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The milestone of genetic screening: Mammalian haploid cells

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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.

Contents

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 one single

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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 limit 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 successively 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 (haEpiLCs) 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

Homozygous 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), resulting in the chromosomes doubling [37]. By adding Wee1 inhibitor PD166285 [37] into the culture medium, haESCs can accelerated G2/M phase transition and prevented entering an irrelevant G1/S phase (Fig. 2A). 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

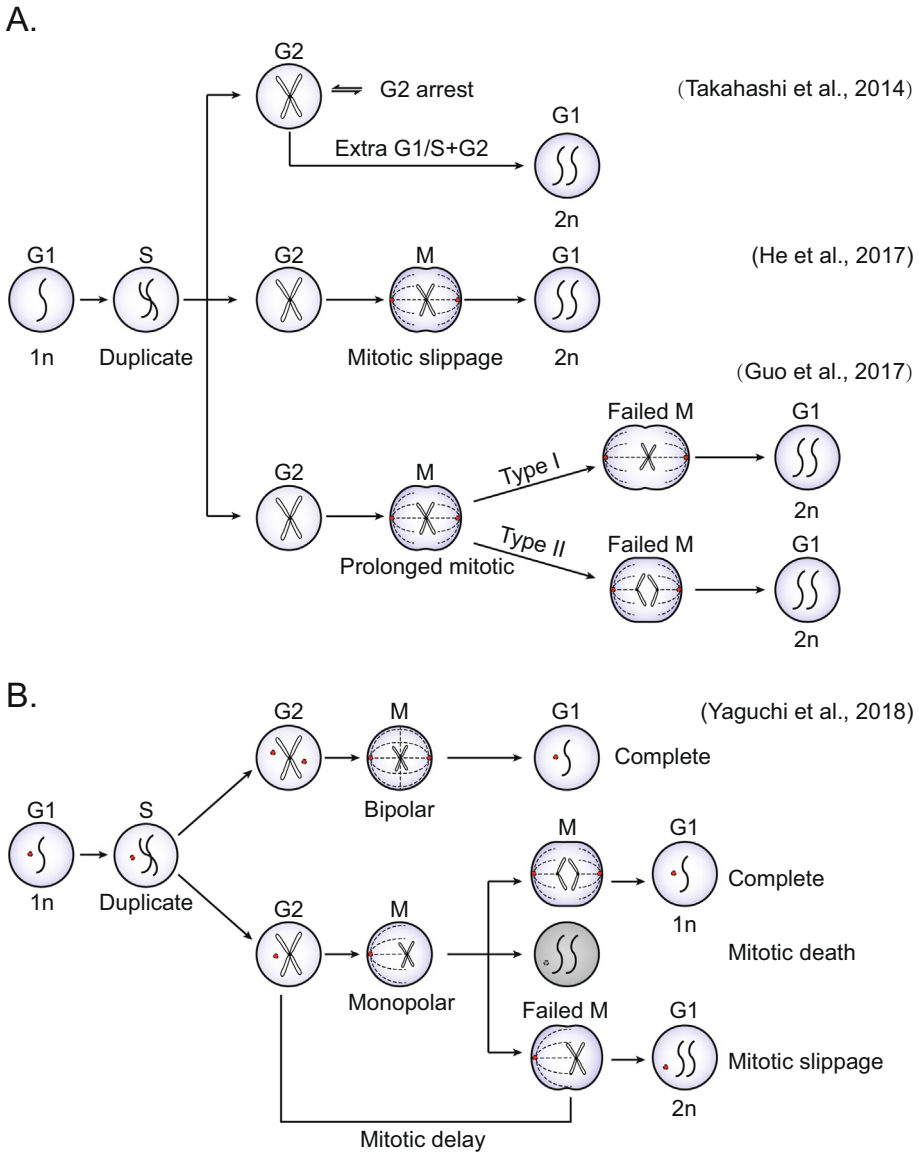

Fig. 1. Mechanisms of self-diploidization A. The main reason of self-diploidization may be caused by a mistaken mitotic cell cycle. The mistaken cells arrest at G2 phase and entry an extra G1/S + G2, resulting in self-diploidization (top) [37]. Some haESCs undergo mitotic slippage, escape from M phase 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 fulfil mitotic, whereas most of them experience mitotic death, mitotic delay and mitotic slippage [40].

