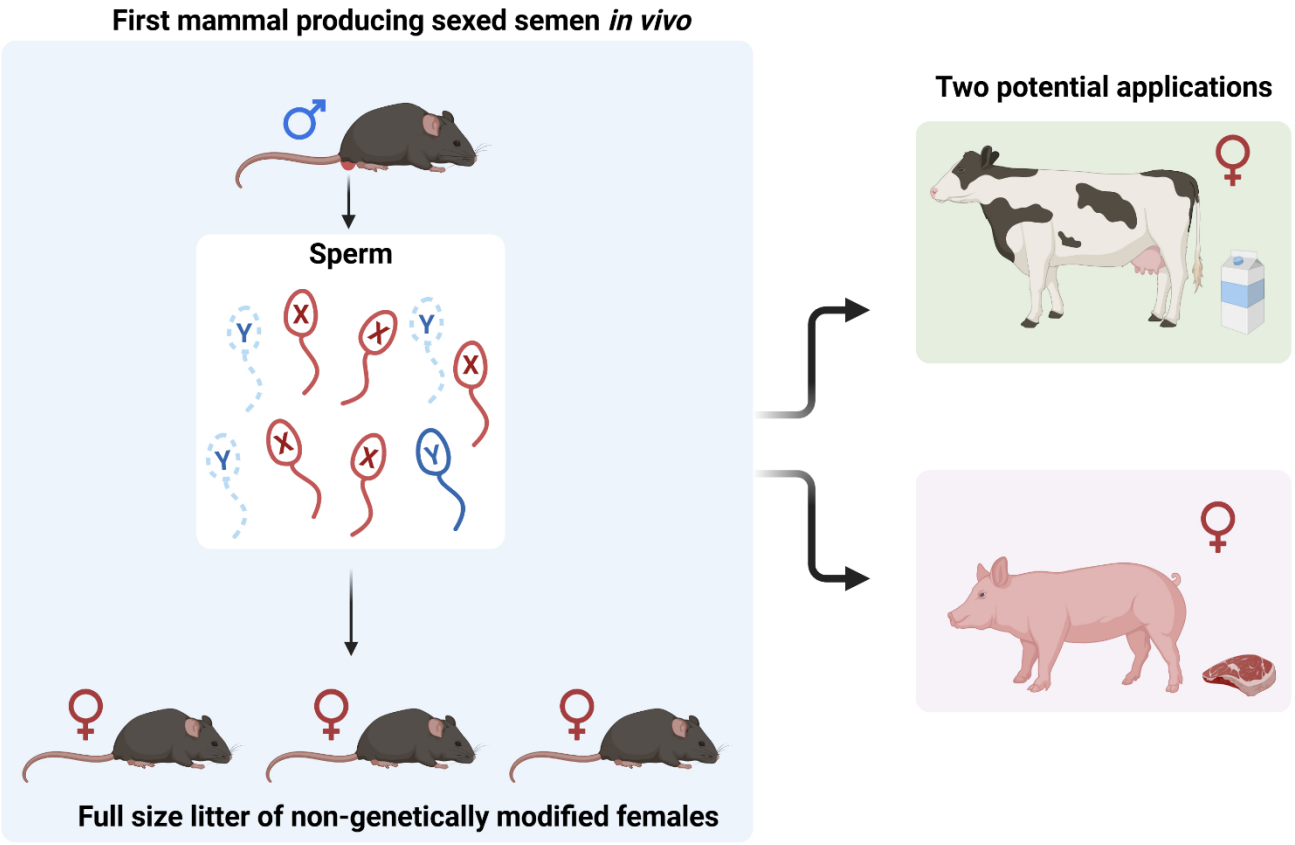


Graphical Abstract. A single line genetic system producing female biased, genetically unmodified progeny in mice by conditionally impairing Y bearing sperm**.**

One sentence summary

Y chromosome manipulation yields mostly female mice, preserving litter size without genetic alteration in females.

Significance statement

This study describes a genetic approach for producing predominantly female offspring in mice without altering litter size or transmitting genetic modifications to the progeny. A cassette inserted into the Y chromosome of males induces a strong female bias in offspring through a mechanism that remains unclear. The female offspring do not inherit the transgene and are genetically unmodified. This system provides a simple and conditional platform for sex ratio control in mammals, with potential applications in animal breeding and welfare.

