Engineering mice for female-biased progeny without impacting genetic integrity and litter size

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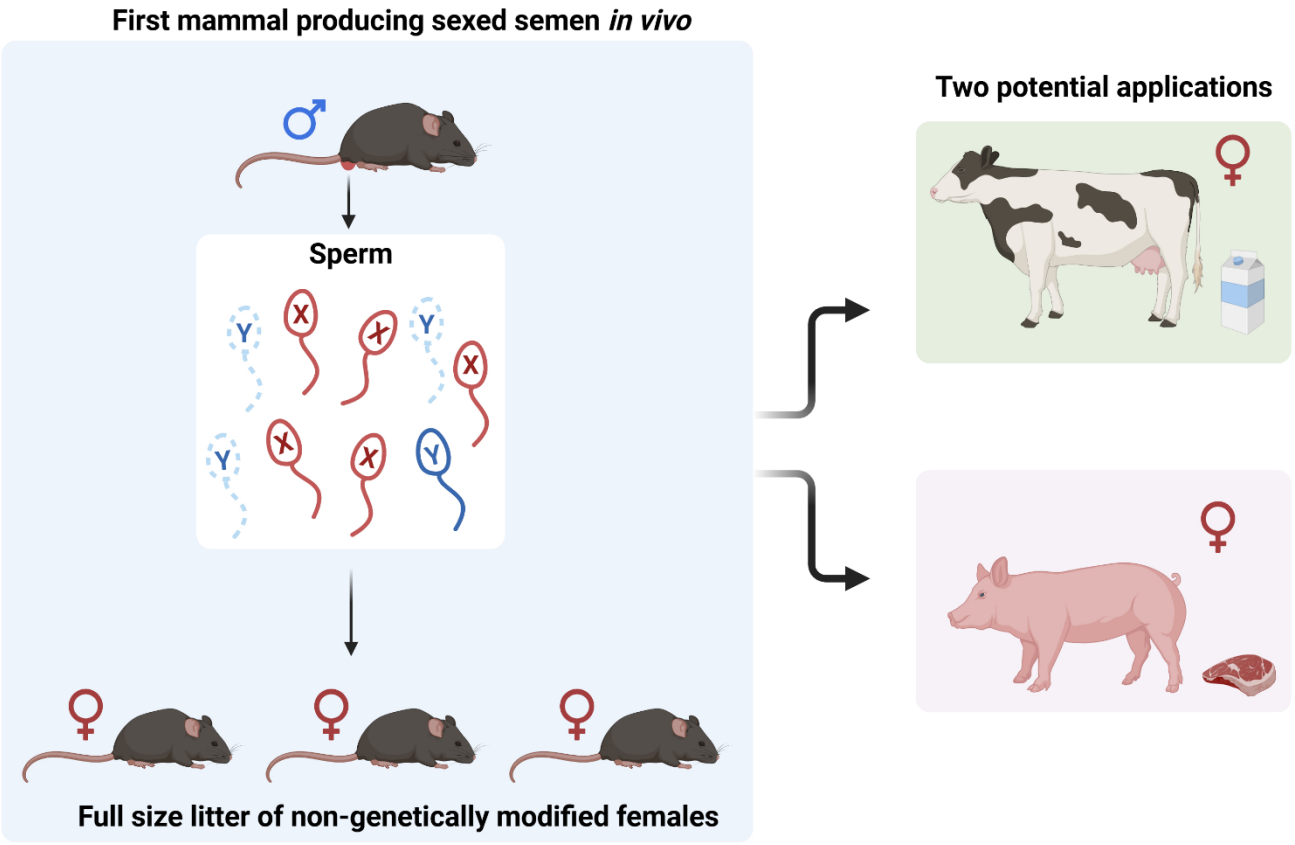

Graphical Abstract. A proof of concept for the first mammal producing sexed semen may revolutionize the way for breeding animals such as cattle for the dairy industry and swine for the meat industry.

One sentence summary

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

Significance statement

This study demonstrates a groundbreaking approach to producing predominantly female offspring in mice without altering the genetic makeup of the females. By engineering a male mouse's Y chromosome to prevent the proper development of male sperm, we can bias the offspring toward females while maintaining normal litter sizes and without genetically modifying the females. This technique has significant potential for reducing waste, labor, and ethical concerns in animal breeding.

