The milestone of genetic screening: Mammalian haploid cells

Shengyi Sun\textsuperscript{a,1}, Yiding Zhao\textsuperscript{a,1}, Ling Shuai\textsuperscript{a,b,c,\textasteriskcentered}

\textsuperscript{a}State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin 300350, China
\textsuperscript{b}Tate Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
\textsuperscript{c}Tianjin Central Hospital of Gynecology Obstetrics / Tianjin Key Laboratory of Human Development and Reproductive Regulation, Tianjin 300052, China

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Abstract
Mammalian haploid cells provide insights into multiple genetics approaches as have been proved by advances in homozygous phenotypes and function as gametes. Recent achievements make ploidy of mammalian haploid cells stable and improve the developmental efficiency of embryos derived from mammalian haploid cells intracytoplasmic microinjection, which promise great potentials for using mammalian haploid cells in forward and reverse genetic screening. In this review, we introduce breakthroughs of mammalian haploid cells involving in mechanisms of self-diploidization, forward genetics for various targeting genes and imprinted genes related development.

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1. Introduction

Not like some lower organisms such as yeast and drone have haploid individuals, most animals are diploid organisms. Diploid genomes have been playing important roles on evolution and species reproduction, mainly due to that they can enrich gene diversity and mask the deleterious recessive mutations [1,2]. However, it is inconvenient for the exploration of recessive gene function for existence of alleles in diploid genomes. Haploid yeasts have only one set of chromosomes, thus are convenient for gene editing and widely used in genetic screening and epigenetic modification [3,4]. Therefore, it is necessary to develop haploid systems in mammals like yeast. With the improvement of culture methods and the application of flow cytometry, haploid embryonic stem cell (haESC) lines in rodents and primates have been established. They show similar pluripotency to diploid ESCs and can also contribute to chimeras including germline, although they have only single
genome [5,6]. However, previous studies claimed that haESCs underwent serious self-diploidization in both daily culture and differentiation, which hindered the application of haESCs in genetic screening for loss of single genome feature [7]. To open massive values of haESCs into uncover recessive gene functions, it is vital to figure out mechanisms underlying self-diploidization.

Besides haploidy and pluripotency, haESCs have a unique function that their nucleus can substitute that of sperms or oocytes in reproduction via intracytoplasmic microinjection, as a matter of retaining their original imprinting [8–10]. With advantages of pluripotency for haESCs, it is convenient to obtain abundant mutant individuals, which upgrades genetic screening from cellular level to organism level. However, obvious loss of imprinting occurred in haESCs during long-term culture, which significantly decrease the developmental efficiency of intracytoplasmic microinjection. Similar loss of imprinting in wild-type (WT) diploid ESCs resulting in low efficiency of tetraploid complementation were reported, either [11,12]. All the evidences proved that suitable expression of imprinted genes was essential for development. Therefore, scientists mainly focused on manifesting mechanisms of self-diploidization and roles of imprinted genes on intracytoplasmic microinjection in last decade. Here, we review the derivations of various haploid cell types in mammals assisted with strategies preventing self-diploidization, achievements of genetic screening with haploid cells and discuss recent findings that modifications of imprinted genes benefit for increased efficiency in intracytoplasmic microinjection.

2. The establishment of mammalian haploid cells

In 1970s, scientists successfully obtained mouse haploid embryos by chemical activation of oocytes or from bisected zygotes [13,14]. The establishment of mouse ESCs from blastocysts in 1981 [15] made it possible to derive haESCs from haploid embryos. Although Kaufman and his colleagues succeeded in derivation ESCs from mouse haploid embryos, they failed to obtain haESCs due to lacking of enrichment ways for haploid cells [16]. In 1980s, near-haploid cell lines were established from leukemia cancer cells, which raised extensive concerns for their single genome feature [17,18]. These unique cell lines (most famous: KBM7 and HAP1) provided good platforms for the screening of anticancer drugs and the studies of unknown gene functions [19–21]. However, near-haploid cells carry massive copy number variations and show genome instability, which limited their applications in many more other areas including development. In 2009, Medaka fish haESCs was the first reported vertebrate haploid pluripotent stem cells, and thus initiated the discovery of haploid cell lines with intact genome in higher species [22]. Two years later, two independent groups achieved in derivation of mouse haESCs from parthenogenetic haploid embryos assisted with fluorescence-activated cell sorting (FACS) [5,6]. These cells have similar transcription characteristics with diploid ESCs, express classical pluripotent markers and possess potentials to differentiate into three germ layers in vitro and in vivo. In 2012, two groups independently proved that mouse androgenetic haESCs could produce live offspring via intracytoplasmic microinjection, which meant that androgenetic haESCs were able to function as sperms in reproduction [8,9]. Besides, Wan et al. validated that nucleus of parthenogenetic haESCs could also replace that of oocyte during fertilization [10]. Subsequently, scientists successfully established haESCs in other species including human, broadening application of mammalian haploid cells in various genetic screening [23–27].