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 *p53* (Fig. 2B) in HAP1 cell lines and mouse haESCs could stabilize haploidy genomes significantly [41]. Similar evidence proved that deletion of *p53* also facilitated derivation of mouse haiTSCs by stabilizing haploidy during conversion [34] A recent study showed that knockout of *p53* can significantly down regulate the expression of apoptosis related genes in haESCs, thus maintaining the stability of haploids [42]. If the apoptosis related gene *p73* was knocked out, haESCs could also maintain haploidy steadily, which suggested that apoptosis is another potential cause of diploidization in haploid cell cultures [42]. Another report found that the methylation level of haESCs was lower than that of diploid ESCs. Overexpression of *Dnmt3b* could improve global DNA methylation level and reduce the self-diploidization in haESCs (Fig. 2B), mainly depending on regulating some G2/M phase related genes [43]. Moreover, overexpression of Aurora B (*Aurkb*) can effectively shorten the M phase and prevent the occurrence of diploidization (Fig. 2B), either [36].

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

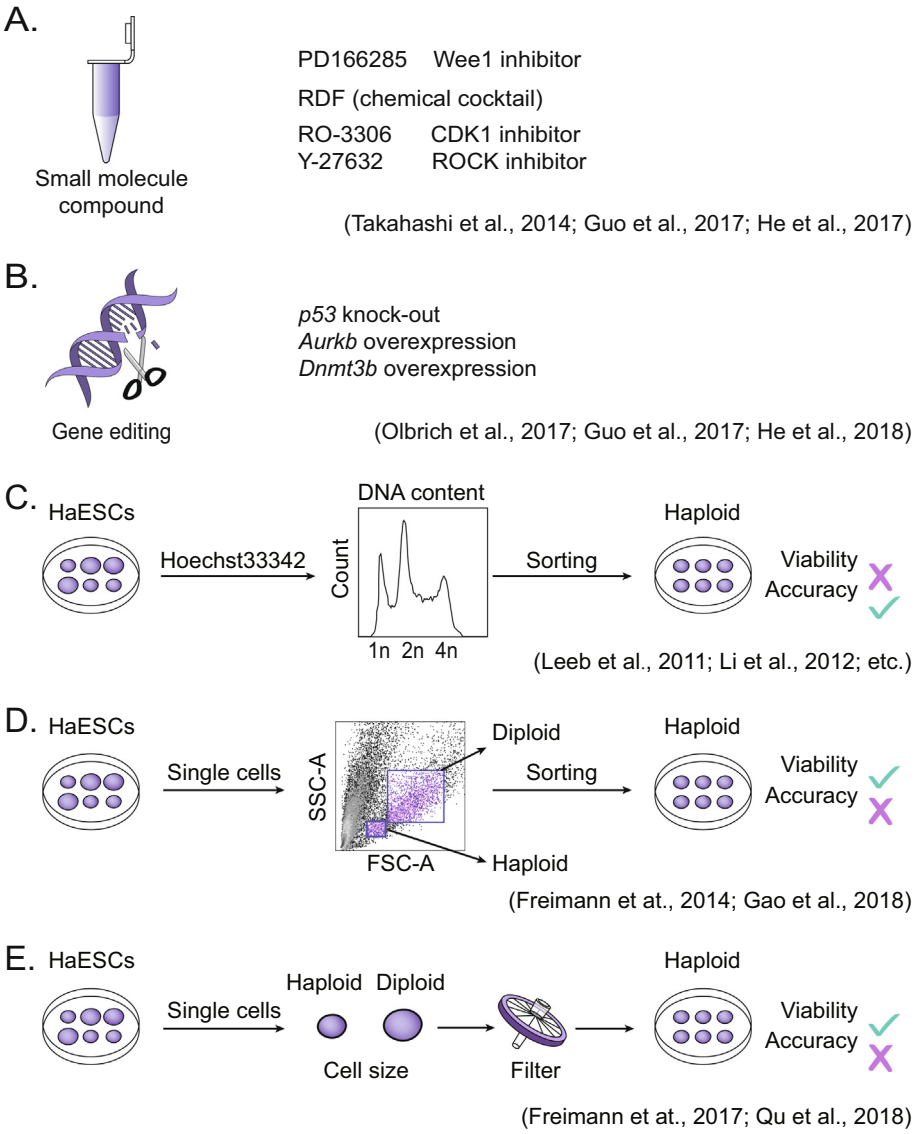

Fig. 2. Different strategies for sustaining and enriching for haploid cells A. Effective chemical inhibitors to reduce self-diploidization of mouse haESCs [32,37], PD166285 (Wee1 inhibitor) repress diploidization *via* promoting G2/M transition; RDF (cocktail of Repsox, DMH1 and Forskolin) can shorten 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].

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 between haploid and diploid cells [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). In 2009, Carette et al. introduced substantial mutations into KBM7 by retrovirus to construct mutant libraries, and identified the host factors essential for influenza infection by forward genetic screening [20]. Using the same strategy, the same group screened out *NPC1* with another near-haploid line (HAP1), as an infection target of Ebola virus [50]. These were approaches of genetic screening with near-haploid cells as platforms. With the progress of mammalian haESCs, scientists