Abstract

The ability to influence the sex ratio of mammalian offspring has applications in agriculture and animal welfare. Here we describe a genetic system in mice that produces predominantly female progeny, reaching approximately ninety percent, without affecting litter size or introducing genetic modifications into the offspring. The system consists of a doxycycline-regulated cassette inserted into the Y chromosome, encoding dCas9 and RNA guides intended to impair the function of Y bearing sperm. While the cassette was originally intended to repress a spermatid maturation gene, our analyses did not detect meaningful changes in gene expression or sperm function that would account for the phenotype. Nevertheless, male mice carrying the cassette consistently produced female biased litters, and the effect was reversed by doxycycline, confirming that it depends on cassette expression. Similar female bias was observed following in vitro fertilization using sperm from transgenic males. These findings demonstrate a reproducible, genetically confined, and conditionally regulated system for sex ratio bias in mice. The strategy may prove adaptable across species, even without a complete mechanistic understanding.

Keywords: Null segregant /sexed semen /sex-ratio /mammals/ Y-chromosome

Introduction

The ability to influence the sex ratio of mammalian offspring holds practical and ethical value in agriculture, biomedical research, and animal welfare. In livestock production, for example, female animals are often preferred for milk or egg yield, whereas in other cases, such as meat production or breeding efficiency, males may be favored. Current practices to address this mismatch between desired and actual sex ratios often involve the early culling of animals of the undesired sex, leading to economic inefficiencies and ethical concerns [1]. In laboratory animal breeding, similar inefficiencies arise when experiments require sex-matched cohorts but produce litters with unbalanced sex distributions.

Various technologies have been developed to bias sex ratios. In mammals, the most widely applied is sexed semen, where X- and Y-bearing sperm are separated by flow cytometry prior to artificial insemination [2,3]. While this method can increase the proportion of offspring of the desired sex, it is technically complex, reduces fertility, and is not suitable for all species [4–6]. Hormonal treatments, which have proven effective in aquatic species for producing single-sex populations [7–11], are not viable in mammals. Genetic strategies have been successfully implemented in insects using CRISPR-based systems or gene drives [12–20], but similar approaches in mammals remain technically challenging and often involve transgene inheritance.

We previously described a two-line genetic system in mice that used maternal expression of Cas9 and paternal inheritance of Y-linked guide RNAs to selectively eliminate male embryos post-fertilization [21]. The fundamental principles elucidated in our work were subsequently refined and replicated by the Turner group, achieving sex bias for both males and females [22]. While effective in biasing the sex ratio, those systems resulted in reduced litter sizes, genetically modified progeny, and required maintaining two genetically modified lines. A simpler and more efficient approach, particularly one that avoids transgene inheritance by the offspring and maintains litter size, would represent a valuable advance.

In this study, we designed a one-line, conditionally regulated genetic system in which a cassette encoding dCas9 and guide RNAs aimed at targeting a spermatid maturation gene was inserted into the Y chromosome. The system was intended to impair the function of Y-bearing sperm following meiosis, thereby skewing offspring sex ratios

toward females. The cassette was placed under control of a tetracycline-responsive element, enabling reversible suppression of its expression. We found that male mice carrying this Y-linked cassette produced female-biased litters, with normal litter sizes and no detectable inheritance of the cassette by the progeny. The sex bias was reversed by administration of doxycycline, confirming that the effect was dependent on cassette expression.

Although the system was designed to function by repressing the targeted gene, our expression and morphological analyses did not reveal changes of sufficient magnitude to account for the phenotype. These findings suggest that the observed sex bias may result from a yet unidentified mechanism, possibly involving subtle impairments in Y-bearing sperm function. This system provides a platform for sex ratio control in mammals that is operationally simple, non-heritable, and conditionally regulated. The work highlights a reproducible and conditionally regulated system with potential applications in breeding and animal welfare.

Results

Generation of transgenic male mice harboring a conditional repressive cassette on the Y chromosome

To generate male mice that would predominantly produce X-bearing sperm, we designed a genetic cassette encoding dCas9 [23] fused to a KRAB repressor domain, along with five RNA guides targeting the *Spem1* gene. This gene is known to be required for proper maturation of spermatids in mice [24]. The entire cassette was placed under the control of a tetracycline-responsive element (TRE), allowing repression of dCas9 expression in the presence of doxycycline [25]. The cassette was flanked by homology arms targeting an intron within the Y-linked *Uty* gene [26], previously shown to be suitable for insertion of genetic elements [21]. The design rationale was that after meiotic segregation, only the Y-bearing gametes would express the cassette and undergo repression of *Spem1*, while X-bearing gametes would be unaffected. As a result, we expected the Y-bearing sperm to be nonfunctional or less competitive, leading to a female-biased progeny (Figure 1).

