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Haploid pluripotent stem cells: twofold benefits with half the effort in genetic screening and reproduction

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Haploid pluripotent stem cells, which are capable of self-renewal and differentiation into other cell types with only one set of chromosomes, have been established in several species from haploid embryos. Compared with diploid embryonic stem cells (ESCs), haploid embryonic stem cells (haESCs) are smaller in size, have a prolonged metaphase, and undergo self-doubling during culture. The monoallelic expression of haESCs provides great convenience for recessive inheritance research. Genetically modified haESCs also provide benefits in replacement of the gamete genomes, which not only facilitates the study of the function of imprinted genes but also potentially removes barriers to same-sex reproduction. In this review, we focus on strategies for obtaining haESCs and their potential applications in genetic screening, genomic imprinting, and unisexual reproduction.

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Introduction

The recent birth of the first normal bimaternal mouse and live bipaternal mouse represented a significant advance in the field of reproductive biology. The genomes of the bimaternal mouse and live bipaternal mouse were generated by combining gametes and hypomethylated haploid embryonic stem cells (haESCs) with specific imprinted region deletions, which removed uniparental reproductive barriers in mice [1••]. The realization of same-sex reproduction in mice is attributable to the development of haESC technology, which not only provides a platform for the study of imprinted genes but also provides a valuable tool for reproductive technologies.

Derivation of the first haESCs from parthenogenetic embryos in mice was accomplished from the development of embryonic stem cells (ESCs) and fluorescence-activated cell sorting (FACS) technology [2••,3••]. Subsequently, haESCs with self-renewal and differentiation abilities were obtained in other species [4•,5••,6•]. In this review, we introduce methods for the derivation and maintenance of diploidy in haESCs, and describe their advantages and applications in genetic screening and reproductive biology, which have great potential for expanding our understanding of genome evolution, developmental biology, and sexual reproduction.

Part 1 from diploid to haploid: derivation and characteristics of haESCs

Derivation of haESCs: why and how

Haploidy refers to the presence of only one set of chromosomes in a cell. Under natural conditions, haploidy tolerance is observed in yeasts and plants, but not in mammals, with one exception of haploid gametes. In vertebrates, the first artificial haploid cell lines, which were obtained from frogs [7], have served as a valuable tool in genetic screening. Mammals have evolved to possess diploid genomes, in which the lethal mutations of recessive traits are neutralized by the other allele of the diploid genome. In contrast, the haploid organism is vulnerable to environmental changes owing to its simpler genome. The differences in genome evolution between haploid and diploid organisms remain poorly understood. Therefore, the establishment of haploid stem cells in mammals is expected to yield novel strategies for clarifying the differences in genome evolution between haploid and diploid organisms.

Because the only available haploid cells in mammals are germ cells, a challenging question is whether stable haploid cell lines can be obtained from diploid mammals. A feasible strategy for the derivation of haploid cells from mammals is to establish cell lines from haploid embryos that only contain one copy of the genome. Haploid blastomeres only exist in the parthenogenetic embryo and in a low percentage of haploid cells [8], which made it difficult to obtain a haploid cell line. However, recent progress in ESC and FACS technology has resolved this issue, and haploid cells in zebrafish have been established and applied for genetic screening [9]. Subsequently,

haploid pluripotent cell lines from medaka fish (*Oryzias latipes)* were established [10].

In mammals, the first mouse PG-haESCs were generated from parthenogenetic embryos [2••,3••]. Thereafter, mouse AG-haESCs were generated from androgenetic embryos containing only the paternal genome set [11••,12••]. Rat [4•], monkey [6•], and human [5••] haESCs were successfully established over the subsequent several years (Table 1). According to the origin of the genome, haESCs are derived from either parthenogenetic (PG) or androgenetic (AG) embryos that contain one set of chromosomes from paternal or maternal genomes, and are classified into androgenetic haESCs (AG-haESCs) and parthenogenetic haESCs (PG-haESCs). PG haploid embryos can be derived via *in vitro* activation of mature oocytes, followed by the exclusion of the second polar body or by the removal of the female pronucleus from the zygote [8,13,14]. In contrast, AG haploid embryos can be generated via microsurgical removal of the female pronucleus from the zygote or the replacement of the chromosome-spindle complex of metaphase II (MII) oocytes with a sperm head [15,16] (Figure 1).