Abstract

Generating mammalian gametes with a skewed sex ratio has thus far eluded empirical confirmation. The utilization of such genetically engineered organisms would offer the potential to curtail the necessity for culling animals of undesirable sex, mitigate resource wastage, and alleviate superfluous labor burdens. In this study, we introduce a transgenic male mouse lineage, which consistently yields predominantly female progeny (comprising up to ~90% of the total offspring). This accomplishment was made possible by integrating a controllable genetic cassette onto the Y chromosome. The cassette encodes dCas9 and RNA guides that selectively silence a spermatid maturation gene. After the separation of X and Y gametes during meiosis, gametes containing an X chromosome develop normally, while those harboring the engineered Y chromosome, subjected to dCas9 silencing of the spermatid maturation gene, do not mature properly. Indeed, some spermatozoa from the transgenic mice exhibit a unique morphology, associated with the absence of the maturation gene. Notably, the resultant female offspring do not inherit the genetically engineered Y chromosome and are thus not genetically modified. Importantly, the litter size of the transgenic mice remains unchanged compared to the wild type. These findings represent the potential of genetic engineering to yield sex-biased litters of full size without compromising genetic integrity, marking a pioneering advancement in this field of study.

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

Introduction

Generating animals of a preferred sex holds substantial advantages for various sectors involved in animal breeding. This includes economically significant farm species such as dairy cattle, egg-laying poultry, swine, and wool-producing sheep, as well as laboratory and companion animals. Sex-biased animal production not only reduces the suffering of animals of the unwanted sex but also provides economic benefits by lowering labor and resource costs.

Laboratory animals, such as mice, rabbits, and rats, offer a particularly relevant example. Many research studies, especially those focused on reproductive biology, require sex-specific models. The ability to produce animals with a controlled sex ratio could help optimize research efficiency while minimizing the ethical and logistical challenges associated with surplus animals. Furthermore, a successful approach in laboratory models could serve as a proof of principle for developing similar strategies in larger farm animals, potentially transforming breeding practices across multiple industries. (*1*).

Farmers can significantly mitigate both economic expenses and the complexities associated with rearing unwanted animals by adopting a strategy that predominantly yields animals of the preferred sex. In agricultural sectors such as dairy farming, where females are the sole choice for milk production, male offspring are often killed prior to weaning due to the financial burden they impose upon the farm (*2*). The adoption of a system that exclusively produces animals of the desired sex may substantially diminish the suffering and mortality of animals belonging to the undesired sex.

In the broader context, females typically represent the bottleneck in population expansion, given that their numerical abundance sets the upper limit on potential pregnancies, while a small number of males suffice for mating with the entire female population. Consequently, to optimize the yield of a given product, such as wool, or for any other intended purpose, favoring females in the population would facilitate the overall herd expansion. Moreover, it is worth noting that males often exhibit heightened aggressiveness towards each other, which can lead to injuries and compromise the overall welfare of the herd (*3*). Hence, in cases where males are not the preferred choice for specific reasons (e.g., male calves in the meat industry due to their superior meat production mass (*1*)), skewing

the sex ratio in favor of females would result in an economic benefit of swift expansion of the animal population with the added benefit of reduced aggressiveness within the herd.

The only method currently employed to influence the sex distribution of domesticated cattle livestock relies on the use of sexed semen, a product obtained through flow cytometry-based sorting of desired gametes (*4*, *5*). This sorting process leverages the slight difference in weight between gametes carrying the X versus the Y chromosome. Sorted sexed semen undergoes approximately 20 procedures, yielding a potential sex bias of up to 90% while losing more than 50% of the total semen. The process is labor-intensive, involves high economic costs, has a reduced fertility rate compared to unprocessed semen, is associated with the production of oxidative stress and DNA fragmentation in the semen (*6, 7*), and has variable effectiveness in achieving the desired sex ratio. Additionally, this method is not prevalent in swine breeding due to the challenges posed by the high volume and count of pig sperm (*8*).

Hormonal-based approaches have been successfully implemented crustaceans (*9*) and fish (*10-13*) to generate litters consisting entirely of one sex. These treatments involve the administration of hormones, which can feminize males or masculinize females, leading to the production of offspring of a single sex. However, these hormonal treatments are not practical or feasible for application in mammals and poultry.

Genetic-based methodologies for inducing sex bias in offspring have been successfully implemented in various insects, including mosquitoes and drosophila flies, through the manipulation of specific genes (*14-22*). In mammals, our research group achieved a pioneering milestone by introducing a genetic system comprising two distinct mouse lines, the mating of which predominantly yielded female offspring (*23*). The fundamental principles elucidated in our work were subsequently refined and successfully replicated by the Turner group, achieving complete sex bias for both males and females (*24*). In our published study, zygote development within the uterus occurred predominantly when fertilized with an x gamete because the y gamete was lethal. Specifically, male sperm carried RNA guides on their Y chromosome, which, in conjunction with the CRISPR-Cas9 protein encoded by the female's autosomal chromosomes, led to the demise of developing male zygotes. This intricate genetic system necessitated the use of two distinct mouse lines: one carrying the Cas9 gene in females and the other

encoding the RNA guides in males. Notably, the litter size resulting from such mating was reduced by approximately half due to the selective abortion of male progeny. Furthermore, it is essential to recognize that the female mice involved in this process were genetically modified organisms (GMOs), as they harbored the Cas9 gene within their genome.