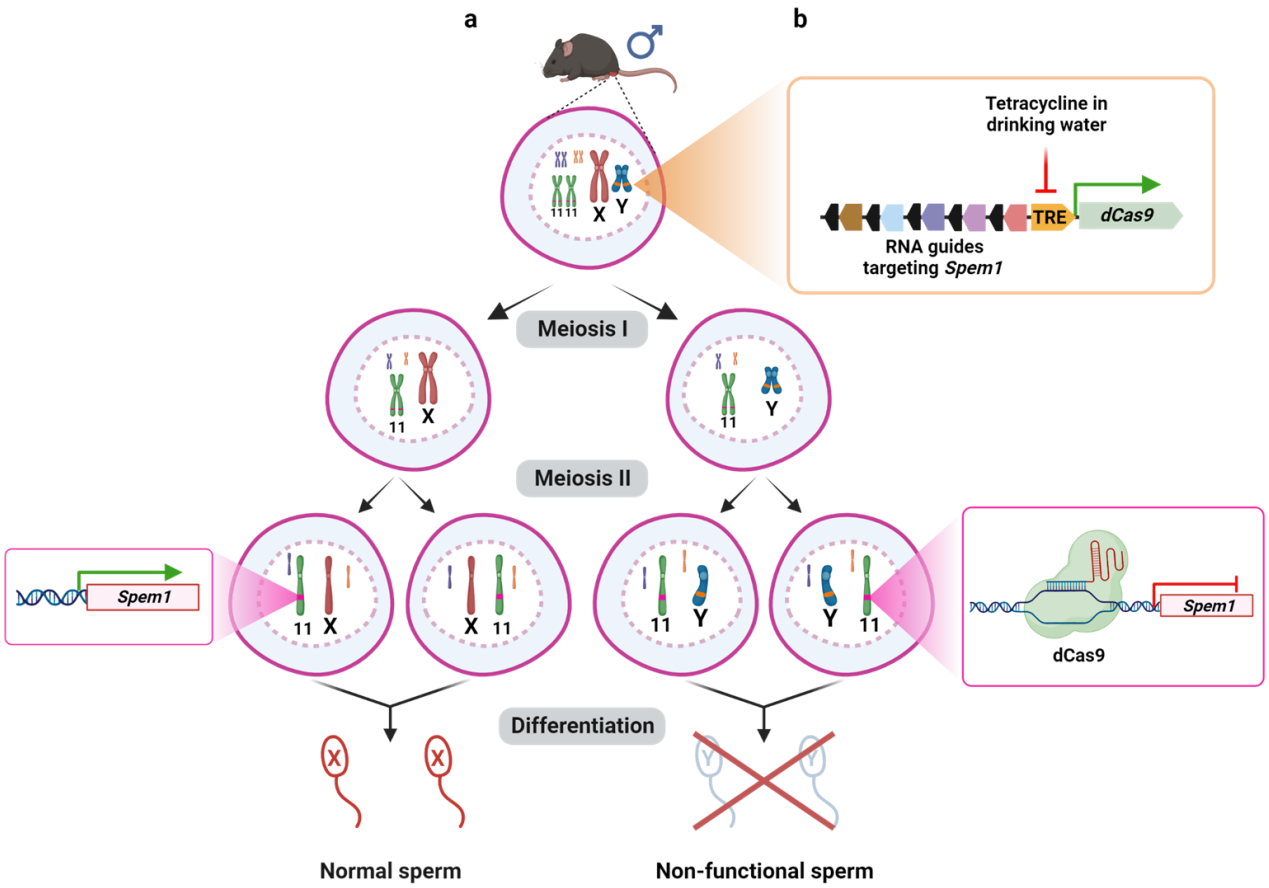


Figure 1. Sexing the semen *in vivo* in a male mouse. (a) A male mouse's Y chromosome is genetically engineered to contain a cassette intended to repress a gene essential for gamete development. After meiosis, the Y-gametes encode and express the cassette, whereas the X-gametes lack it. As designed, the Y-gametes are expected to fail to mature while the X-gametes develop normally.
(b) A schematic representation of the cassette inserted into the Y chromosome. dCas9 is expressed from a promoter encoding a tetracycline-responsive element (TRE), repressed if tetracycline/doxycycline is present in the drinking water. The cassette also encodes five RNA guides that target the *Spem1* gene encoded on chromosome 11. The representation is not drawn to scale. Additional elements of the cassette are described in Materials and Methods.