Characteristics of haESCs: common features and differences

As a specific type of stem cell, haESCs share common characteristics with diploid ESCs including self-renewal and differentiation abilities, supporting the development of the three germ layers and transmission to the germline. Similar characteristics are observed in the rat [4•], monkey [6•], and human [5••]. In addition, haESCs also exhibit a gamete-like function similar to that of haploid sperm or oocytes, and yield live mice after intracytoplasmic injection into reconstructed embryos [12••,17•].

However, the haESCs showed a lower capacity for development and a more unstable genome than diploid ESCs, as well as persistent dosage imbalance between the autosomes and the X chromosome, which hints at the differential regulation of X chromosome inactivation compared with that in diploid ESCs. haESCs have a smaller cell size and prolonged metaphase compared with diploid cells, making them prone to duplication without cytokinesis [5••,18•].

Other haploid cell types

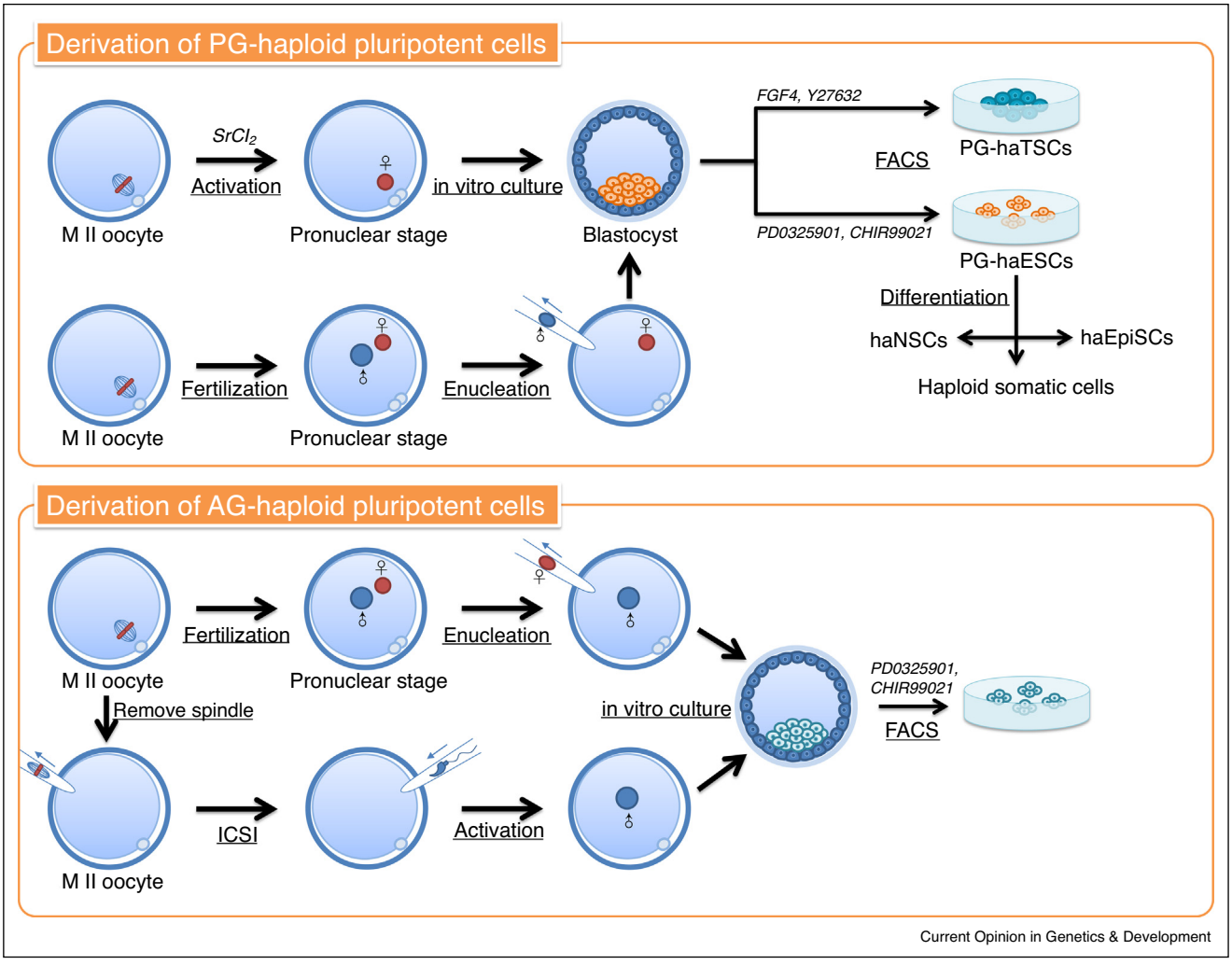
In addition to haESCs, other types of mammalian haploid cells can be obtained *in vitro* (Figure 1, Table 1). The first haploid epiblast stem cells (haEpiSCs) were derived from differentiated mouse haESCs [19•]. Furthermore, researchers have obtained haploid neural stem cells (haNSCs) or induced neural-like stem cells (ha-iNSC) in mice [20•,21] and monkeys [22•], which has broadened our understanding of neural development through genetic screening. Haploid somatic cells corresponding to all three germ layers were also obtained via induction from haESCs [20•]. Cui *et al.* established haploid trophoblast stem cells (haTSCs) from parthenogenetic blastocysts by using FGF4 and ROCK inhibitors, yielding an *in vitro* model for studying gene