Securing approvals for GMOs intended for human consumption is a stringent and intricate process. GMOs destined for consumption must conform to a set of criteria outlined in the United States Food and Drug Administration (FDA) Act to ensure their safety and effectiveness. These criteria encompass several key aspects: safety for human consumption; safety for the modified organism; demonstration of enhanced traits, and minimal environmental impact. It is worth noting that the FDA has granted approvals for certain GMOs, including salmons that exhibit accelerated growth compared to their non-GMO counterparts (*25*), as well as genetically engineered pigs (*26*). However, it is important to acknowledge that these approvals may not necessarily be universally accepted as safe, either by the public or by regulatory agencies outside the purview of the FDA. Consequently, the availability of non-GMO animals remains highly advantageous, particularly from a commercialization perspective.

The development of an efficient genetic system capable of yielding null segregants (non-GMO) progeny with regular litter sizes represents a leap forward in the field. This system leverages natural reproduction processes, obviating the need for cumbersome technological interventions, hormone treatments, or other costly and intricate methods. Of particular significance is the fact that normal litter sizes effectively double the production of animals of the desired sex. This doubling of output translates into profitability, especially within sectors such as laying hens, the dairy and the swine industry, where efficiency and yield are pivotal.

In this study, we present a proof-of-concept, marking the first instance of achieving normal-sized litters with a sex bias in mammals. This accomplishment entails the production of a null segregant (non-GMO) female offspring from genetically modified males. Crucially, the litter size of the resulting progeny remains within the typical range. Moreover, our system stands out for its simplicity in both production and maintenance when compared to previous genetic systems. Notably, genetic modification is exclusively applied to the male parent, leaving the female parent unaltered, thus

enabling the maintenance of a single transgenic lineage. This streamlined approach underscores the practicality and feasibility of implementing this methodology.

Results

To generate males whose sperm exclusively contains X-gametes, we engineered a cassette on the Y chromosome producing a repressor of a gene essential for gamete development. Our rationale was that after the separation of the X gametes from the Y gametes during meiosis, the Y gametes would fail to mature due to the repression of this maturation gene, while the X gametes, lacking the repressor post-meiosis, would undergo normal development. Consequently, these males would predominantly produce X gametes, leading to the generation of mostly female offspring (Figure 1a). The cassette itself was designed to be repressed through a genetic switch to allow production of Y gametes for maintaining the male lineage.

To genetically engineer these mice, we constructed a plasmid encoding dCas9, a modified version of Cas9 capable of gene silencing without DNA cleavage (*27*). To enhance its repression activity, we fused dCas9 with the KRAB domain. The cassette featured five RNA guides that directed dCas9 to silence the *Spermatid maturation 1* (*Spem1*) gene, an essential component of spermatid maturation in mice (*28*). Furthermore, a tetracycline-trans activator repressed dCas9-KRAB (hereafter referred to as dCas9) expression in the presence of tetracycline/doxycycline (*29*) (Figure 1b). To facilitate integration into the Y chromosome, the entire cassette was flanked by sequences homologous to an intron in the *Uty* gene encoded on the Y chromosome (*30*), an intron previously targeted by us for insertion of RNA guides (*23*).

