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'Artificial spermatid'-mediated genome editing†

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Abstract

For years, extensive efforts have been made to use mammalian sperm as the mediator to generate genetically modified animals; however, the strategy of sperm-mediated gene transfer (SMGT) is unable to produce stable and diversified modifications in descendants. Recently, haploid embryonic stem cells (haESCs) have been successfully derived from haploid embryos carrying the genome of highly specialized gametes, and can stably maintain haploidy (through periodic cell sorting based on DNA quantity) and both self-renewal and pluripotency in long-term cell culture. In particular, haESCs derived from androgenetic haploid blastocysts (AG-haESCs), carrying only the sperm genome, can support the generation of live mice (semi-cloned, SC mice) through oocyte injection. Remarkably, after removal of the imprinted control regions *H19*-DMR (differentially methylated region of DNA) and *IG*-DMR in AG-haESCs, the double knockout (DKO)-AG-haESCs can stably produce SC animals with high efficiency, and so can serve as a sperm equivalent. Importantly, DKO-AG-haESCs can be used for multiple rounds of gene modifications in vitro, followed by efficient generation of live and fertile mice with the expected genetic traits. Thus, DKO-AG-haESCs (referred to as 'artificial spermatids') combed with CRISPR-Cas technology can be used as the genetically tractable fertilization agent, to efficiently create genetically modified offspring, and is a versatile genetic tool for in vivo analyses of gene function.

Key words: haploid embryonic stem cells, androgenetic haESCs, 'artificial spermatids', semi-cloned mouse (SC mouse), CRISPR-Cas9, gene editing.

Introduction

In mammals, sperm and oocytes are gametes: specialized haploid cells that combine at fertilization to form a diploid totipotent zygote, which undergoes development. In adult sexual organs, gametes are developed through the process of meiosis. This fluctuation between diploid and haploid phases lays the foundation for sexual

reproduction, which is important for biodiversity, adaption to the environment and evolution [1–3]. The formation of haploid germ cells (particularly sperm) and the process of fertilization provide a unique method to deliver exogenous DNA to descendants [4–6]. Initial reports of sperm-mediated gene transfer (SMGT) in 1989 showed that sperm could associate with and transfer exogenous DNA molecules through in vitro fertilization to produce transgenic offspring [7]. Similarly, transgenic mice were efficiently generated by co-injection of sperm heads and exogenous DNA, particularly so when sperm membranes were disrupted via physical or chemical methods [8]. SMGT has been proven as a simple, low-cost and rapid strategy to produce transgenic animals; however, subsequent studies have indicated that the final fate of the transgenes is unpredictable in the progeny [4–6]. Moreover, sperm are terminally differentiated cells that cannot divide, possess a highly dense nucleus, and are so far inaccessible for complex gene modifications such as multiple-gene editing and gene-targeting experiments.

The advent of haploid embryonic stem cells (haESCs) derived from sperm-originated androgenetic haploid blastocysts (AG-haESCs) has overcome these problems [9–11]. The AG-haESCs can divide rapidly and infinitely in vitro, maintain haploidy via periodic cell sorting, and sustain pluripotency [12, 13]. These cells partially maintain the gamete-specific imprinting pattern and retain the ability to mimick the sperm genome and support the full-term embryonic development through injection into mature oocytes (intracytoplasmic AG-haESC injection; ICAHCI) and lead to live animals (semi-cloned mice; SC mice). Importantly, removal of the differentially methylated regions (DMRs) of *H19* and *IG* in AG-haESCs mimics the imprinted state of sperm, decreases expression of *H19* and *Gtl2*, and creates DKO-AG-haESCs ('artificial spermatids') capable of efficient and stable production of SC mice. These 'artificial spermatids' are amenable to complex gene modifications in culture and efficiently generate mouse models with the expected genetic traits in one step via ICHACI [14, 15]. Combined with CRISPR-Cas9 technology, they enable one-step generation of mice with different mutations and provide an efficient tool for genetic screening [14, 16]. Taken together, 'artificial spermatid'-mediated genome editing opens new avenues for functional genetic analysis in vivo [9–11, 17–19].

