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Male gamete copies to characterize genome inheritance and generate progenies

Philip Xie[1], Takumi Takeuchi[2], Stephanie Cheung[1], Zev Rosenwaks[1] & Gianpiero D. Palermo[1]✉

Male factor infertility accounts for approximately 30% of infertility cases. When spermatozoa are extremely scarce, replicating the male gamete to fertilize a large cohort of oocytes is ideal. Additionally, patients with inherited disorders currently rely on pre-implantation genetic testing (PGT) to select healthy embryos, which raises ethical concerns owing to the generation of multiple embryos to select one healthy conceptus. Therefore, it would be beneficial to decode the genetics of a single sperm cell before conceptus generation. In this study, we demonstrated the feasibility of replicating the sperm genome via androgenesis and selecting the desired gamete before fertilization to preserve a specific paternal genotype, as confirmed by phenotypic observations and genetic testing in a murine model. We achieved satisfactory pre-implantation development rates with replicated male gametes and generated healthy offspring. Specifically, using 8-cell stage androgenetic embryos, a single spermatozoon can yield up to three conceptuses carrying an identical paternal haplotype.

Male infertility affects approximately 30% of the infertile population and 9–15% of the overall population.[1] Intracytoplasmic sperm injection (ICSI) offers a promising solution by allowing a single selected spermatozoon to fertilize an oocyte regardless of the quantity, type of motility and morphology of the male gamete.[2,3] Even in cases where the spermatozoa are functionally impaired, such as globozoospermia, ICSI can grant fertilization when used in conjunction with assisted oocyte activation.[4] However, in cases where spermatozoa are extremely scarce, it would be ideal to replicate male gametes to fertilize a large cohort of oocytes. In addition, some patients have inherited disorders, and the current treatment plan is solely dependent on the selection of embryos available through pre-implantation genetic testing (PGT). This is ethically debatable, because several embryos are generated to select a normal conceptus.[5] Therefore, it is ideal to decipher the genetics of a single sperm cell prior to conceptus generation.

There are situations in which inherited disorders manifest due to the heterogeneity of the germline. This gamete heterogeneity is relevant in cases of structural chromosomal abnormalities, which occur in 5–15% of infertile men.[6] These abnormalities include balanced and unbalanced translocations, where the affected individual generates a heterogeneous sperm population, whether healthy or abnormal.[7–9] In contrast, gamete heterogeneity can also be characterized based on monogenic diseases, which are estimated to affect approximately 6% of the human population.[10] In autosomal recessive disorders, phenotypically healthy carriers produce a heterogeneous population of germ cells because of their heterozygosity.[11] Nonetheless, even in individuals who are not carriers of diseases, de novo germline mutations may occur and accumulate over many years at a frequency of 45–60 per genome per generation[12]. For example, unaffected individuals may pass on pathological de novo variants of alleles such as the X-linked *TEX11* mutation and the autosomal dominant *PTPN11* mutation responsible for the Noonan Syndrome.[13–15]

This awareness has stimulated attempts to replicate the sperm genome to identify healthy gametes. Indeed, androgenetic embryos can be generated by microinjection of a single spermatozoon into an enucleated oocyte,[16] which can yield haploid embryonic stem cells (haESCs) to study a recessive phenotype, or be utilized in reproductive semi-cloning.[17,18] The generation of haESCs has been successful; however, as expected, the utilization of the haESC lines is hindered by a low level of blastocyst development, averaging 12.8%, and an even lower efficiency of haESC derivation of up to 40%.[19] Therefore, to obtain one haESC line, at least 20 haploid embryos must be sacrificed, even under optimal conditions. In addition, haploidy in mammalian somatic cells is unstable and tend to self-diploidize due to endo-cycling and failed cytokinesis, which becomes more manifested where the uncoupling of DNA replication and centrosome duplication cycles are more common.[20–23] Once

[1]The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Cornell Medicine, 1305 York Avenue, New York, NY Y72010021, USA. [2]Reproduction Clinic Tokyo, Shiodome City Center 3F, 1-5-2 Higashi-shimbashi, Minato-ku, Tokyo, Japan. ✉email: gdpalerm@med.cornell.edu

haESCs are achieved and purified, the utilization of such cells as a source of male gametes was able to yield offspring of the desired genotype, albeit at a very low rate at ~ 4.5%.[24,25] Nevertheless, concerns exist about the epigenetic profiles of the replicated male gametes and consequential genetic imprinting disorders after its use.

In this study, we replicated murine spermatozoa by injecting a single spermatozoon into an enucleated oocyte and subsequently used these replicated gametes to fertilize oocytes and generate conceptuses. We closely tracked the pre-implantation development of the conceptuses using time-lapse microscopy, and monitored the post-implantation development by assessing the health and reproductive abilities of the offspring. To confirm the provenance of the paternal genome, we sequenced the DNA of replicated pseudo-gametes and compared them with the genomes of the resulting offspring. We also assessed the uniformity of the genotype among siblings originating from a single spermatozoon.

Methods

Study design

Here, we conducted a preliminary study to assess the ability of haploid androgenetic and gynogenetic constructs to develop into blastocysts in comparison with a mouse ICSI control. Next, we assessed the ability of haploid androgenetic pseudo-blastomeres to generate conceptuses as sources of male gametes at different cleavage stages. Haploid pseudo-blastomeres were isolated from 2-cell, 4-cell, and 8-cell stage embryos. The resulting constructs were assessed for full pre-implantation development. Chromosomal analyses were performed on some embryos to confirm their ploidy. To demonstrate the developmental competence of these biparental zygotes, hatching blastocysts were transferred to pseudo-pregnant mice. This allowed us to assess the development up to adulthood and the related reproductive potential. In a subsequent series of experiments aimed at confirming the origin of the male genome, we generated haploid androgenetic embryos as a source of male gametes using GFP-transgenic male mice (Fig. 1). This study was approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine (protocol number: 0605-493A). All animal studies were carried out in accordance with institutional guidelines and regulations. This study was conducted in accordance with the ARRIVE guidelines. The animals used in this study were purchased from the Jackson Laboratory and the Charles River Laboratory.