The cassette was introduced into C57BL/6 mouse embryonic stem cells through homologous recombination, and properly targeted clones were used to generate chimeric mice [27]. The resulting F_0 transgenic males exhibited poor mating efficiency, which necessitated the use of in vitro fertilization (IVF) to establish the line. All male founders

115 carried the transgene on their Y chromosome, as validated by PCR (Figure S1), and were
116 otherwise viable and morphologically normal. Male offspring derived from IVF were
117 backcrossed to wild type females to generate F_1 and subsequently F_2 transgenic males,
118 which were used for the breeding experiments described below. These progeny mice
119 could breed normally. One consistent difference was a reduction of approximately 25%
120 in body weight relative to wild type mice at 8 weeks of age (Table S1), though this
121 phenotype had not been previously associated with *Spem1* knockout [24]. Nevertheless,
122 this was not expected to compromise the strategy, as the male mice are used solely for
123 breeding, and the objective is to produce predominantly female offspring. Aside from the
124 weight difference, the transgenic mice developed normally, with no physiological or
125 morphological abnormalities (Figure S2a). In this regard, it is important to note that the
126 female offspring of the transgenic mice maintained normal body weight, as determined
127 by comparing their growth curve to the published reference values for wild-type
128 C57BL/6 females (Figure S2b).

129 **Breeding experiments reveal highly female-biased litters from transgenic males**

130 To test whether the transgene could bias the sex ratio as designed, we carried out a series
131 of controlled matings. We first mated six wild type C57BL/6N males with two wild type
132 females each, resulting in a total of 61 pups. As expected, the sex distribution in this
133 group was close to even, with 43% males and 57% females (Figure 2a). We next mated
134 15 transgenic males, each with two wild type C57BL/6N females. These matings yielded
135 157 pups in total, with a striking female bias of 89% females and 11% males. All male
136 progeny in this group were positive for the Y-linked transgene, whereas all female pups
137 tested negative, as determined by PCR (Figure S1). Importantly, the average litter size in
138 this group was not significantly different from that of wild type controls (Figure 2b),
139 indicating that the sex bias was not the result of selective embryonic loss.
140 To test whether the observed bias was indeed dependent on expression of the
141 cassette, we administered doxycycline into the drinking water of a separate group of eight
142 transgenic males prior and throughout the mating period. These animals were housed
143 individually with two females each, and the resulting 56 pups displayed a sex ratio of
144 46% males and 54% females, a result statistically indistinguishable from wild type

controls (Figure 2). This observation supports the conclusion that cassette activity is responsible for the sex bias and that the effect can be conditionally turned off by doxycycline.