Table 1

Representative studies reporting the derivation of haploid stem cells from different species

Cell types	Representative studies	Speice	Origin	Application	References
haESC	PG-haESCs from mouse	Mouse	PG-embryos	Genetic screening of DNA mismatch repair pathway; ricin toxicity; self-renewal pathway; X chromosome inactivation	[2••,3••,28,30]
	AG-haESCs from mouse	Mouse	AG-embryos	Generation of transgenic or mutant mouse models in One-step; substitute sperm	[11••,12••]
	AG-haESCs from rat	Rat	AG-embryos	Functional genetic studies; production of transgenic rat	[4•]
	PG-haESCs from monkey	Monkey	PG-embryos	Generation of genome modified haploid cell lines by drug-resistance library	[6•]
	PG-haESCs from human	Human	PG-embryos	Differential regulation of X chromosome inactivation and oxidative phosphorylation pathways	[5••]
Other haploid cell lines	haEPiSCs from mouse	Mouse	PG-embryos	haEpiSCs can support embryonic development until midgestation	[19•]
	ha-NSCs from mouse	Mouse	PG-embryos	Genetic screening of neural and retinal development; Mn^{2+}-mediated toxicity in neural cell and identified the Park2 gene; genome-wide mutant libraty in neural cell	[20•,21]
	haNSCs from monkey	Monkey	PG-embryos	Gene targets of neural toxicants (B4GALT6); NSC differentiation pathways	[22•]
	ha-TSCs from mouse	Mouse	PG-embryos	Genome-wide screening in the trophoblast lineage	[23•]
	haploid somatic cell line from mouse	Mouse	PG-ESCs	Derived haploid somatic cells from all three germ layers	[20•]

Figure 1



Derivation of haploid pluripotent stem cells from parthenogenetic (PG) or androgenetic (AG) haploid embryos.

function in placental biology [23•]. The establishment of various haploid cell lines has provided a diversified platform for genetic screening in mammals, and it is anticipated that more types of haploid cells will be derived in the future.

Part 2 from haploid to diploid: how to avoid self-diploidization and offer potential for genetic screening?