Ovarian stimulation, oocyte collection, and oocyte preparation

To harvest mouse metaphase II oocytes, 8–12-week-old B6D2F1 mice (Jackson Laboratory) were stimulated with an intra-peritoneal (IP) injection of 0.1–0.2 cc of ready-to-use pregnant mare serum gonadotropin/inhibin superovulation reagent (CARD HyperOva®; Cosmo Bio, Japan). Forty-eight hours after stimulation, oocyte maturation and ovulation were triggered using an IP injection of 7.5IU of human chorionic gonadotropin (hCG; Sigma-Aldrich, St. Louis, MO, USA). Up to 16 h after hCG treatment, the mice were euthanized by cervical dislocation and dissected to retrieve oviducts containing cumulus-oocyte complexes (COCs).

The COCs were isolated from the ampullae of the oviducts and treated with 80IU/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), followed by mechanical denudation using a microcapillary. Oocytes were serially washed three times in potassium simplex optimized medium (KSOM; Cosmo Bio, Japan) and placed in an incubator at 37 °C and 5% CO_2 in equilibrated KSOM for subsequent use.

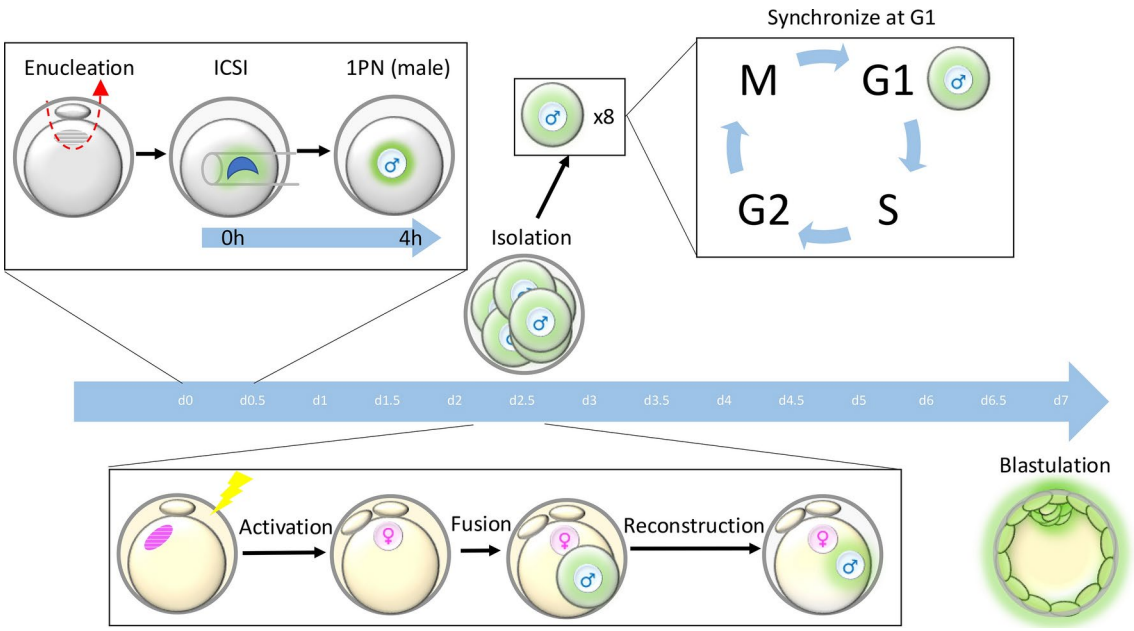


Fig. 1. Timeline—8-cell stage haploid male pseudo-blastomere NT.

Spermatozoa collection and preparation

Euthanasia was performed by cervical dislocation on male B6D2F1 or heterozygous B6-EGFP mice aged 12–16 weeks. The lower abdomen was sanitized with betadine, an incision was made surgically, and the bilateral cauda epididymis was identified and excised. The samples were placed in modified human tubal fluid (HTF) medium (Cosmo Bio, Japan) on a heat block for transport. Spermatozoa were extracted from the cauda epididymis and diluted to 3 million/mL for insemination.

Preparation of ooplasts for haploid androgenetic embryos

To generate haploid androgenetic embryos, mature oocytes from B6D2F1 mice were enucleated using techniques based on previous studies.[26–28] Briefly, denuded oocytes were transferred into HEPES-buffered M2 medium (Sigma-Aldrich, St. Louis, MO, USA) droplets supplemented with 5 µg/mL cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA) on specialized glass dish (FluoroDish™; WPI, Inc., Sarasota, FL, USA). Instead of nuclear staining, which is commonly used in cloning, oocyte metaphase II spindles were visualized under a stain-free polarizing microscope (Oosight®; Hamilton-Thorne, Beverly, MA, USA). The oocyte was then held using a holding pipette (Vitrolife, Gothenburg, Sweden) and rotated to have the spindle located at the 3 o'clock position. A laser (LYKOS®; Hamilton-Thorne, Beverly, MA, USA) or piezo pulse (PMM-150; PrimeTech, Japan) was then applied to breach the zona. Next, a micropipette (Vitrolife, Gothenburg, Sweden) was advanced into the perivitelline space through the breach and gentle suction was applied to remove the spindle. The enucleated oocytes were then placed in KSOM for at least 1 h until the next procedure.

Generation of haploid androgenetic embryos

Piezo-actuated ICSI was performed to generate haploid androgenetic embryos using enucleated oocytes. The piezo-ICSI procedure was performed according to a standard protocol.[29] A single mouse spermatozoon from B6D2F1 or heterozygous B6-EGFP mice was mechanically decapitated by rubbing the flagella with the tip of micropipette, and the isolated head was suctioned into a micropipette in 7% polyvinylpyrrolidone (Fujifilm Irvine Scientific, Santa Ana, CA, USA). An individual ooplast, placed in HEPES-buffered M2 medium, was then positioned using a holding pipette (Vitrolife, Gothenburg, Sweden) and rotated so that the previously created breach was placed at the 3 o'clock position. An injection micropipette containing the spermatozoa was advanced into the perivitelline space of the ooplasts, creating an invagination. Finally, a weak piezoelectric pulse was applied to penetrate the oolemma, enabling the sperm head to be deposited into the ooplasm.