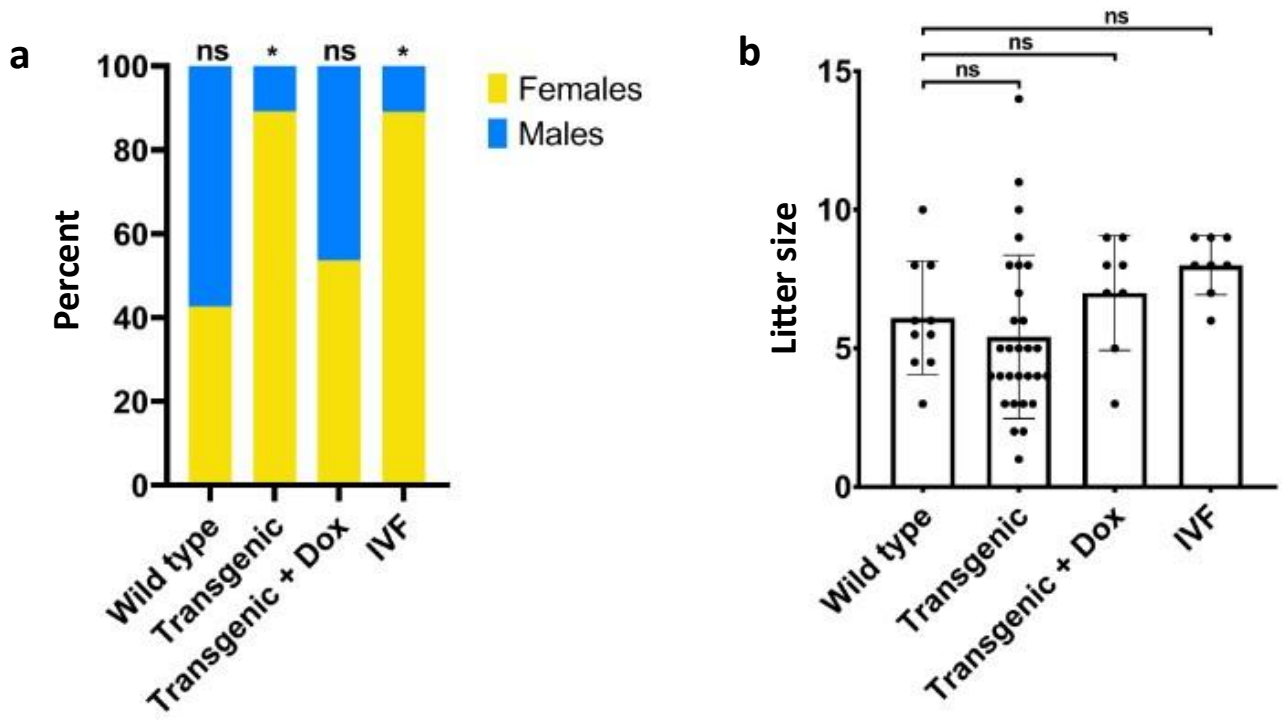


Figure 2. (**a**) The sex distribution of the total pups from crosses between C57BL/6N females with wild type C57BL/6N males (n=61), F_2 transgenic males (n=157), F_2 transgenic males treated with 2mg/ml doxycycline (Transgenic + dox) (n=56), and F_2 transgenic sperm through IVF (n=64). (**b**) Litter sizes of the crosses indicated in panel a. Bars represent average litter size ± Standard Deviation. Significance was determined by using unpaired parametric two-tailed t-test. *, $P < 0.0001$; ns, not significant.

Female bias is preserved in vitro and not associated with inheritance of the transgene

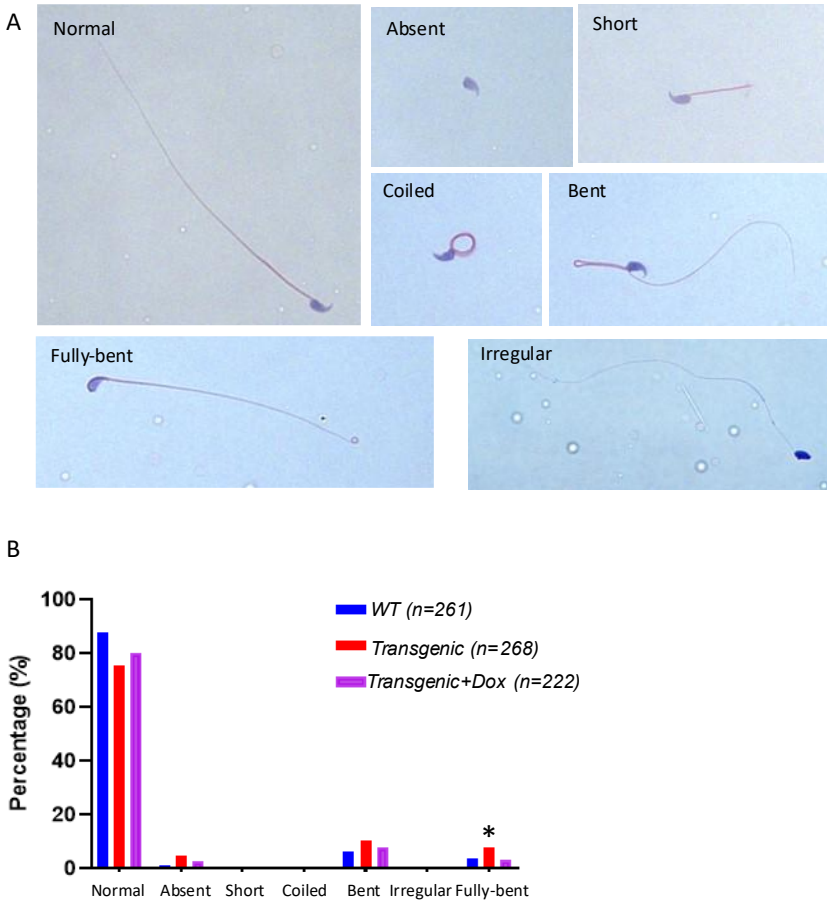
To assess whether the observed sex bias could be recapitulated in vitro, we performed IVF using sperm isolated from four transgenic males. These sperm were used to inseminate oocytes collected from wild type females. The fertilized zygotes were