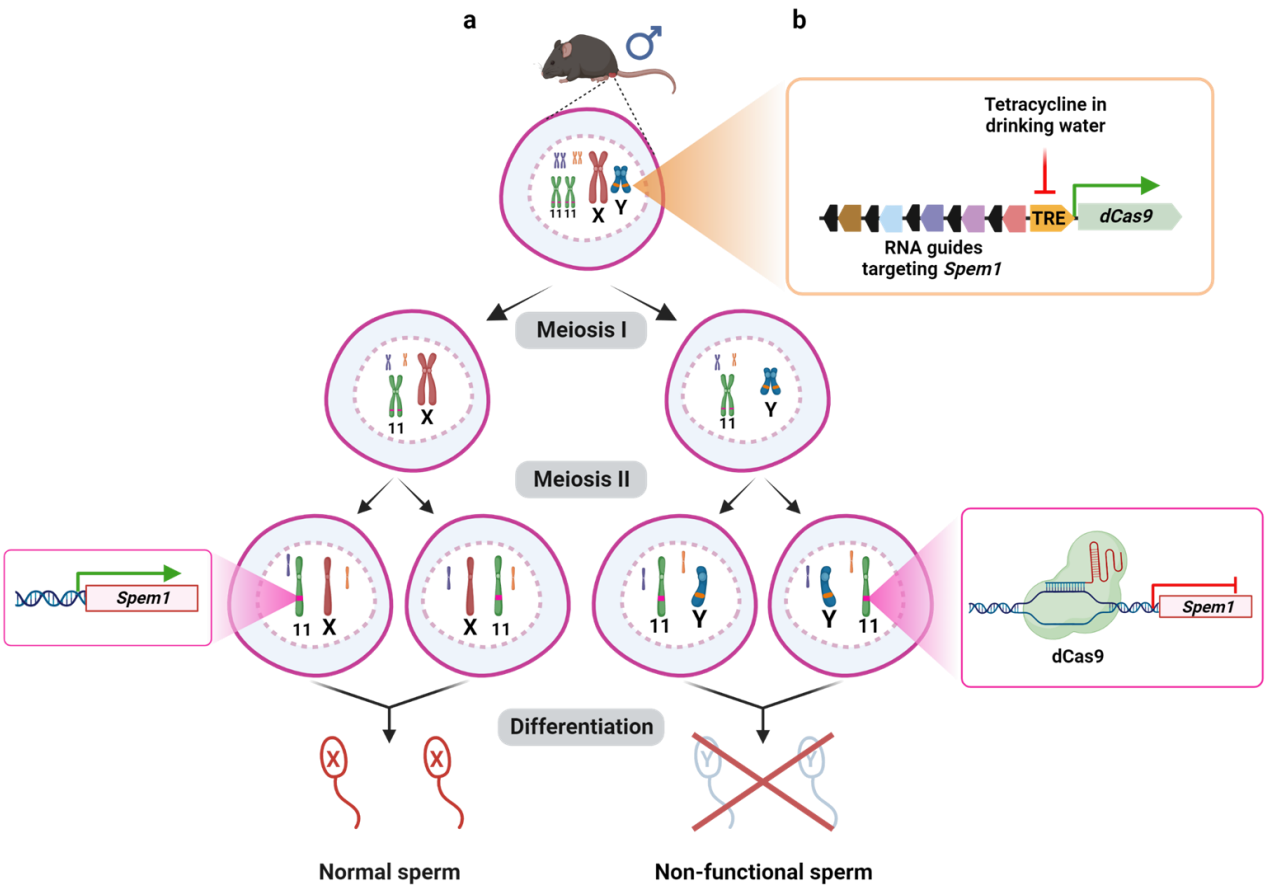

Figure 1. Sexing the semen *in vivo* in a male mouse. (**a**) A male mouse's Y chromosome is genetically engineered to contain a cassette repressing a gene essential for gamete development. After meiosis, the Y-gametes encode and express the repressing gene whereas the X-gametes lack them. Consequently, the Y-gametes do not mature whereas the X-gametes mature normally. (**b**) A schematic representation of the cassette inserted to the Y chromosome. dCas9 is expressed from a promoter encoding a tetracycline responsive element (TRE), repressed if tetracycline/doxycycline are present in the drinking water. The cassette also encodes 5 different RNA guides that target the *Spem1* gene encoded on chromosome 11. The representation is not adjusted to the actual scale. Additional elements of the cassette are described in Materials and Methods.

We introduced this cassette into mouse embryonic stem cells (mESC) and achieved integration into the Y chromosome through homologous recombination. Subsequently, we used these modified mESCs to generate transgenic mice using a proprietary technology (*31*). An issue that we encountered is that the F0 transgenic mice mated inefficiently. We therefore used in vitro fertilization (IVF) to

successfully yield progeny. The produced male mice all carried the transgene on their Y chromosome, as validated by PCRs (Figure S1).

Another noticeable finding was that at 8 weeks of age, the average weight of the transgenic mice was ~25% lower than that of the wild type mice (Table S1). This phenotype was not previously reported for *Spem1* knockout mice (*28*), but it should not impact the main goal of producing mostly-female offspring, as the male mice are merely carriers of sperm and not the desired final product. Aside from the weight difference, the transgenic mice developed normally, with no physiological or morphological abnormalities, consistent with previous reports on *Spem1* knockout mice (Figure S2).

We quantified Spem1 expression by PCR and, unexpectedly, found that it is only slightly and insignificantly reduced in transgenic mice compared to wild type mice (Figure S3). However, we considered the possibility that this effect may be partially obscured by expression of Spem1 from the X gametes. To further investigate, we proceeded with sperm characterization, comparing sperm from three transgenic mice, three transgenic mice treated with doxycycline (to repress cassette expression), and three wild type mice. The testes of transgenic mice, whether treated or untreated with doxycycline, were morphologically similar to those of the control group (Figure S4a) but were smaller even after adjusting for overall weight differences (Figure S4b). Importantly, despite the size difference, sperm count per epididymis remained comparable across all three groups (Figure S4c). The testes and the cauda epididymis appeared similar in histological staining (Figure S5), though a few of the seminiferous tubules in the transgenic mice (either treated or not with doxycycline) appeared abnormal.