Post-ICSI oocytes were then cultured in KSOM for 24 h, 36 h, or 48 h to generate 2-cell, 4-cell, or 8-cell haploid androgenetic embryos, respectively. In our modified experiment using spermatozoa from B6-EGFP mice, haploid androgenetic embryos were cultured in G-TL medium (Vitrolife, Gothenburg, Sweden) designed for time-lapse microscopy. Each haploid androgenetic embryo was transferred into an EmbryoSlide (Vitrolife, Gothenburg, Sweden) microwell and covered with mineral oil (Vitrolife, Gothenburg, Sweden), and the EmbryoSlide culture dish was then loaded into the EmbryoScope to capture images every 10 min for time-lapse microscopy to observe embryo morphokinesis (Supplementary video 1). Haploid androgenetic embryos displaying extensive fragmentation (> 35%), abnormal morphokinetics, or arrested development were excluded from the study.

Preparation of control zygotes

Intact MII oocytes from B6D2F1 mice were microinjected with spermatozoa from B6D2F1 male to generate control zygotes. The piezo-ICSI procedure was similar as aforementioned. Injected oocytes were checked for survival 15 minutes after piezo-ICSI, transferred into an EmbryoSlide, and cultured in KSOM up to 96 h to obtain blastocyst while monitoring embryo morphokinesis.

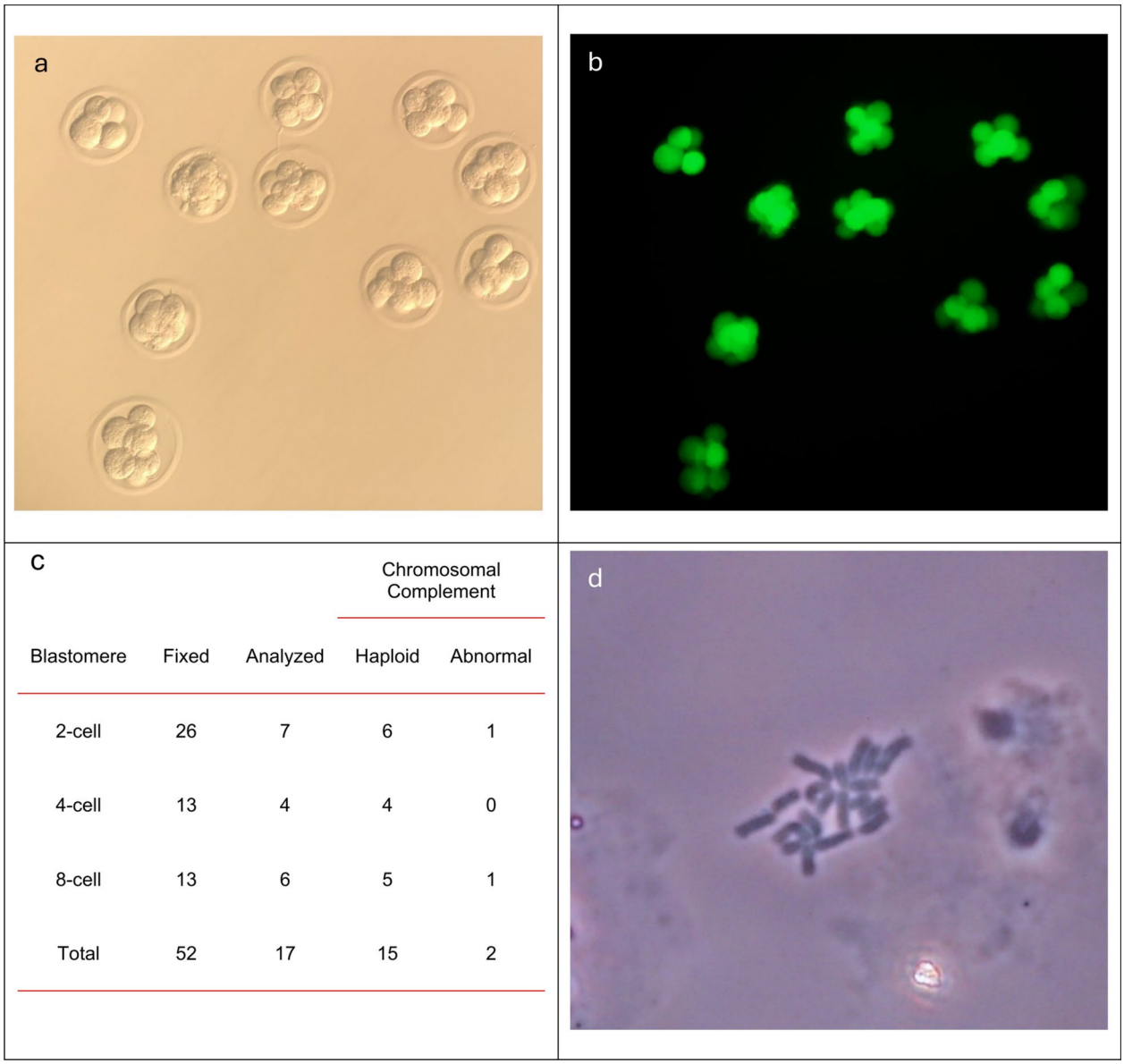
Recipient oocyte preparation and generation of biparental zygotes

To prepare recipient oocytes for the replicated male gamete, mature oocytes from B6D2F1 mice were chemically activated using a 2.5-h incubation in calcium-free CZB medium supplemented with 10 mM $SrCl_2$ (Sigma-Aldrich, St. Louis, MO, USA). These oocytes were cultured in KSOM for an additional 2–3 h until a female pronucleus was present and a second polar body was extruded.[30]

In our early experiments using B6D2F1 androgenotes, a single pseudo-blastomere was isolated from 2-cell, 4-cell, or 8-cell haploid androgenetic embryos with each pseudo-blastomere (15 µm in diameter) and transferred into the perivitelline space of an activated parthenote. Each grafted oocyte was aligned between two microelectrodes (ECF-100; BTX, Cambridge, UK) connected to an electrocell manipulator (BTX 200; BTX, Cambridge, UK). The grafted construct was positioned such that the axes of the oocyte and pseudo-blastomere were aligned parallel to the electrodes. Electrofusion was conducted using a single or double 1.0 kV/cm DC pulse with a 50–99 µs duration within electrolytic M2 medium (Sigma-Aldrich, St. Louis, MO, USA). After washing and culturing for 3 min, reconstructed zygotes were monitored for fusion and survival.