In order to apply powerful haploid system in lineage specific genetic screening, scientists attempted to obtain more haploid cell lines in various cell types. However, these attempts seemed very difficult for overall self-diploidization existing during the differentiation processes. Nevertheless, haploid epiblast stem cells-like cells (haEpLCs) were generated by differentiation of haESCs in vitro, assisting with optimized culture medium and FACS [9,28]. Mouse haploid neural stem cells [29] and monkey haploid neural progenitor cells [30] were also derived via modified differentiation protocols. Besides, haploid neurons were generated by differentiation of haESCs in mouse [31] and human [25]. However, it is difficult to maintain haploid state during differentiation, especially in terminal differentiated cell types [31]. Subsequently, He et al. induced mouse haESCs to differentiate into haploid neurons, astrocytes, cardiomyocytes and pancreatic progenitors by using chemical inhibitors (details see the next section) to maintain haploidy [32]. Except for achievements of haploid cell types in embryonic lineages, mouse haploid trophoblast stem cells (haTSCs) were generated from haploid blastocysts, which could maintain haploidy and differentiation potentials relying on ROCK inhibitor (Y27632) and F4H (FGF4 and heparin) [33]. Meanwhile, mouse haESCs could also be converted to haploid induced trophoblast stem cells (haITSCs) by inducible overexpression of Cdx2 and knockout of p53 [34].

3. Characteristics and application of haploid cells

3.1. Self-diploidization: the obstacle for culturing the haploid cells

Homzygous genotype with one set of chromosomes is the biggest advantage of haploid cells. However, most haploid cells trend to double back to diploid genomes not only in daily culture, but also in the differentiation, which is of course a major obstacle hampering application of their advantages. By labeling haESCs with different marker genes, scientists figured out that diploidization was caused by the failure of cell division instead of cell fusion [35], suggesting that self-diploidization may be caused by mistaken cell cycle. The evidence showed that the main reason for the self-diploidization was that the haploid cells went through a prolonged M phase (Fig. 1A). Part of haESCs failed to divide into daughter cells during mitosis caused the self-diploidization [36]. The addition of chemical cocktail RDF (R, Repsox, an inhibitor of the TGF-β pathway; D, DMH1, an inhibitor of the BMP4 pathway; F, Forskolin, an adenylate cyclase activator) can effectively inhibit the diploidization by shortening the time of M phase (Fig. 2A) [36]. Another study found that these cells could not smoothly alternate from G2 phase to M phase, instead, they undergo G2 arrest or directly entered an extra G1/S phase (Fig. 1A). It made haESCs stable for more than four weeks without FACS [37]. Whereas, another hypothesis demonstrated the self-diploidization was due to the occurrence of mitotic slippage. Briefly, haESCs re-entered the G1 phase of the next cell cycle, without segregating chromosomes and cytokinesis (Fig. 1A) [32]. Therefore, haploidy of haESCs and haTSCs were stabilized when their culture mediums were supplemented with CDK1 inhibitor (RO-3306) and ROCK inhibitor (Y-27632) (Fig. 2A), which could prevent mitotic slippage effectively and delay the self-diploidization process to some extent [32,33]. Similarly, the combination of 2i inhibitors (PD0325901 and CHIR99021), PD166285 and RDF could also inhibit the self-diploidization by shortening the S-G2/M phase (Fig. 2A), and simultaneously guarantee the pluripotency of haESCs [38]. Besides, 10-Deacetyl-baccatin-III (DAB) was selected out to enrich the haploid cells in HAP1 or mouse haESCs cell cultures, by promoting mitotic arrest in a ploidy-dependent manner [39]. Yaguchi et al. compared human near-haploid cells (HAP1) in
different ploidy, and found that the rate of centrosomes replication in haploid cells was often lower than that in diploid cells, which led to cell death, mitotic slippage and cytokinesis failure (Fig. 1B). They questioned that the incompatibility between centrosome and DNA replication might be an important reason for haploid instability [40].