Computer assisted sperm analyses for the sperm of the three groups indicated no difference in direct motility, progressive motility, path velocity, progressive velocity, or track speed for sperm from all groups (Figure S6). Furthermore, Y-chromosome probe labeling of the causa epididymal sperm, showed even distribution of Y-gametes to X-gametes in the transgenic mice and, as expected, also in the wild type group (Figure S7).

The morphology of the sperm showed some differences between the transgenic group and the wild type group. Importantly, we observed that the significant differences in the sperm of the transgenic mice compared to that of the wild type mice and the doxycycline treated mice stemmed from distinct bending of the head toward the tail (fully bent morphology) (Figure 2). Bent heads were observed as a

hallmark morphological signature in the lack of *Spem1* (*28*). These findings support the hypothesis that some gametes fail to develop properly due to Spem1 silencing. Moreover, the fact that the observed percentage of bent morphology is lower than the expected 50%, if Spem1 is completely silenced in all Y-gametes, suggests that the phenotype results from partial, rather than complete, reduction of Spem1.

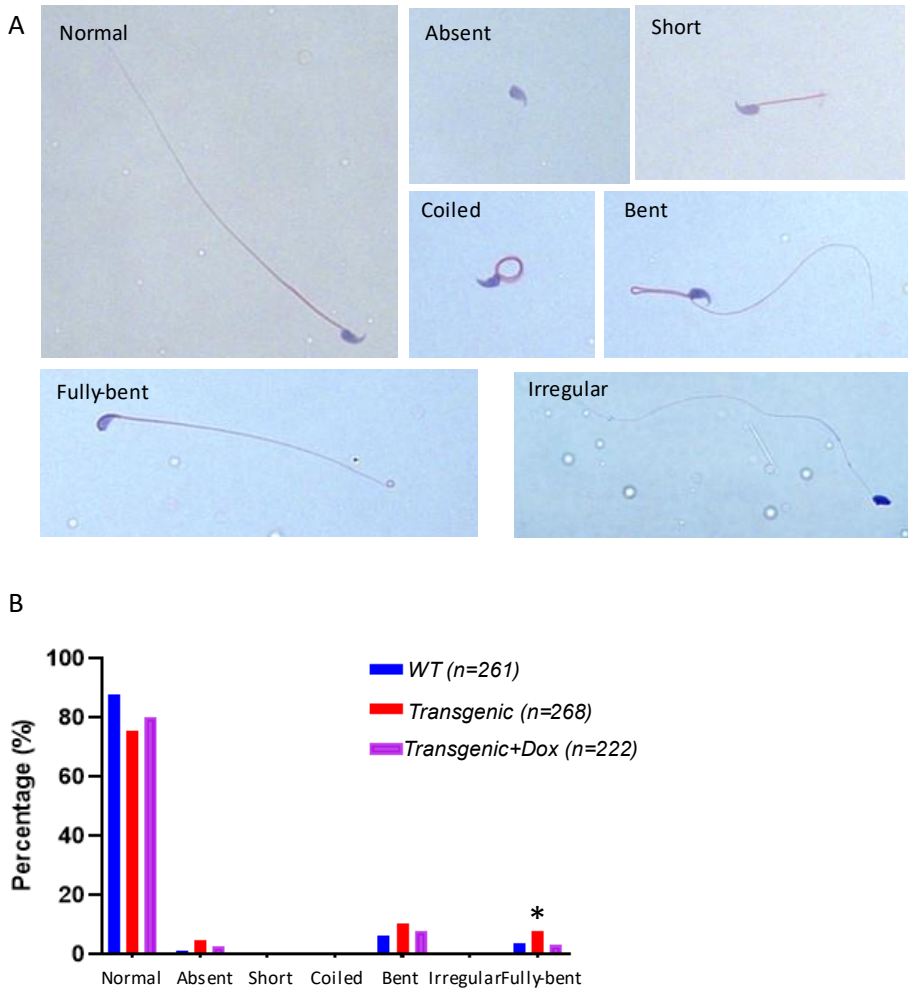

Figure 2. Sperm morphology of WT mice, transgenic mice, and transgenic mice treated with doxycycline. A. Representative images of sperm from the cauda epididymides of different mice showing the indicated morphologies. B. Distribution of the different morphologies in the different groups. *, P<0.05

Based on the morphological defects of the sperm of the transgenic mice compared to the control mice, we hypothesized that at least some of their Y gametes are selectively defective. If indeed this is the case, then their progeny should show bias toward females. Furthermore, based on the construction of the cassette, we expected that in the presence of doxycycline, dCas9 would be repressed, leading to a weaker effect of the cassette and consequently a Y-bearing sperm production pattern that is closer to the expected unbiased distribution.