160 transferred to pseudopregnant recipient females, and a total of 64 offspring were
161 obtained. Of these, 89% were female, a distribution nearly identical to that observed *in*
162 *vivo* (Figure 2). As with the *in vivo* litters, none of the female pups carried the Y-linked
163 cassette. Thus, the cassette reliably biases offspring toward females without being
164 inherited by the progeny.

165 **_Spem1_ expression and sperm phenotype do not fully explain the observed effect**

166 Although the phenotype was statistically robust and reproducible, we were surprised to
167 find that *Spem1* expression in the testes of transgenic males was only slightly and
168 insignificantly reduced compared to wild type, as determined by quantitative PCR
169 (Figure S3). Since X-bearing and Y-bearing gametes coexist in the same tissue, and
170 *Spem1* is expressed late in spermatid maturation, we considered the possibility that
171 transcripts from X-bearing cells may mask the reduction in Y-bearing gametes.
172 We further analyzed the morphology and function of sperm from three transgenic
173 males, three transgenic males treated with doxycycline, and three wild type controls. The
174 testes of transgenic mice, whether treated or untreated with doxycycline, were
175 morphologically similar to those of the control group (Figure S4a) but were smaller even
176 after adjusting for overall weight differences (Figure S4b). Importantly, despite the size
177 difference, sperm count per epididymis remained comparable across all three groups
178 (Figure S4c). The testes and the cauda epididymis appeared similar in histological
179 staining (Figure S5), though a few of the seminiferous tubules in the transgenic mice
180 (either treated or not with doxycycline) appeared abnormal. Computer assisted sperm
181 analyses for the sperm of the three groups indicated no difference in direct motility,
182 progressive motility, path velocity, progressive velocity, or track speed for sperm from all
183 groups (Figure S6). Furthermore, Y-chromosome probe labeling of the causa epididymal
184 sperm, showed equal proportions of X- and Y-bearing sperm in the transgenic and wild
185 type groups (Figure S7). These findings rule out skewed gamete production or major
186 defects in motility as causes for the observed bias.
187 Morphological analysis did reveal a specific difference: a higher proportion of
188 sperm in transgenic mice exhibited a tightly bent head structure (8.9% ± 1.5%), compared
189 to 3.8% ± 0.9% in wild type and 4.5% ± 1.8% in doxycycline-treated transgenic mice

190 (Figure 3). This morphological defect has been previously associated with *Spem1*
191 deficiency [24], but the frequency of affected sperm was far below the expected 50% if
192 all Y-bearing gametes were impaired. Thus, while the morphology data are compatible
193 with partial impairment of Y-bearing sperm, they do not explain the striking magnitude
194 of the observed female bias.



195 **Figure 3. Sperm morphology of WT mice, transgenic mice, and transgenic mice treated with**
196 **doxycycline.** A. Representative images of sperm from the cauda epididymides of different mice
197 showing the indicated morphologies. B. Distribution of the different morphologies in the different
198 groups. *, P<0.05

199 ## Discussion

200 In this study, we established a genetic system in mice that biases offspring toward
201 females without reducing litter size and without transmitting any genetic modification to
202 the progeny. This outcome was achieved by expressing a repressive cassette from the Y

203 chromosome of male mice, though the precise mechanism by which this cassette acts
204 remains to be elucidated. The cassette was designed to impair the maturation of Y-
205 bearing gametes by targeting a gene known to be required for spermatid differentiation.
206 The system was conditionally regulated through a tetracycline-responsive promoter,
207 allowing for suppression of cassette expression by doxycycline administration.