Table 1
Haploid cells for genetic screening.

Approaches	Screening purpose	Cell type	Mutant method	Target gene	Reference
Genetic screening with near-haploid cells	Host factors essential for infection with influenza	Human KBM7	retrovirus	*SLC35A2, CMAS*	Carette et al., 2009 [20]
	Ebola virus receptor	Human HAP1	retrovirus	*NPC1*	Carette et al., 2011 [50]
	Adeno-associated virus receptor	Human HAP1	retrovirus	*KIAA0319L*	Pillay et al., 2016 [80]
	Required for ERAD	Human KBM7	CRISPR/Cas9	*TXNDC11*	Timms et al., 2016 [56]
Genetic screening with haESCs using retrovirus	Ricin toxicity	Mouse haESCs	retrovirus	*Gpr107*	Elling et at., 2011 [6]
	X-chromosome inactivation	Mouse haESCs	retrovirus	*SPEN*	Monfort et al., 2015 [53]
	Resistance to 6-TG	Human haESCs	retrovirus	*NUDT5*	Sagi et al., 2016 [25]
Genetic screening with haESCs using PB	Resistance to 6-TG	Mouse haESCs	PB	*Msh2, Hprt*	Leeb and Wutz, 2011 [5]
	Resistance to Olaparib	Mouse haESCs	PB	*Parp1*	Pettitt et al., 2013 [54]
	Detection of mutation efficiency	Monkey haESCs	PB	*PRKD1 et al.*	Yang et al., 2013 [24]
	Exit from self-renewal	Mouse haESCs	PB	*Zfp706, Pum1*	Leeb et al., 2014 [52]
Genetic screening in other haploid cell types	Resistance to 6-TG	Mouse haESCs	EMS	*Hprt*	Josep et al., 2017 [63]
	Mn^{2+} toxicity	Mouse haNSCLSs	PB	*Park2*	He et al., 2017 [32]
	Tetrodotoxin-like toxicant	Monkey haNPCs	PB	*B4GALT6*	Wang et al., 2018 [30]
	Blocker for spongiotrophoblast specification	Mouse haiTSCs	PB	*Htra1*	Peng et al., 2019 [34]
	Resistance to 6-TG	Mouse haTSCs	PB	*Hprt*	Cui et al., 2019 [33]
Genetic screening with haESCs *in vivo*	Related to bone development	Mouse haESCs	CRISPR/Cas9	*Zic1, Clec11a, Rln1* and *Irx5*	Bai et al., 2019 [58]
	Related to stability of DND1 protein	Mouse haESCs	CRISPR/Cas9	4 amino acids of DND1	Li et al., 2018 [59]

The enclosed manuscript entitled "The Milestone of Genetic Screening: Mammalian Haploid Cells" introduces recent breakthroughs of mammalian haploid cells involving 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***.