In the modified experimental protocol using B6-EGFP androgenotes, the resulting 8-cell haploid embryos were examined for the presence of a GFP signal (Fig. 2a,b). The B6-EGFP strain was chosen due to its ubiquitous expression of green fluorescent protein, giving a visual cue to allow androgenotes selection based on phenotype. GFP-positive androgenotes were selected and isolated by micromanipulation, similarly as above described. Instead of electrofusion, a modified virus-mediated method was used. Each haploid androgenetic pseudo-blastomere was first coated with inactivated Sendai virus (HVJ-E; Cosmo Bio, Japan) and subsequently transferred into the perivitelline space of an activated parthenote[31,32] (Supplementary video 2). The reconstructed zygotes were monitored for fusion 15 min after micromanipulation.

All reconstructed zygotes were cultured in G-TL medium in EmbryoSlide culture dishes. Embryonic morphokinesis was recorded and evaluated (Supplementary video 3). Embryos with extensive fragmentation



Blastomere	Fixed	Analyzed	Chromosomal Complement	
			Haploid	Abnormal
2-cell	26	7	6	1
4-cell	13	4	4	0
8-cell	13	6	5	1
Total	52	17	15	2

Fig. 2. Haploid androgenetic embryos and karyotyping. (**a**) Eight-cell Haploid androgenetic embryos generated from B6-GFP sperm. (**b**) The same embryos under fluorescence microscope. (**c**) Karyotyping demonstrated a consistent haploidy rate at 88.2% throughout early androgenesis from 2-cell to 8-cell stage. (**d**) Chromosome spread of haploid androgenetic blastomere.

(> 35%), direct uneven cleavage, or delayed morphokinetics were excluded. Blastocysts were collected and transferred into the uterine cavity of 2.5-day-post-coitus CD1 mice previously mated with male vasectomized CD-1 mice.

Karyotyping and genetic analysis

In the early set of experiments, karyotyping with Giemsa staining was used to assess ploidy.[33] Haploid pseudo-blastomeres or blastomeres from reconstructed embryos were mechanically isolated using micromanipulation and transferred into media containing 0.06 µL/mL Colcemid (Sigma-Aldrich, St. Louis, MO, USA) to pause mitosis. After overnight culture, the blastomeres were transferred to a hypotonic solution containing 0.1% sodium citrate and 0.6% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) for 15 min on a glass slide, subsequently fixed in methanol:acetic acid (3:1) and stained with a 2% Giemsa solution (KaryoMAX™, Gibco, Grand Island, NY, USA).[34] The chromosomal spread was observed under a microscope at 100× magnification.

To confirm the inheritance of the paternal genome, WES was employed to first identify genetic discrepancies between sibling spermatozoa from a wildtype control mouse to validate the sensitivity of the test. We then use the same genotyping method to identify genetic similarities between sibling haploid blastomeres from the same haploid androgenetic embryo. DNA was extracted and amplified using a commercial kit (Repli-G Single Cell; Qiagen, Hilden, Germany). Purified DNA was normalized to 20 ng/µL and sent to an external laboratory

(Genewiz; Azenta Life Sciences, South Plainfield, NJ, USA) where 150-bp paired-end exome sequencing was performed on an Illumina (San Diego, CA, USA) HiSeq 2500 platform, as previously described.[35] Quality control was performed for each sample using quantitative polymerase chain reaction, and poor-quality nucleotides were removed (based on an error rate < 0.01). Variant detection was performed using the CLC Genomics Server 9.0, and the detected variants were annotated to identify unique gene mutations. All genomic coordinates were based on the reference mouse genome assembly, GRCm38 (mm10).

Statistical analysis and bioinformatics

The chi-square test was used to compare fertilization, embryo development, and pregnancy outcomes. Statistical analysis was performed using IBM SPSS statistical software (IBM, Armonk, NY, USA). A *P*-value < 0.05 was considered significant. Copy number variant determination and the annotation of gene mutations were performed using CLC Genomics Server 9.0 modules with next-generation sequencing core tools. Integrative Genomics Viewer (IGV, version 2.17.0) was used to visualize copy number variants with comparison to the GRCm38 (mm10) reference genome.[36]

Results

Generation of haploid androgenetic embryos to replicate the sperm genome

In a series of 17 experiments involving 85 mice, a total of 535 oocytes were enucleated, with a survival rate of 97.0% (519/535). Sperm microinjections were performed on all enucleated oocytes, resulting in 471 mononucleated constructs (90.8%, Table 1). In the female counterparts (parthenogenetic embryos, after mature oocyte artificial activation), 895 intact metaphase II oocytes were activated, and 96.4% (863/895) developed a single pronucleus and extruded a second polar body. Control oocytes were fertilized at a rate of 97.8%. A decrease in development from the 4-cell stage onward was apparent in both haploid embryo types and this became pronounced in the blastocyst stage, particularly in androgenotes. The same experiment was repeated in the heterozygous transgenic strain (B6-EGFP) and the results followed the same trend as those observed for B6D2F1 androgenotes (Table 1). The resulting haploid androgenetic embryos expressed GFP (Fig. 2a,b). To verify the haploidy status of the embryos generated using a single male genome, karyotyping was performed on blastomeres from 2-cell, 4-cell, and 8-cell embryos, which demonstrated a haploidy rate of 88.2% (Fig. 2c,d).