208 The transgenic males produced strongly female-biased litters, with up to 89% of
209 offspring being female, while maintaining normal fertility and average litter sizes. The
210 bias was reversed by providing doxycycline in the drinking water, supporting the
211 conclusion that the cassette is directly responsible for the effect. Importantly, female
212 offspring did not inherit the transgene, confirming that the cassette is not transmitted
213 through the X-bearing sperm. These findings demonstrate that the sex bias is linked to the
214 presence and expression of the cassette on the Y chromosome.

215 We initially reasoned that the cassette would function by repressing *Spem1*, a
216 gene required for proper spermatid maturation [24]. However, our measurements showed
217 only a slight and statistically insignificant reduction in *Spem1* expression in the testes of
218 transgenic males. Morphological analysis of sperm revealed a small but significant
219 increase in abnormal sperm with bent head morphology, previously associated with
220 *Spem1* deletion [24]. Nonetheless, the frequency of these defects was low and not
221 sufficient to account for the magnitude of the observed female bias. Sperm motility,
222 gamete counts, and the ratio of X- and Y-bearing gametes were not significantly altered.
223 Thus, although the phenotype is reproducible and cassette-dependent, the mechanism
224 underlying the selective impairment of Y-bearing sperm remains unclear.

225 While the initial design of the system relied on the hypothesis that repression of
226 *Spem1* would impair Y-bearing sperm, our findings suggest that the observed phenotype
227 is unlikely to be mediated by substantial reduction in *Spem1* levels. One possibility is that
228 the cassette affects a tightly regulated or spatially confined process during
229 spermiogenesis, in a manner that is not detectable by our standard assays. Alternatively,
230 the burden of expressing dCas9 and guides from the Y chromosome itself may
231 compromise Y-bearing sperm in a subtle but effective way. It is also possible that
232 unintended interactions between the expressed RNA guides and off-target genomic

regions contribute to the phenotype. Future studies will be required to dissect whether epigenetic, transcriptomic, or structural perturbations contribute to the observed sex bias. The ability to generate sex-biased litters without compromising fertility or transmitting transgenes opens the possibility of adapting similar systems to other mammalian species. In principle, a comparable strategy could be constructed by identifying a late-stage expression gene required for spermatogenesis in the target species and placing a repressive cassette on the Y chromosome under conditional control. The choice of target gene and promoter would require species-specific validation, but it would be of interest to test whether similar constructs can produce equivalent outcomes in other species. Such systems may find future applications in agriculture, where skewing the sex ratio toward the more economically relevant sex could improve efficiency and reduce the need for postnatal sex sorting.

245 # Materials and Methods

246 ## Targeting vector for transgene insertion

247 The targeting vector VB201210-1271-bpw was synthesized by VectorBuilder (USA). It
248 includes the following elements: Uty homology arms (a 4.6-kb 5′ arm and 2.5-kb 3′ arm) for
249 insertion into intron 2 of Uty; cassette of 5 sgRNA against *Spem1*; Kruppel-associated box
250 (KRAB) domain fused to dead Cas9 with a nuclear localization signal (KRAB-dCas9-NLS);
251 Self-deleting Neomycine (SDN) cassette; tetracycline-controlled transactivator (tTA); a
252 negative selection marker - diphtheria toxin A (DTA) cassette downstream of the 3′ homology
253 arm. The full sequence and annotations of the vector can be found here:
254 https://en.vectorbuilder.com/vector/VB201210-1271bpw.html.

255 ## Housing and husbandry

256 All mice were housed and bred under specific pathogen-free conditions maintained in the
257 Cyagen Transgenic Animal Center. Animals were housed in exhaust ventilated cage systems or
258 individually ventilated cage systems. Air in the facility was controlled by fully enclosed
259 ventilation systems. Temperature was kept at 24±2^{0}C. Humidity was kept at 40-70%. Light was
260 automatically set from 6AM to 6PM. Pre-sterilized diet was manufactured according to the
261 standard published by the Chinese government (No. GB 14924.3-2010, Laboratory Animals:
262 Nutrients for Formula Feeds). The quality of diet was monitored via periodic testing by state-
263 approved third-party test providers. Water was filtered from the city's public water system,
264 placed in bottles with sipper tubes and autoclaved before use. The bedding was placed in cages
265 and autoclaved before use. Water was added with either 0.5 mg/ml or 2mg/ml Doxycycline
266 hyclate (TCI, catalog number D4116) where indicated and replaced weekly. Mating was carried
267 out by placing one male with two female mice in a cage with nesting materials. The weight of
268 the males was monitored weekly. Copulatory plugs, pregnancies, and births were monitored
269 every three days.