In addition, gene editing is an efficient strategy to stabilize haploidy either. An approach proved that knock-out of \( \text{p53} \) (Fig. 2B) in HAP1 cell lines and mouse haESCs could stabilize haploidy genomes significantly [41]. Similar evidence proved that deletion of \( \text{p53} \) also facilitated derivation of mouse haTSCs by stabilizing haploidy during conversion [32]. Prolonged M phase further cause two types of division failures: chromosome division failure and failure of cytokinesis (bottom) [36]. B. The genomic instability of human near-haploid cells is due to incompatibility of centrosome and DNA replication. Therefore, only a few haploid cells can fulfill mitotic, whereas most of them experience mitotic death, mitotic delay and mitotic slippage [40].

The viability of diploid cells is better than that of haploid cells [41], so it is necessary to purify haploid cell cultures periodically. Up to date, the most widely used method for haploid enrichment is Hoechst33342 staining-based FACS technology (Fig. 2C), which is very accuracy to enrich haploid cells [5,6,44]. However, DNA staining and physically sorting in this technology would bring great harm to haESCs, resulting in low survival efficiency. It was difficult to expand haESCs quickly to a massive cell count suitable for genetic screening by Hoechst33342 staining-based method, therefore, new methods for better viability was quite in need. As

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**Fig. 1.** Mechanisms of self-diploidization A. The main reason of self-diploidization may be caused by a mistaken mitotic cell cycle. The mistaken cells arrest at G2 phase and entry an extra G1/S + G2, resulting in self-diploidization (top) [37]. Some haESCs undergo mitotic slippage, escape from M phase and enter the next G1 phase without chromosome segregation or cytokinesis (middle) [32]. Prolonged M phase further cause two types of division failures: chromosome division failure and failure of cytokinesis (bottom) [36]. B. The genomic instability of human near-haploid cells is due to incompatibility of centrosome and DNA replication. Therefore, only a few haploid cells can fulfill mitotic, whereas most of them experience mitotic death, mitotic delay and mitotic slippage [40].
The cell size of haESCs was smaller than that of diploid ESCs, specific haploid populations with lower FSC and SSC values could be determined to distinguish haploid cells and diploid cells (Fig. 2D). However, this method lacked strict diploid control and the accuracy of gating haploid cell population needed for improvement [29,45]. Above methods needed complicated flow cytometry, which were time-consuming and hard to handle. Two groups independently developed a sorting method just with microporous filtration (Fig. 2E), based on cell size differences [46,47]. This method simplified the sorting process and avoided the cell damage caused by Hoechst33342 staining, but whether it could be widely used still needs further investigation. Nevertheless, the exact mechanism inducing self-diploidization has not been elucidated yet. Only if the scientists addressed the exact mechanism of self-diploidization, could they find a way to avoid self-diploidization completely.

### 3.2. The haploid cells in functional genomics

Forward and reverse genetic screening are two widely used high-throughput strategies to study functional genomics [48,49]. Haploid cells advanced themselves in forward genetic screening because their homozygous genotypes took advantages in gain or loss of function traits. In the past decade, haploid cells were extensively utilized to identify specific biological phenotypes or uncover functions of recessive genes (Table 1).