To evaluate our hypotheses, we first mated a group of 6 wild type C57BL/6N mice, which produced a typical sex ratio of ~43% males and ~57% females (n=61) (Figure 3a). Next, we mated a group of 15 F2 transgenic mice, which exhibited a striking female-biased sex ratio of ~89% females and only ~11% males (n=157). The sex of all progenies was determined through physical examination and PCR testing, confirming that all male offspring carried the transgene on the Y chromosome, while all females tested negative for it (Figure S1). Notably, this strong sex bias was observed despite the incomplete silencing of Spem1, suggesting that even a subtle reduction in its expression can lead to a highly significant effect on progeny sex ratio. To further confirm that this effect was specifically driven by the cassette and dCas9 activity, we treated a group of 8 transgenic mice with doxycycline to repress the cassette's expression. As expected, this group produced a normal sex ratio of ~54% females and ~46% males (n=56), statistically indistinguishable from the wild type ratio. This result demonstrates that the observed sex bias is directly dependent on the expression of the cassette, allowing production of males to continue the line when required.

The average litter sizes across all four groups are comparable to those of the wild type control group (Figure 3b). These findings indicate that the transgenic mice exhibit normal litter sizes, irrespective of doxycycline treatment.

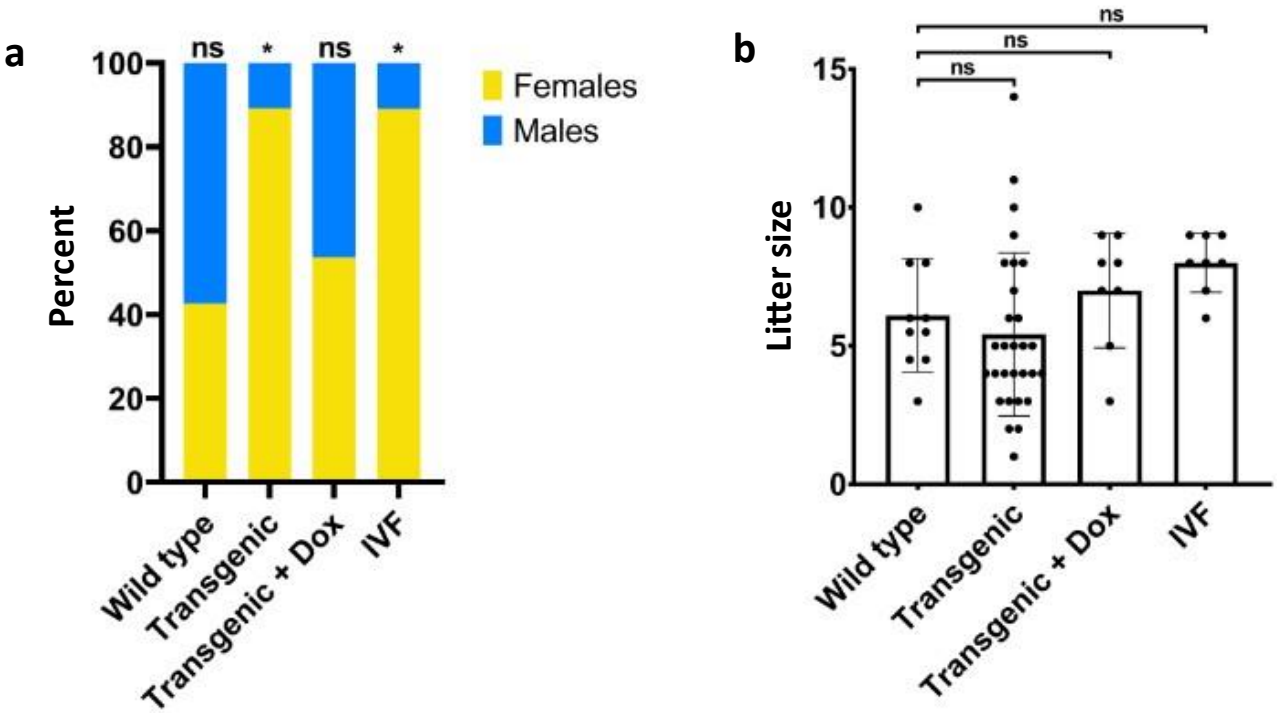

Figure 3. (**a**) The sex distribution of the total pups from crosses between C57BL/6N females with wild type C57BL/6N males (n=61), F_2 transgenic males (n=157), F_2 transgenic males treated with 2mg/ml doxycycline (Transgenic + dox) (n=56), and F_2 transgenic sperm through IVF (n=64). (**b**) Litter sizes of the crosses indicated in panel a. Bars represent average litter size ± Standard Deviation. Significance was determined using unpaired parametric two-tailed t-test. *, $P < 0.0001$; ns, not significant.