Haploid androgenetic blastomeres as gametes

Subsequently 2-cell, 4-cell, and 8-cell stage androgenetic pseudo-blastomeres were used as male gametes to generate biparental constructs, and additional sets were generated using 8-cell blastomeres from B6-GFP mice (Table 2). Interestingly, the reconstruction (survived grafting) and fusion (presence of 2PN in the biparental constructs) rates were extremely high in all categories of constructs, reaching more than 95%, similar to the respective ICSI survival and fertilization rates of the control zygotes. Biparental zygotes generated from all types of blastomeres were capable of supporting full pre-implantation development. While, overall, experimental constructs presented embryo development rates similar to those of control zygotes, in a sub-analysis comparing each type of conceptus, lower rates of compaction ($P < 0.001$) and blastulation ($P < 0.0001$) rates were observed in constructs created from 8-cell blastomeres from B6-EGFP mice. In experiments using B6-EGFP gametes, the resulting biparental embryos expressed GFP (Fig. 3). To confirm the ploidy status of these biparental embryos, chromosome spreads were prepared from the biparental constructs and results revealed a diploidy rate of 83.3%.

Haploid androgenetic blastomeres as gametes can support livebirth and maintaining the paternal haplotype

To demonstrate post-implantation developmental ability, overall, 240 blastocysts generated at different stages of the haploid blastomeres were transferred to 25 surrogates, resulting in 17 (17/25, 68.0%) pregnancies. A total of

	Control ICSI (2PN/2 PB)	B6D2F1 Androgenetic (1PN/1 PB)	*P*-Value	B6-EGFP Androgenetic (1PN/1 PB)	*P*-Value	B6D2F1 Parthenogenetic (1PN/2PN)	*P*-Value
Oocyte enucleated	–	535		421		–	
Enucleation survival (%)	–	519/535 (97.0)		415/421 (98.6)		–	
Oocytes activated	–	–		–		895	
Fertilization/1PN development (%)	437/447 (97.8)	471/519 (90.8)	< 0.0001	351/415 (84.6)	< 0.0001	863/895 (96.4)	NS
2-cell development (%)	426/437 (97.5)	445/471 (94.4)	NS	322/351 (91.7)	NS	116/119¶ (97.5)	NS
4-cell development (%)	410/437 (93.8)	129/158 (81.6)*	< 0.0001	281/351 (80.1)	< 0.0001	102/119 (85.7)	< 0.01
8-cell development (%)	405/437 (92.7)	53/72 (73.6)†	< 0.0001	208/351 (59.3)	< 0.0001	91/119(76.4)	< 0.0001
Morula development (%)	390/437 (89.2)	29/43 (67.4)ƒ	< 0.0001	183/330 (55.5)‡	< 0.0001	88/119 (73.9)	< 0.0001
Blastocyst development (%)	353/437 (80.8)	4/43 (9.3)	< 0.0001	37/330 (11.2)	< 0.0001	33/119 (27.7)	< 0.0001

Table 1. Embryo development of haploid androgenetic and haploid parthenogenetic embryos versus control embryos. *287 2-Cell male haploid embryos allocated for zygote reconstruction. †57 4-Cell male haploid embryos allocated for zygote reconstruction. ƒ10 8-cell male haploid embryos allocated for zygote reconstruction. ‡21 8-cell male haploid embryos allocated for zygote reconstruction. ¶744 1PN parthenotes allocated for zygote reconstruction.

Conceptuses	Control	B6D2F1 (2-cell)	*P*-Value	B6D2F1 (4-cell)	*P*-Value	B6D2F1 (8-cell)	*P*-Value	B6-EGFP (8-cell)	*P*-Value
Oocyte activation (%)	—	330/346 (95.3)		222/223 (99.6)		30/30 (100)		162/172 (94.2)	NS
ICSI/**Reconstruction** (%)	*447/481 (92.9)*	**254/261 (97.3)**	NS	**115/120 (95.8)**	NS	**24/24 (100)**	NS	**145/148 (97.9)**	NS
2PN/**Fusion** (%)	*437/447 (97.8)*	**248/254 (97.6)**	NS	**111/115 (96.5)**	NS	**23/24 (95.8)**	NS	**140/145 (96.6)**	NS
2-Cell Development (%)	426/437 (97.5)	248/248 (100)	NS	111/111 (100)	NS	23/23 (100)	NS	135/140 (96.4)	NS
4-Cell Development (%)	410/437 (93.8)	248/248 (100)	NS	103/111 (92.8)	NS	23/23 (100)	NS	132/140 (94.3)	NS
8-Cell Development (%)	405/437 (92.7)	246/248 (99.2)	NS	101/111 (91.0)	NS	23/23 (100)	NS	128/140 (91.4)	NS
Morula Compaction (%)	390/437 (89.2)	238/248 (96.0)	NS	96/111 (86.5)	NS	23/23 (100)	NS	112/140 (80.0)	< 0.001
Blastocyst Development (%)	353/437 (80.8)	199/248 (80.2)	NS	90/111 (81.1)	NS	18/23 (78.3)	NS	85/140 (60.7)	< 0.0001

Table 2. Preimplantation development of reconstructed zygotes using blastomeres isolated from different stages of haploid androgenetic embryos and comparison to that of control.

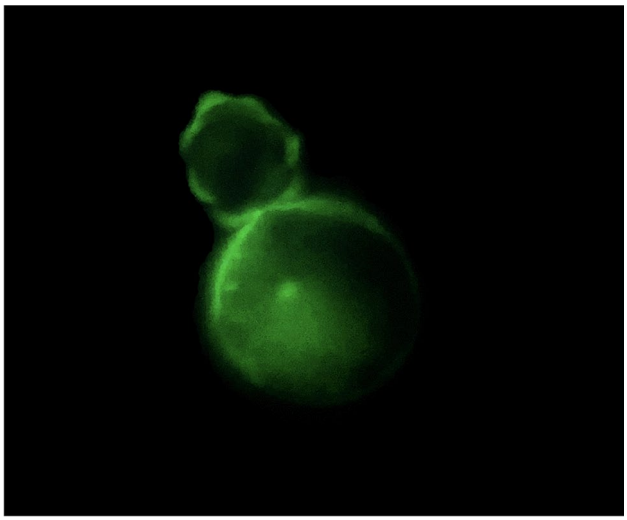


Fig. 3. Hatching blastocyst generated from embryo reconstruction using haploid pseudo-blastomere generated using B6-GFP sperm.