Self-diploidization of haESCs

Compared with diploid genomes, the haESCs genome is unstable and progressively undergoes spontaneous and irreversible diploidization due to the longer duration of mitosis in haESCs [18•,24]. Haploid cells undergo diploidization during the differentiation process, both *in vivo* and *in vitro*. The avoidance of self-diploidization is a key challenge in the maintenance of haploidy. haESCs exhibit an increased duration of mitosis in both the mouse [18•] and human [5••]; therefore, researchers have attempted to regulate the cell cycle of haESCs to repress self-diploidization.

haESCs of rodents rapidly undergo diploidy during differentiation and thus cannot yield haploid cells; in comparison, human haESCs spontaneously differentiate toward haploid somatic fates, both *in vitro* and in *vivo* [5••]. The derivation of haploid cells from rodent haploid embryonic stem cells is challenging. He *et al.* showed that the spontaneous diploidization of haESCs occurs in metaphase, owing to mitotic slippage. The inhibition of CDK1 and ROCK suppresses the spontaneous diploidization of haESCs; on this basis, haploid somatic cells were generated from all three germ layers for genome-wide genetic screening [20•]. The addition of MEK and GSK3 inhibitors PD0325901 and CHIR99021, respectively, to the

embryonic stem cell culture medium is critical for the stabilization of the haploid karyotype [25•]. Because of the prolonged metaphase of haESCs [18•], the rate of spontaneous diploidization can be reduced by accelerating G2/M phase transition using the Wee1 kinase inhibitor PD166284 [26•]. p53 deletion [24] has been shown to promote the survival of mouse haESCs, and the downregulation of G2/M-related genes via the overexpression of Dnmt3b was sufficient to stabilize the genome of haESCs [25•].

Rapid genetic screening using haESCs

Random mutagenesis is a common method used for genetic screening, although its efficiency is low in mutant generation of recessive phenotypes. haESCs have only one copy of the genome, which provides considerable benefits in terms of the induction of biallelic mutations during gene editing; accordingly, the efficiency of screening for gene function and generating a transgenic animal model is greatly improved by the addition of small molecules combined with FACS. Genomic engineering is applied to produce mutant libraries through transposon-mediated insertion or nuclease-mediated targeting modification technologies. Haploid cell-based genetic screening in mice [2••,12••], rats [4•], monkeys [6•], and humans [5••] has been broadly applied to investigate DNA repair, drug toxicity and target identification [3••,20•], X-chromosome inactivation [27••,28], lineage specification [19•,23•], and clinical research [29] (Table 1) in combination with gene-editing technologies such as the piggyBac transposon system and the clustered regularly interspaced short palindromic repeats/Cas (CRISPR/Cas) approach [29]. In addition to mutagenic screening strategies, tailormade reporter screening systems for investigating signaling pathways have been employed recently, and hold potential for investigating molecular interactions via specific lineage tracing in mammalian cells [30,31] (Table 1). Therefore, haESCs offer the potential to circumvent the difficulties encountered in the generation of genome-wide homozygous mutant libraries with one set of chromosomes, and enable the establishment of an alternative screening platform to diploid stem cells, with a high efficiency, with applications in the investigation of human disorders.

Part 3 from haploid to haploid: replacement of the gamete genome for developmental studies

The role of parental genomes in embryonic development

Considering that haESCs are haploid and their genomes originate from only one of their parents, it is of interest to determine whether PG-haESCs or AG-haESCs can replace the genome of oocytes or sperm, respectively, and act as gametes to support reconstructed embryo development. Both sperm and oocytes are structurally specialized cells that cannot be genetically manipulated *in vitro* or *in vivo*, which also makes haESCs a good alternative to paternal or maternal genomes for generating parental-specific modified animals.