Lastly, we wished to investigate the viability of extracted sperm for fertilization, akin to the application of sexed semen in cattle. To this end, sperm was extracted from the epididymis of 4 F_2 transgenic mice (all of them used in the experiment above) and employed in IVF procedures with 8 different wild type female counterparts. Notably, these IVFs resulted in the successful development of 8 pregnancies out of 8 IVF procedures, yielding a total of 64 offspring (average litter size of 8 pups). Subsequent analysis of the offspring's sex composition revealed that 89% of the progeny (57 out of 64 pups) were females (Figure 3a). These results effectively demonstrate that the sperm is functional, and

further extends the potential applications of transgenic animals to include the production of sexed semen.

Altogether, these results demonstrate, for the first time, a genetic engineering of a mammal male resulting in the production of sexed semen *in vivo*. The sexed semen produces a null segregant (non-GMO) bias sex ratio toward females. Importantly, a full litter size is observed, doubling the production of the desired sex.

Discussion

We present the creation of a genetically engineered mammal with *in vivo* sexed semen, effectively eliminating most of the Y gametes. This demonstration represents a significant advancement compared to the current state-of-the-art method of *ex vivo* sexed semen for producing sex-biased litters. Sexed semen technology has been employed for generating offspring of a preferred sex in several species, including cattle and humans, but is not feasible in certain species such as swine. The success rate of sex bias using sexed semen technology reaches up to 90%, with a typically lower fertility compared to unsexed semen (*32*, *33*). In our approach, we achieve a similar bias with minimal ongoing effort after the initial engineering of the mammal. The genetically modified male can be propagated for at least three generations without the need for additional labor or complex equipment. Importantly, this technology does not affect the litter size, because the males are not terminated in utero but rather the gametes producing males are eliminated in the sperm. The genetic manipulation on the male's Y chromosome does not pass to the females, maintaining the null segregant (non-GMO) status of the female offspring. These qualities align this technology with the positive aspects of sorted sexed semen while avoiding its disadvantages.

Despite the high percentage of female biasing, only a minority, not constituting 50% of sperm, displayed a morphology indicative of complete *Spem1* inactivation (*28*). It was expected that a higher percentage of morphology-defective cells, i.e., most of the Y gametes, would show this morphology. The low incidence, exhibiting this phenotype may arise from the incomplete suppression of *Spem1*, leading to other subtle morphological alterations challenging to discern microscopically. Indeed, quantitative PCR showed that Spem1 expression is only slightly reduced, explaining the observed phenotype. Despite being undetected, these subtle alterations likely make the gametes non-functional, suggesting an explanation for the difference between the malformed gametes observed and the resulting female bias. This also suggests that further suppression of Spem1 expression will result in even higher bias toward females.

Transitioning to the production of single-sex males can be achieved straightforwardly by transferring the genetic construct to the X chromosome in the male parent. A similar approach, switching the transgene to the opposite sex chromosome to yield single-sex litters of the opposite sex,

has been previously demonstrated by Turner and colleagues (*24*). This method offers a versatile means of manipulating offspring sex ratios to suit specific breeding objectives.

The proof-of-principle described here holds promise for application in other mammalian species, including cows within the dairy industry. A similar genetic cassette can be designed and constructed on the Y chromosome of a bull, necessitating only minor modifications. Specifically, the *Spem1* gene can be targeted for silencing, given its high degree of conservation across mammals, with homologs identified in rat, dog, cow, chimp, and human (*28*). Targeting an additional gene with similar properties (see criteria discussed below) simultaneously may provide even enhanced efficiency in female production (i.e., close to 100%). Once established, the sperm from the genetically engineered bull may be utilized to fill insemination straws for fertilizing cows. This adaptability underscores the versatility and potential widespread applicability of the approach across various mammalian species.

Several crucial features are required from a silenced gene, such as *Spem1*, to effectively eliminate sex-specific gametes: 1. Testis-specific expression: The gene should be exclusively required only for gamete development and expressed solely within the testis. Silencing a gene whose expression is required in other tissues could lead to undesired potential developmental defects in the animal. *Spem1* and numerous other testis-specific genes fulfill this criterion (*36*-*38*). Notably, knockout studies of *Spem1* have demonstrated that mice develop normally in its absence, except in their sperm maturation (*28*). 2. Essentiality for gamete formation: The gene must play a pivotal role in gamete formation and be indispensable for the process. In the case of *Spem1*, its absence leads to the production of deformed sperm with characteristic structural and motility abnormalities, ultimately resulting in male infertility (*28*). 3. Expression timing during meiosis: The stage of meiosis during which the gene is silenced is also important. Silencing of the maturation gene should occur after the separation of gametes to ensure that only the targeted sex gamete is affected. Spermatogenesis involves several distinct stages, such as mitoses and two meiotic divisions (*39*-*41*). The earliest point at which gamete selection, based on specific silencing, can be effectively implemented is after the first meiotic division, where the X and Y chromosomes segregate. However, even at this stage, the presence of cytoplasmic bridges, which exist early in meiosis and persist into the spermatid stage, can potentially allow for organelles and protein transfer between gametes (*42*, *43*). Consequently, *Spem1*, chosen for its exclusive expression in the late stages of spermatid formation, aims to reduce the potential transfer of