Conceptuses	Control	B6D2F1 (2-cell)	B6D2F1 (4-cell)	B6D2F1 (8-cell)	B6-GFP (8-cell)
Blastocysts transferred	94	81	58	16	85
Total number of recipient	8	7	5	1	12
Pregnant recipient	8	6	5	1	5
Implantation (%)	53/94 (56.4)	17/81 (20.1)	16/58 (27.6)	6/16 (37.5)	15/85 (17.6)
Full-term developed (%)	48/94 (51.1)	13/81 (16.0)	12/58 (27.6)	0/16 (0)	12/85 (14.1)
Live offspring (%)	48/94 (51.1)	11/81 (13.6)	12/58 (27.6)	0/16 (0)	10/85 (11.1)
Male	25 (52.1)	4 (36.4)	6 (50.0)	0	3 (30.0)
Female	23 (47.9)	7 (63.6)	6 (50.0)	0	7 (70.0)
Mean weight (g)	1.76 ± 0.17	1.69 ± 0.31	1.66 ± 0.12	N/A	1.51 ± 0.22
Weaned	42/48 (93.8)	6/11 (54.5)	10/12 (83.3)	N/A	9/10 (90.0)
Abnormal/**Cannibalized**	*0*/**6**	*3*/**2**	*2*/**0**	N/A	*1*/**0**

Table 3. Pregnancy outcomes after embryo transfer into 2.5-dpc CD-1 surrogate.

54 experimental embryos were implanted (ET/sacs, 22.5%), 37 of which were full-term embryos (37/240, 15.4%) and 33 were live births (33/239, 13.8%). Of note, the offspring tended to be female, as only 13 (13/33, 39.4%) were male and 20 (2/33, 60.1%) were female. When comparing pregnancy outcomes between experiments using different types of haploid blastomeres, we observed that the live birth rate decreased as the haploid blastomere stage advanced (Table 3). In the experiment with GFP mice, all pups (10/10) expressed green fluorescence (Fig. 4).

To confirm the inheritance and conservation of the original paternal genome, the exome regions of replicated sperm from male haploid blastomeres, trophectoderm cells from replicated sperm blastocysts, and skin from

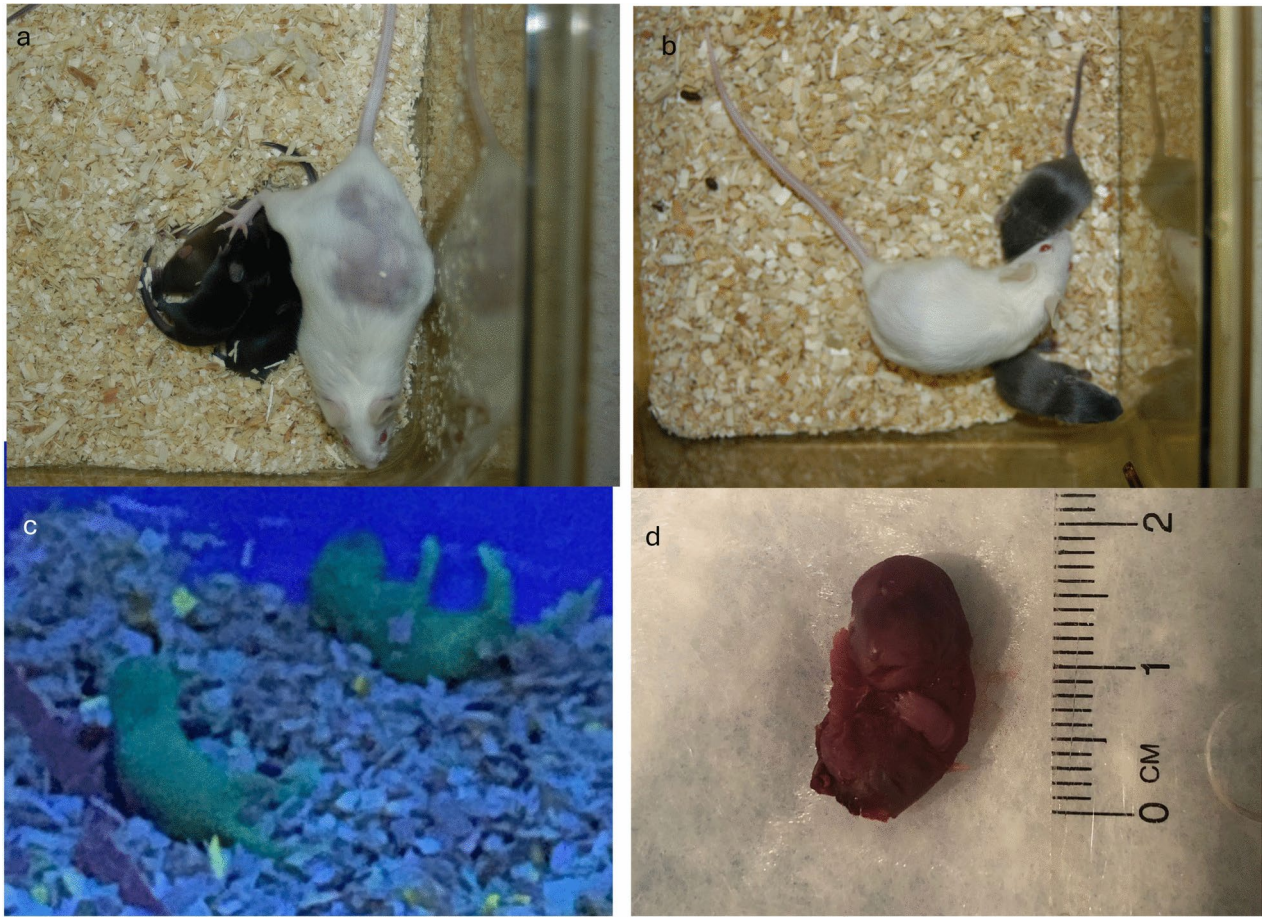


Fig. 4. Pups generated from reconstructed embryos using (**a**) B6D2F1 2-cell androgenotes, (**b**). B6D2F1 4-cell androgenotes, (**c**). B6-GFP 8-cell androgenotes and (**d**) dead pup with abnormality.