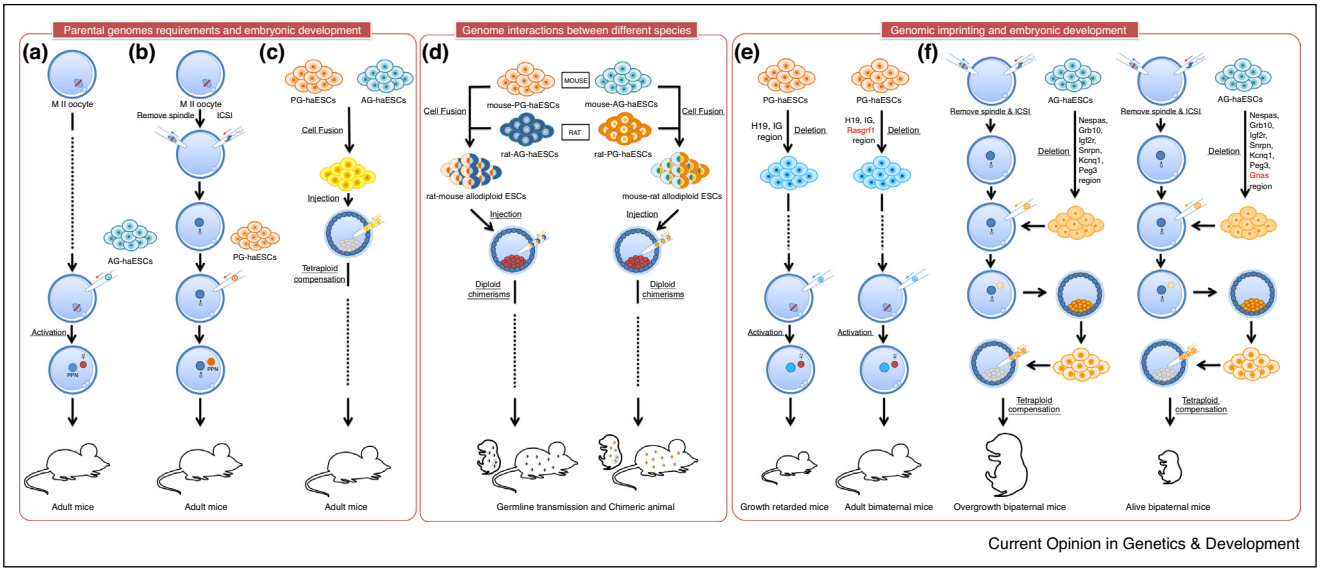
Some groups have reported that the injection of AG-haESCs that carry paternal imprints into oocytes, in place of sperm, has led to the successful generation of fertile live mice (Figure 2a) [11••,12••,32•]. In addition, the genomes of PG-haESCs can also be directly delivered to support embryonic development when substituted for the maternal genome (Figure 2b) [17•]. Although haESCs can be substituted for gametes to support embryonic development and lead to the birth of offspring, their efficiency is quite low (0.7–2% of transferred embryos) compared with that of sperm and oocytes, which may be attributed to changes in imprinting during passage and culture.

Previous research has shown that the paternal genome is not necessarily associated with the maternal genome within the same nucleus before the eight-cell stage [33]. Whether the participation of both genomes is required in later embryonic stages for full-term development is an important question in developmental biology. Li *et al.* established fused ESCs (fESCs) via the fusion of AG-haESCs and PG-haESCs. The fESCs developed to full-term mouse embryos after tetraploid complementation assay, which demonstrated that co-participation of the paternal and maternal genomes before the blastocyst stage is not essential. This research also allows *in vitro* functional analysis of the dynamics of DNA methylation within each haploid parental genome, and then in the diploid nucleus after their assembly (Figure 2c) [34].

Genetic isolation and interactions between different species

With the successive establishment of haESCs in different species, the interesting question arises of whether is it possible to establish a diploid cell line to study the genetic isolation and interactions between different species. To investigate the differences in gene regulatory networks between species and study the evolution of gene regulatory systems, we established a unique ESC line, referred to as mouse-rat allodiploid ESCs, via a cell-fusion technique based on the establishment of mouse and rat haESCs. The allodiploid ESCs were pluripotent and possessed a stable diploid genome with mid-parent and species-biased gene expression patterns, which was useful for studying the genes that regulate phenotypic differences between the mouse and rat. Additionally, these hybrid cells have proven to be an ideal tool for X chromosome inactivation and species-based pluripotency maintenance. The mouse-rat allodiploid ESCs offers a cellular model for studying the reproductive isolation of species and genomic dialogue between different species, thus providing a reference for the study of species evolution and reproductive isolation (Figure 2d) [27••].