**Fig. 2.** Different strategies for sustaining and enriching for haploid cells. A. Effective chemical inhibitors to reduce self-diploidization of mouse haESCs [32,37], PD166285 (Wee1 inhibitor) repress diploidization via promoting G2/M transition; RDF (cocktail of Repsox, DMH1 and Forskolin) can shorten mitosis [38], RO-3306 (CDK1 inhibitor) and Y-27632 (ROCK inhibitor) can significantly reduce the mitotic slippage [32]. B. p53 KO can stabilize haploidy of mouse haESCs [41]. Overexpression Aurkb can promote the mitosis progression of haESCs [38], Dnmt3b overexpression in AG-haESCs can effectively improve DNA methylation level, and reduce the high incidence of self-diploidization [43]. C. Common used haploid enrichment method is Hoechst33342 staining-based FACS technology [5,9], which is accurate but harmful to the survival cells. D. A novel cell sorting method for haploid cells is sorting according to cell size [29,45], but this method lacks accurate diploid control. E. Haploid cell cultures separate from diploid cells with a filter, basing on the cell size differences [46,47].
began to put haESCs into genetic screening combined with high-throughput mutation protocols. Cprt7, a potential key targeting gene of ricin toxicity, was figured out by screening with mutant mouse haESCs [6]. The group further addressed the relationship between glycosylation modification and ricin target protein [51]. Targeting genes of other vital biological including pluripotency exiting [52] and X chromosome inactivation regulating [53] were also uncovered by screening with mutant mouse haESCs. However, these approaches used piggyBac (PB) transposon to introduce genome-wide gene trapping. Many more groups preferred to choose PB-based trapping system to bring numerous mutations into mammalian haploid cells for genetic screening [23,24,54]. Recently, Mao et al. developed an inducible self-inactivating PB system, which facilitates rapid construction of a whole-genome mutant haESCs library, with one copy mutation in a single cell [55]. Therefore, retrovirus and PB transposon were two main strategies to introduce gene trapping in mammalian haploid cells. CRISPR-mediated mutation based on the sgRNA library is another convenient method. Timms et al. compared the efficiencies of genome-widely CRISPR/Cas9-mediated forward genetic screens and gene-trap mutagenesis screen in KMB7 cells. They found that the two approaches showed great concordance (>70%) and successfully identified the gene TXNDC11 related to glycoprotein endoplasmic reticulum-associated degradation (ERAD) [56]. Similarly, CRISPR-mediated mutagenesis could also induce high-throughput mutations into haESCs to form a mutation library, which was beneficial for generating different genome-modification semi-cloning (SC) pups or genetic screening [57]. With genetic screening in mutation SC pups, four bone-development-related genes: Zic1, Clec11a, Rhi1 and Inx5 was be screened out [58]. CRISPR-mediated base editing system was also used in identifying critical amino acids for primordial germ cell development in SC pups generated from haESCs [59]. Unlimited proliferation ability and haploid feature of haESCs make themselves powerful tools to generate tremendous homozygous mutation pools. In 2017, Elling et al. established a biobank of mouse haESCs called Haplobank, which contained more than 100,000 individual lines targeting 16,970 genes with genetic barcodes, conditional and reversible mutations. It was very easy to address out candidate genes by screening with such an identified mutant biobank [60]. Remarkably, mutant haploid cells are still homozgyous even if they undergo self-diploidization. Two groups independently proved that arrayed homozygous mutant libraries could be obtained using mutant mouse haESCs [61,62], which provided useful cell resources for future researchers to discover key regulatory genes. Besides biological mutation protocols, chemical mutagens such as ethyl methanesulfonate (EMS) could also introduce massive mutations into haESCs, and the mutant libraries were validated useful in finding the targets of 6-TG toxicant [63]. Recent established haploid cell lines in other cell types also showed great values in lineage specific functional genomics. Resistant gene of neurotoxin Mn2+ (Park2) was figured out with mouse haNSCLCs mutant libraries [32]. Target genes of a tetrodotoxin-like toxicant A803467 (B4GALT6) were uncovered using monkey mutant haploid neural progenitors [30]. In addition, Peng et al. screened out the blocker gene (Htra1) for spongiorrhapholoblast specification with mouse haiTSCs [34]. In conclusion, all mammalian haploid cell lines show great advantages in distinct forward genetic screening, which benefits for human health and disease researches in the future.