270 ## Transgenic mice

271 Transgenic mice were produced by Cyagen Biosciences Inc., Suzhou, China. The linearized
272 targeting vector VB201210-1271-bpw was introduced into Cyagen's proprietary
273 TurboKnockout embryonic stem (ES) cells from C57BL/6N by electroporation. Clones that

274 underwent homologous recombination were isolated using positive (neomycin) and negative
275 (DTA) selection. 96 G418 resistant clones were picked and amplified in 96-well plates. A
276 duplicate of this 96-well plate served for DNA isolation and subsequent PCR screening for
277 homologous recombination. Of the 41 initially targeted ES clones, 18 were randomly selected
278 for recovery and expansion. From these, 5 clones showing good cell morphology were selected,
279 frozen, and subjected to Southern blot and karyotype analysis to confirm correct targeting and
280 chromosomal integrity. Six targeted ES clones were injected into pre-blastocyst-stage albino
281 B6N embryos and transplanted into CD-1 pseudo pregnant mice. This procedure yielded 24 F_0
282 mice with 100% chimerism and 3 F_0 mice with approximately 90% chimerism, as judged by
283 coat color. Three independent F_0-derived lines were established, and male founders were
284 selected randomly. Males were genotyped as described below for validating transgene insertion
285 and germline transmission.

286 **Sperm extraction and analysis**

287 Four-month-old male mice were euthanized by cervical dislocation. Following previously
288 described methods (46), epididymides were harvested and minced in Dulbecco's Modified
289 Eagle Medium (DMEM). The tissue fragments were incubated for 30 minutes at 37°C in a
290 humidified incubator to allow sperm release. Sperm concentration was determined using a
291 hemocytometer.

292 For morphological analysis, sperm smears were prepared on slides, fixed, and stained with H&E
293 (Phygene, PH0516) according to the manufacturer's instructions. Morphological abnormalities
294 were assessed by examining at least 200 spermatozoa per mouse.

295 To evaluate sperm motility, multiple incisions were made in the epididymis, which was then
296 placed in human tubal fluid supplemented with 10% FBS and incubated at 37°C for 5 minutes.
297 Sperm motility parameters were analyzed using Computer Assisted Sperm Analysis (CASA).

298 **Genotyping and sex determination**

299 Genomic DNA templates for PCR reactions were extracted from mice tails. The transgene and
300 sex of the pups were determined by 3 PCRs of the Y chromosome using oligonucleotides
301 described in Table S2 and LongAmp DNA polymerase (New England Biolabs, USA).
302 Thermocycling conditions were as follows: 94 °C (3 min), 34 X cycles of 95 °C (30 s), 60 °C

303 (30 s), 65 °C (50 s/kb) followed by 72 °C (3 min) and 4 °C (hold). Sex was further confirmed at
304 day 7 and at weaning by observing the genitals.

305 ***In vitro* fertilization**

306 Sperm was collected from different transgenic mice and stored in liquid nitrogen. Oocytes were
307 obtained from female mice 15-17 hours following chorionic gonadotropin injection. The
308 fertilization dish containing the cumulus-oocytes complexes (COCs) was incubated on CARD
309 medium for 30-60 min. before insemination. 20 µL of the sperm was thawed, diluted in
310 FERTIUP sperm dish and incubated for 30 min. 10-20 µL of sperm was then added to the COCs
311 and incubated for 3 h. The oocytes were then washed 3 times in a fresh mHTF medium. 6 H
312 after insemination, oocytes that had only one pronucleus were removed. After overnight, the 2-
313 cell stage embryos were then transferred to female mice.

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Author Contributions

Conceptualization, U.Q.; Methodology, I.Y., H.B-J. R.S., A.M., M.G., and U.Q.; Investigation, I.Y., J.C., H.B-J. R.S., A.M., M.G., and U.Q.; Writing – Original Draft, U.Q.; Writing – Review & Editing, I.Y., T.M., R.S., A.M., M.G., and U.Q.; Funding Acquisition, A.M., M.G., and U.Q.

Competing interests

I.Y., M.G., and U.Q. have submitted a patent request on a related technology in January 2019.

I.Y., A.M., M.G, and U.Q. have submitted a patent request on the described technology in August 2023.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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