replicated sperm pups, were fully analyzed. Whole-exome sequencing (WES) revealed an identical nucleotide sequence in genes *Ahr, Gas1, Nlrp2*, and *Tg*, all of which demonstrated unique base pairs compared to the GRCm38 mm10 reference genome, while sibling spermatozoa from a hybrid control demonstrated different nucleotide sequences in those genes (Fig. 5).

Discussion

Our study demonstrated the feasibility of replicating the sperm genome via androgenesis and this would theoretically allow selecting the desired gamete before fertilization to preserve a specific paternal genotype. This was confirmed by phenotypic observations and corroborated by genetic testing. We achieved a satisfactory pre-implantation embryo developmental rate of conceptuses generated using replicated male gametes and were able to generate healthy offspring. Specifically, in our experiment using 8-cell stage androgenetic embryos, a single spermatozoon had yielded up to three conceptuses that carried an identical paternal haplotype.

Of note, the development of haploid pseudo-embryos reported in the first part of the study, whether androgenetic or parthenogenetic (Table 1), demonstrated a trend similar to that observed in previous studies, particularly those focused on digyneic and dispermic embryos, which underscores the importance of the interdependence and complementation of both paternal and maternal genomes in mammalian embryogenesis.[37] Once the haploid androgenetic pseudo-blastomeres were used as gametes, the embryos generated demonstrated embryo development comparable to that of the controls (Table 2) when 2- or 4-cell stage blastomeres were used. The utilization of 8-cell stage blastomeres was less successful, possibly due to intrinsic imprinting or epigenomic heterogeneity between sibling blastomeres, as mouse embryo polarization occurs at the 8-cell stage.[38–41] However, more recent studies have suggested that the loss of totipotency in mouse can occur even in the earlier stages, at the 2-cell stage, in ~ 30% of blastomeres,[42] being further amplified in 4-cell-stage embryos.[43] Therefore, when an differentiated 8-cell-stage pseudo-blastomere was used as a male gamete, embryos generated by such cells may experience a slightly higher incidence of embryo development arrest, implantation failure, and post-implantation development arrest.

Another explanation for why 8-cell androgenetic pseudo-blastomeres yielded fewer blastocysts per embryo reconstruction may be the higher level of epigenomic asynchrony between the native maternal genome and the replicated paternal genome. During murine preimplantation development, zygotic genome activation occurs at

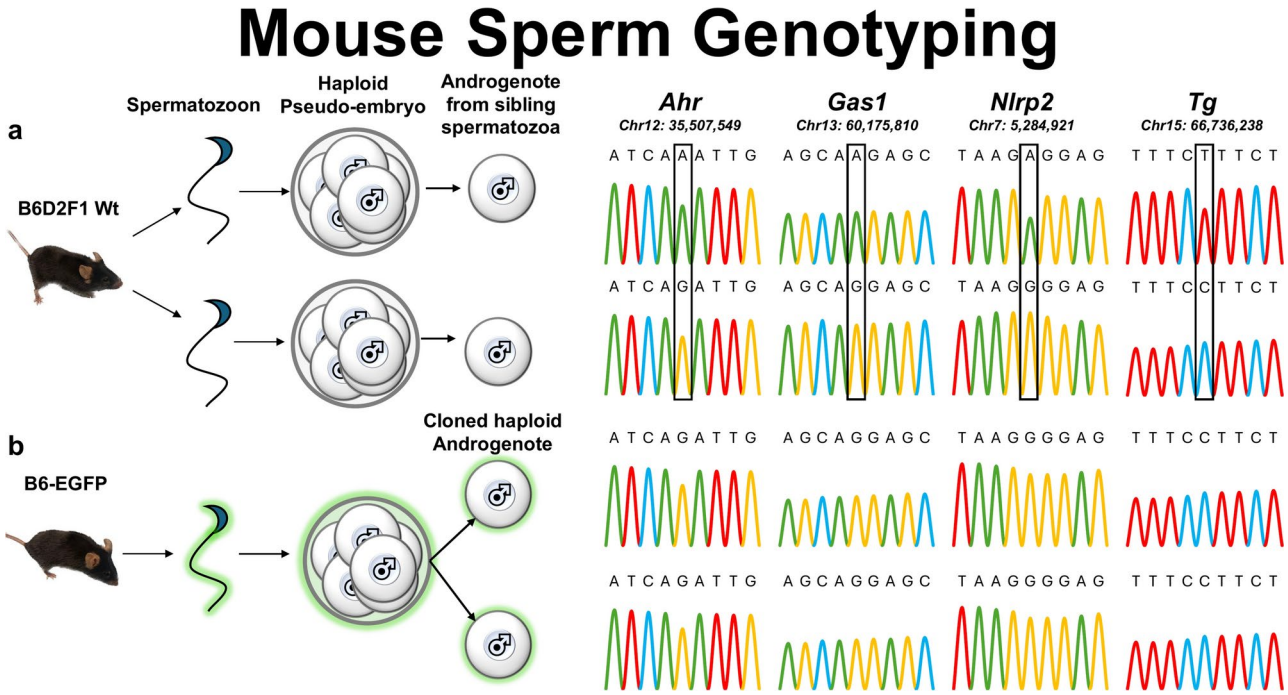


Fig. 5. To confirm the inheritance of the paternal genome, WES was employed to first identify genetic discrepancies between sibling spermatozoa from a wildtype control mouse to validate the sensitivity of the test. We then use the same genotyping method to identify genetic similarities between sibling haploid blastomeres from the same haploid androgenetic embryo. Chromatogram demonstrated: (**a**) different nucleotide sequence between sibling spermatozoa in a hybrid wildtype mouse, while (**b**) cloned spermatozoa displayed identical nucleotide sequence.