### 3.3. Mouse haESCs produce offspring via semi-cloning

Given that mammalian haploid cells are convenient tools for functional genomics, mouse haESCs are advanced in studying phenotypes at animal level for their potentials to produce offspring via intracytoplasmic microinjection (also named semi-cloning). Mouse androgenetic haESCs (aHaESCs) could function as sperm to support full term development by intracytoplasmic aHaESCs injection (ICAI), and thus were called ‘artificial sperm’ [64]. The mice derived through ICAI procedure were called semi-cloned mice (SC mice) [8]. Genomic modification could be transmitted from aHaESCs to individual mice in one step [8,9], which put insights to transgenic animal research by this novel method. However, the overall birth rate of alive transgenic pups was very low (~2%), and it was attributed to the loss of imprinting in aHaESCs, including critical imprinted genes such as H19 [Fig. 3A]. To figure out whether the genome of oocytes could be replaced by partheno-

### Table 1

| Approaches | Screening purpose | Cell type | Mutant method | Target gene | Reference |
|------------|------------------|-----------|---------------|-------------|-----------|
| Genetic screening with near-haploid cells | Host factors essential for infection with influenza | Human KMB7 | retrovirus | SLC35A2, CMAS | Carette et al., 2009 [20] |
| Genetic screening with haESCs using retrovirus | X-chromosome inactivation | Mouse haESCs | retrovirus | SPEN | Monfort et al., 2015 [53] |
| Genetic screening with haESCs using PB | Resistance to 6-TG | Mouse haESCs | PB | Hprt | Leeb and Wutz, 2011 [5] |
| Genetic screening in other haploid cell types | Resistance to 6-TG | Mouse haESCs | EMS | Hprt | Josey et al., 2017 [63] |
| Genetic screening with haESCs in vivo | Related to bone development | Mouse haESCs | CRISPR/Cas9 | Zic1, Clec11a, Rhi1 and Inx5 | Bai et al., 2019 [58] |

The enclosed manuscript entitled “The Milestone of Genetic Screening: Mammalian Haploid Cells” introduces recent breakthroughs of mammalian haploid cells involved in haploidy maintaining mechanisms and improvement of developmental efficiency in intracytoplasmic microinjection. Mammalian haploid cells are extensively concerned, mainly due to their advantages of homozygous phenotypes and functions as gametes in reproduction. This manuscript is a response to the invitation of Dr. Gianni Panagiotou (Editor-in-Chief of CSBJ). We believe this review is of immediate interest to many people related to forward genetic studies and transgenic animals producing, thus would like to submit it for publication consideration by Computational and Structural Biotechnology Journal.
Fig. 3. Summary of intracytoplasmic microinjection of mouse haESCs. A. Generation of viable mice by ICAI procedure with WT-ahaESCs, the full-term birth rate of which is about 2–3% [8,9]. B. Generation of viable mice via co-injecting sperm and WT-phaESCs into enucleated oocytes, the full-term birth rate of which is about 1% [10]. C. Generation of viable mice by ICAI procedure with DKO-ahaESCs, the full-term birth rate of which is about 20%[57]. DKO: double knockout of regions of imprinted genes including H19-DMR and IG-DMR. D. Generation of viable mice by ICPI procedure with DKO-phaESCs, the full-term birth rate of which is about 9%–16%[68,69]. DKO: double knockout of regions of imprinted genes including H19-DMR and IG-DMR. E. Generation of viable bi-paternal mice through tetraploid complementation, the donor cells are androgenetic diploid ESCs derived from bi-paternal embryos. The bi-paternal embryos were constructed via injecting sperm and 7KO-ahaESC into enucleated oocytes, the full-term birth rate of which is about 3%[65]. 7KO: Knock-out of 7 imprinting regions of imprinted genes including Nespas, Grb10, Igf2r, Snrpn, Kcnq1, Peg3 and Gnas.