Figure 2



The application of haESCs in rodent embryonic development and reproduction. **(a)** Parental genomes could be substituted by fusion of PG-haESCs and AG-haESCs before the blastocyst stage to support full-term development of mice. **(b)** AG-haESCs could replace sperm to support the adulthood development of mice. **(c)** PG-haESCs could replace maternal genomes to support the adult development of mice. **(d)** The mouse-rat allodiploid ESCs showed germline transmission and enabled generation of adult chimaeras. **(e)** Generation of bimaternal mice by imprinting-modified PG-haESCs. **(f)** Generation of bipaternal mice by imprinting-modified AG-haESCs.

The study of genomic imprinting using haESCs

Embryonic development requires both maternal and paternal contributions, and the two sets of parental chromosomes exhibit genetic asymmetry in imprinted genes. Imprinted genes, which showed monoallelic and parental origin-specific expression patterns, are considered important obstacles to same-sex reproduction. The molecular mechanisms that regulate imprinting in different regions are complex and have not been fully elucidated. Both AG-haESCs and PG-haESCs specifically retain genomic imprinted genes, which makes them a good model for studying genomic imprinting in both maternal and paternal genomes.

To elucidate the role of imprinted genes in same-sex reproduction, Kono *et al.* deleted the H19 imprinted region in immature oocytes and produced the first parthenogenetic mice that were able to develop to adulthood [35••]. Furthermore, removal of H19 and Gtl2 differentially methylated regions (DMRs) in AG-haESCs improved the birth rate of sperm replacement mice [32•], which indicates that imprinted genes play an important role in reproduction. Zhong *et al.* reported that, following the deletion of H19 and IG DMRs, PG-haESCs exhibit a gamete capacity comparable to round spermatids and retain stable developmental potential in bimaternal offspring [36••]. Subsequently, our research showed that MII oocyte-derived PG-haESCs can efficiently be used to generate full-term bimaternal pups following the deletion of a specific imprinted region in the IG-DMR, but not the H19-DMR, indicating that the Dlk1-Dio3 region has a more profound effect on fetal growth [37••]. Both studies established a convenient and efficient approach to generate bimaternal mammals from imprinting-modified PG-haESCs, which provides a novel platform for genomic imprinting studies and offers a novel strategy in the field of reproductive technology (Figure 2e).

The genomic imprinting differences between parental genomes may be the main obstacle to the uniparental reproduction of mammals; how to overcome bipaternal reproduction barriers in mammals is a relevant question that needs to be addressed. Li *et al.* utilized haESCs to overcome uniparental reproductive barriers in mice and observed parent-specific erasure of DNA methylation in PG-haESCs and AG-haESCs. PG-haESCs and AG-haESCs show differential demethylation dynamics. By combining gametes and hypomethylated haploid ESCs with certain imprinted region deletions (Nespas, Grb10, Igf2r, Snrpn, Kcnq1, Peg3, and Gnas), live bipaternal mice have been generated [1••]. This research reports the exciting message that the barriers to bipaternal reproduction can be overcome with genomic imprinting-modified haESCs (Figure 2f). In contrast to bimaternal offspring, bipaternal offspring do not survive to adulthood,

which suggests that the bipaternal genomes exist greater reproductive barriers. In addition, the causes of imprinting barriers in same-sex reproduction in mammals need to be further clarified.

Conclusion

haESC technology has enabled an in-depth understanding of the role of parental genomes in embryonic development, the gene dialogue between different species, and the function of imprinted genes. Diploid organisms cannot reproduce unisexually, and the obstacles posed by imprinted genes and other aspects such as epigenomic changes need to be further explored. In contrast to traditional sexual reproduction, asexual reproduction based on haESC technology in mice is expected to usher in a new technological revolution in the field of mammalian reproduction. In addition, further research on diploidization of haESCs will refresh the existing understanding of genomic evolution from haploid to diploid organisms.

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Conflict of interest statement

Nothing declared.

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