the 2-cell stage;[44] is heavily regulated by paternal chromatin.[45] This observation may be supported by the fact that the haploid genome of the earlier-stage pseudo-blastomere did not undergo extensive demethylation compared to the later stage embryos;[46] therefore, the 2-cell and 4-cell stage pseudo-blastomeres retained a more gamete-like epigenome and, when used as a gamete, yielded higher rate of pre-implantation development and embryo implantation comparing to the 8-cell counterpart. Nonetheless, the use of 8-cell-stage pseudo-blastomeres is still preferred because more male gamete copies can be used to fertilize a larger cohort of oocytes.

When considering the use of this approach in higher mammals, and even in human reproduction, this method of gamete replication is preferred over the utilization of haploid embryonic stem cells (haESCs). Conventionally, haESCs are generated from haploid androgenetic embryos, which have limitations, including a low blastulation rate of 10–20%, reported in our study at 11.2% (Table 1) comparable to previous studies;[24,47] a low cell line derivation rate of approximately 20%;[24] and susceptibility to a rapid loss of haploidy that cannot be blocked by fluorescence-activated cell sorting purification.[48,49] Comparing to haESCs which displayed a low haploidy rate of 2–13%,[18] androgenetic blastomeres retained their haploidy at a higher rate (88.2%; Fig. 2).

Additionally, Y-chromosome-bearing haploid androgenetic blastocysts cannot yield haESC lines, further inhibiting the application of haESCs in reproductive semi-cloning[50,51]. In contrast, our technique using haploid androgenetic blastomeres as gametes generated male offspring. However, we did observe a trend towards more viable female offspring (60.6%) comparing to males (39.4%), which may be explained by the fact that an X-chromosome-bearing haploid blastomere had higher embryo competence than its Y-chromosome-bearing counterpart possibility due to the requirement of Xist RNA product that is only produced by the X-chromosome.[51] This trend was indeed observed in earlier studies of androgenetic and parthenogenetic embryos, where androgenones (two male pronuclei, generated by injection of enucleated oocytes with 2 spermatozoa) were arrested earlier, whereas parthenogenetic (1PN/2 PB construct) and gynogenetic (2 maternal PN without the extrusion of second PB) embryos were arrested post-implantation, implicating the important role of the X chromosome in these peculiar embryonic constructs.[52]

During the generation of haploid androgenetic embryos, sperm DNA is decondensed in the ooplasm at the pronuclear stage. The availability of an unraveled sperm genome creates a conducive environment for heritable genome editing experiments. This strategy can ensure the uniformity of the eventual gamete genotype, and the subsequent use of edited gametes can, therefore, prevent the genetic mosaicism sometimes observed following CRISPR-Cas9-mediated genome editing in diploid embryos.[53,54]

One major concern with this technique is species specificity. This tailored technique is currently optimized for murine gametes, with limited generalizability to human gametes because of the differences in physiology between rodent and primate reproduction. One major uncertainty is the role of the sperm centrosome. In murine reproduction, the zygotic spindle is formed from the oocyte's own microtubule organization center

(MTOC), while the mitotic spindle of human zygotes is contributed by the sperm proximal centriole.[55,56] Therefore, whether the human haploid blastomere will carry a functional MTOC remains to be investigated. In terms of epigenetic modifications, we hypothesized that our technique would perform better on human gametes. Mouse zygotic genome activation occurs as early as the 2-cell stage, which explains why embryos generated from 8-cell-stage blastomeres developed at a lower rate. In contrast, higher mammals, including primates, have a later zygotic genome activation, occurring at the 8-cell embryo stage.[57] Therefore, the maintenance of a gamete-like epigenome may compensate for the development of conceptuses generated using our technique.

An additional technical concern that needs to be addressed, while translating from lower mammals to humans, is represented by the utilization of the Sendai virus. Sendai virus is not known to cause disease in humans and is considered safe for research purposes; nonetheless, preliminary experiment in humans has successfully yielded euploid embryos[58] and in a clinical trial on spindle transfer has generated livebirths.[59] Indeed, we agree that long-term follow-up studies are still required. For these reasons, we are exploring alternatives to void the use of viral particles, such as fusogenic cationic phospholipid.[60]

Considering the clinical applications of male gamete replication, our technique represents an alternative to the traditional PGT of embryos. Screening gametes before fertilization may be more ethically palatable than embryo selection using PGD. Our proposed method has been applied on a murine model of Marfan syndrome and has achieved a preliminary success in identifying healthy gametes to generate exclusively healthy embryos using replicated unaffected gametes.[61] The ability to identify healthy gametes before insemination can prevent the generation of excess embryos under pathological genetic conditions, thereby reducing embryo wastage. Moreover, this technique may benefit men with severe oligozoospermia and cryptozoospermia. By replicating scarce spermatozoa, the abundance of sperm copies permits insemination of an entire oocyte cohort using only a few available male gametes.

In conclusion, haploid androgenetic blastomeres functioned as replicated spermatozoa, demonstrated satisfactory blastocyst development, and yielded healthy offspring. This replication of the sperm genome allowed us to select gametes of the desired genotype prior to embryo generation.

Data availability

The datasets generated and/or analysed during the current study are available in the NCBI repository, SAMN44827087, SAMN44827088, SAMN44827089, SAMN44827090, SAMN44827091, SAMN44827092, SAMN44827093, SAMN44827094.

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Author contributions

P.X. and T.T. performed the experiments, derived the models and analyzed the data. S.C. performed DNA extraction and sequencing. P.X. and G.P. wrote the manuscript. G.P. and Z.R. supervised the project and were in charge of overall direction and planning.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to G.D.P.

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