To rescue function of ahaESCs as sperms in long-term culture, the ahaESCs could yield viable SC offspring at a much higher efficiency (~20%, Fig. 3C) by modification of H19-differentially methylated region (DMR) and IG-DMR [57,66]. The birth efficiency of ICAI with modified ahaESCs was close to that of embryos from intracytoplasmic injection of round spermatids, which tremendously promoted the application of ahaESCs. As bi-deletion of H19-DMR and IG-DMR in reconstructed oocytes could result in higher birth rate of bi-maternal mouse [67], this method was also suitable for production of SC mice with phaESCs. The embryos derived by WT-phaESCs injection into MII oocytes could not develop beyond embryonic day 13.5 (E13.5). However, phaESCs with double knock-out (DKO) of the same two imprinted regions could produce live offspring at efficiency of 9%–16% (Fig. 3D) via ICPI procedure [68,69]. Although many approaches have realized bi-maternal reproduction, whether full-term offspring could be generated from bi-paternal embryos was not addressed [70]. To across the bi-paternal reproduction barriers, Li et al. investigated modification of several imprint regions in ahaESCs on bi-paternal development. Knock-out of 7 imprint regions of imprinted genes including Nespas, Grb10, Igf2r, Snrpn, Kcnq1, Peg3 and Gnas enabled the ahaESCs to replace the maternal genome of a zygote (Fig. 3E). The 7KO-ahaESC was co-injected with a sperm into a denucleated oocyte to reconstruct an embryo, which was further utilized to derive ESCs. Two full-term bi-paternal mice were generated via tetraploid complementation from these ESCs [65]. The two pups showed no obvious defects but dead in 48 h, indicating that some other unknown imprint genes also played critical roles on development of bi-paternal embryos. These reports showed that uniparental reproduction was reasonable through property gene modification on imprint regions. However, how the global epigenonomics modulates embryonic development needs more investigations.

3.4. Semi-cloned mice promises transgenic animal researches

As the birth rate of SC mouse was improved significantly, ahaESCs were widely applied to generate mutant mice combined with advanced gene editing technology. Gene targeting mice of p53 KO and Tet family KO were obtained separately via ICAI from DKO-ahaESCs [57], which were useful gene KO mice of interest in many fields. In their approach, a DKO-ahaESC line expressing Cas9 and sgRNA was established using lenti-viruses, and applied in ICAI procedure to produce mutant mice. Numerous homozygous
mutant mice were obtained by this strategy. Given that the homozygous mutant mice could be attained, genetic screens based on mutant mouse library were upgraded robustly. With this system, 72 candidate genes related to bone development were addressed out, 4 key genes of which were validated essential in the regulation of bone development during embryogenesis [58]. Furthermore, ahaESCs could produce heterozygous mutant mice without long-term mating [57]. Four single allele deletion (Sfnb2, Jade1, Gab1 and Smoc1) mice were successfully constructed and applied to study the function of imprinted genes [71]. Single deletion of these genes can effectively improve the pup rates of SCNT [71].

Recently, the discovery of the CRISPR mediated base editor (BE) allowed single-base editing in the genome without double-strand breaks, providing precise base editing systems to introduce point mutations [72–76]. Combined with SC technology, it is possible to screen key amino acids of specific proteins in animal level. Li et al. inserted a modified third-generation BE system into DKO-ahaESCs, using 77 sgRNAs targeting Dmd1 to construct a Dmd1 point mutation library. They generated a mouse mutation library with homozygous point mutations efficiently through ICAI. After screening PGC function in vitro, four amino acids E59, V60, P76 and G82 were found closely related to the stability of DND1 protein [59]. It was proved that the combination of haploid and single base editing system could select related bases during the individual development, and establish a system for efficiently obtaining targeted base editing animals. As many human diseases are caused by single-base mutations of a gene, we can use this system to produce single-base mutation animal models mimicking patients, which can help us to predict related genetic diseases [77]. There are more than 22,000 genes encoding proteins in human genome [78], and the protein functions are the keys to biological activities. In order to acquire large-scale tagged mouse libraries, researchers started the genome tagging project (GTP) [79], ICAI procedure with DKO-ahaESCs was an ideal strategy for this project [57,64]. With this project, we could quickly construct an artificial sperm bank containing various gene modifications in vitro, and produce numerous mice carrying specific mutations and tags efficiently. This project of course facilitated progresses in study of proteome and protein interaction [79].

4. Perspective

The mammalian haploid cells are powerful tools for genomewide screening to identify the functions of unknown recessive genes. In addition, semi-cloning is a reliable way to generate numerous mutant animals with DKO-ahaESCs, which is an advanced technology to uncover gene function at animal level. Many efforts have been made to reduce self-diploidization of haESCs, whereas the exact mechanism underlying it has not been addressed yet. In the future, more convenient and accurate separation methods, or more specific inhibitors are in need to address the issue of self-diploidization. Only solving the problem of self-diploidization completely can explore mammalian haploid system to many more fields, which is helpful for studies of human genetic diseases. To figure out whether primate haESCs can also function as gametes is quite essential in the future, which would shed light on assistant reproduction technology for developing brand-new strategies to give birth.

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