dCas9 into X gametes through these cytoplasmic bridges (*28*). These stringent criteria ensure that the silencing of the gene selectively and effectively targets the desired gametes, minimizing off-target effects and preserving the overall integrity of the organism's development.

Considering the presence of cytoplasmic bridges and the potential for proteins transfer, there has been speculation regarding whether the separation of X and Y gametes is sufficient to enable the selective elimination of one gamete while preserving the other. Some published literature supports the notion that X and Y gametes may indeed differ in their content, despite the existence of these bridges [e.g., (*44-47*)]. Conversely, others have expressed doubts, suggesting that the cytoplasmic content of the gametes is balanced by the cytoplasmic bridges (e.g., (*39*, *43*)). Our system provides empirical evidence that, at the very least, certain elements—specifically, dCas9 and the RNA guides—may be differentially distributed between gametes. We speculate that the presence of a nuclear localization signal (NLS) attached to the dCas9 protein localizes it within the nucleus of the Y gametes where it is encoded, while it remains absent from the X gametes. Thus, despite the presence of cytoplasmic bridges, it is plausible that proteins carrying an NLS are differentially distributed within the gametes.

The principles and considerations applied in the design, production, and optimization of the mouse line should serve as a valuable framework for transferring this technology to bulls and other animals. The potential to generate a null segregant (non-GMO), sex-biased litters with normal litter sizes holds the promise of revolutionizing animal breeding practices across various facilities engaged in large-scale animal production. In certain scenarios, this innovation could lead to a doubling of the desired yield, eliminate the ethically challenging practice of culling animals of undesired sex, and significantly conserve resources, energy, and labor. This groundbreaking approach has the potential to usher in a new era of efficiency and ethics within the field of animal breeding, offering a sustainable and humane solution to enhance productivity while minimizing waste and resource consumption.

Materials and Methods

Targeting vector for transgene insertion

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

Housing and husbandry

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

Transgenic mice

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

isolated using positive (neomycin) and negative (DTA) selection. 96 G418 resistant clones were picked and amplified in 96-well plates. A duplicate of this 96-well plate served for DNA isolation and subsequent PCR screening for homologous recombination. The PCR screening identified 41 potential targeted clones, from which 5 were expanded and further validated to contain the insert by Southern blot and Sanger sequencing. Targeted ES clones were injected into albino B6N embryos and transplanted into CD-1 pseudo pregnant mice to generate F_0 mice with 100% chimerism. F_0 and F_1 males were genotyped as described below for validating transgene insertion and germline transmission.

Sperm extraction and analysis

Sperm extracted from the epididymis was washed twice with PBS, resuspended in 200 μl of PBS. Ten μl of the suspension, or dilutions thereof, were placed on slides and smeared with an additional slide, as described (*48*). The samples were air-dried, immersed in 75% ethanol and-air dried again. The preparations were viewed under the microscope and at least 100 spermatozoa were counted. The percentage of spermatozoa with heads that were bent backward (only those with an unequivocal morphology) was calculated from the total spermatozoa.

Genotyping and sex determination

Genomic DNA templates for PCR reactions were extracted from mice tails. The transgene and sex of the pups were determined by 3 PCRs of the Y chromosome using oligonucleotides described in Table S2 and LongAmp DNA polymerase (New England Biolabs, USA). Thermocycling conditions were as follows: 94 °C (3 min), 34 X cycles of 95 °C (30 s), 60 °C (30 s), 65 °C (50 s/kb) followed by 72 °C (3 min) and 4 °C (hold). Sex was further confirmed at day 7 and at weaning by observing the genitals.

***In vitro* fertilization**

Sperm was collected from different transgenic mice and stored in liquid nitrogen. Oocytes were obtained from female mice 15-17 hours following chorionic gonadotropin injection. The fertilization dish containing the cumulus-oocytes complexes (COCs) was incubated on CARD medium for 30-60 min. before insemination. 20 μL of the sperm was thawed, diluted in FERTIUP sperm dish and incubated for 30 min. 10-20 μL of sperm was then added to the COCs and incubated for 3 h. The oocytes were then

washed 3 times in a fresh mHTF medium. 6 H after insemination, oocytes that had only one pronucleus were removed. After overnight, the 2-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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