began to put haESCs into genetic screening combined with high-throughput mutation protocols. *Gpr107*, 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 KBM7 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, Rln1* and *Irx5* 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 haploidy 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 genetical 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 homozygous 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. Beside 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 Mn^{2+} (*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 spongiotrophoblast 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 sperms to support full term development by intracytoplasmic ahaESCs injection (ICAI), and thus were called 'artificial sperms' [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-

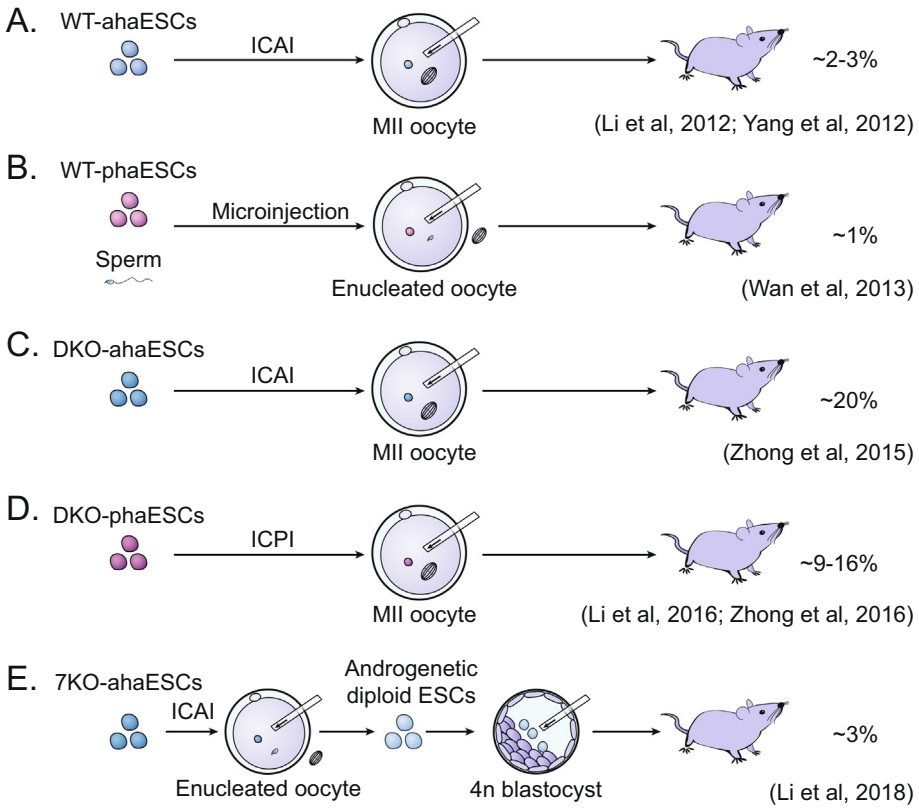

Fig. 3. Summary of intracytoplasmic microinjection of mouse haESCs A. Generation of viable mice by ICAI procedure with WT-ahaESCs, the full-term birth rate of which is about 2–3% [8,9]. B. Generation of viable mice *via* co-injecting sperm and WT-phaESCs into enucleated oocytes, the full-term birth rate 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 *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 *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-ahaESCs 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*.

genetic haESCs (phaESCs), a modified semi-cloned experiment was performed to validate it. A sperm and nuclei of a phaESC were microinjected simultaneously into an oocyte, the procedure of which was named intracytoplasmic phaESCs injection (ICPI) [10]. The reconstructed embryos could produce fertile mice although the full-term birth rate was quite low (Fig. 3B). The low efficiency may be caused by the manipulations and the different epigenetic states between phaESCs and oocytes. Interestingly, alive birth rate in both ICAI and ICPI procedures was decreased when used late-passage haESCs as donor cells [9,65]. It meant that the epigenetic states of important genes changed with passages in long-term culture.

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 mice [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 imprinted regions in ahaESCs on bi-paternal development. Knock-out of 7 imprinted 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 imprinted genes also played critical roles on development of bi-paternal embryos. These reports showed that uni-parental reproduction was reasonable through property gene modification on imprinted regions. However, how the global epigenomics 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 mutation mice could be attained, genetic screens based on mutant mice 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 (*Sfmbt2*, *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 *Dnd1* to construct a *Dnd1* 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 genome-wide 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.

Acknowledgements

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