SMGT

In 1971, sperm cells were first reported to be capable of incorporating heterologous genomes and carrying them into oocytes upon fertilization [20]. Unfortunately, this discovery was ignored for years until it was rediscovered in 1989 by two independent groups [7, 21]. In one report, incubation of mature mouse sperm cells with exogenous DNA prior to in vitro fertilization resulted in the birth of germline-competent transgenic founders [7]. These studies led to the development of SMGT: the introduction of foreign genetic information to produce genetically modified animals, facilitated by the ability of sperm cells to bind and deliver exogenous DNA to oocytes [22]. Several months later, a second report claimed that the original protocol could not be successfully reproduced and raised controversy over SMGT [7, 23], but the obvious ease and low-cost of the protocol has drawn attention from the scientists around the world. Many more SMGT reports have been published since: some have reported stable genomic modifications in resultant animals, others that stable transgene integration has rarely been detected in SMGT productions [6]. Numerous efforts have been made to evolve SMGT, including the use of linearized DNA (instead of circular plasmids) [7], incubation of DNA before intracytoplasmic sperm injection (ICSI) [24], disruption of sperm membranes before injection of DNA [8], optimization of DNA transfer through the use of transfection reagents [25, 26], and treating sperm heads with antibodies reactive to a surface antigen [27]. In spite of these efforts, the efficiency and outcome of SMGT are still unpredictable in single experiment, probably for the following reasons: sperm are highly specialized, non-dividing cells, so it is impractical to select those with integrated foreign DNA before fertilization, and a large number of founders must be screened for the presence of the transgene and its subsequent transmission to progeny; even when transgene presence is confirmed in founders and F_1 progeny, it may remain in an extrachromosomal form rather than integrating into the host genome [28]. The fact that the interaction between sperm cells and exogenous DNA, expected to be mediated by specific factors, is poorly understood contributes to the lack of consistent results with SMGT [5, 29–31]. Consequently, the foreign transgene may be inherited in a non-Mendelian ratio, expressed in a mosaic distribution or be lost after serial cell divisions [5]. While SMGT has not yet been conventionally used as a reliable tool for transgenesis, the concept of using sperm or sperm replacement as the delivery system for rapid transgenesis is illuminating and deserves more effort toward improvement.

Generation of AG-haESCs—the sperm replacement

Haploidy—a single chromosomal set per nucleus—is rare in vertebrates, restricted exclusively to mature gametes in mammals and yet is an essential feature of a sperm replacement [32]. Haploidy permits rapid analysis of recessive mutations in genetic screening without interference of a second allele. Unicellular eukaryote yeast can grow in a haploid fashion [33], and haploid-cell-based loss-of-function screening has made significant contributions to our understanding of the fundamental mechanisms of biology and disease [34–36]. In spite of the absence of haploid mammalian individuals, haploidy does occur in some mammalian somatic cells in a pathological condition: during cancer development, human tumor cells may incur duplication or loss of chromosomes, the latter of which has allowed human near-haploid cells to be isolated [37–39]. As with haploid yeast, these human near-haploid cells can be genetically screened to understand biological processes [40–50]. However, unknown genomic alterations in near-haploid tumor cells limit their usefulness in downstream applications [51]; generation of haploid cells with an intact genome is essential.

The observation that haploid and diploid cells coexist in the parthenogenetic blastocysts generated from activated oocytes implies that they could be used to establish haploid cell lines [52]. The first attempt to establish haploid embryonic stem cell (ESC) lines from parthenogenetic mouse blastocysts was reported in 1983: mature oocytes were artificially activated to extrude the second polar body, leading to haploid embryos that developed into blastocysts used to derive pluripotent cell lines. Unfortunately, all four of the resultant cell lines were diploid with a normal mouse karyotype of 40 chromosomes [53]. Years later, a group from Singapore successfully derived medaka fish haploid ESCs from gynogenetic embryos [54]. The medaka fish haploid ESCs could sustain pluripotency, genomic integrity, and importantly, support the production of SC fish when transplanted into mature oocytes [54]. Inspired by this groundbreaking study, mouse researchers tried

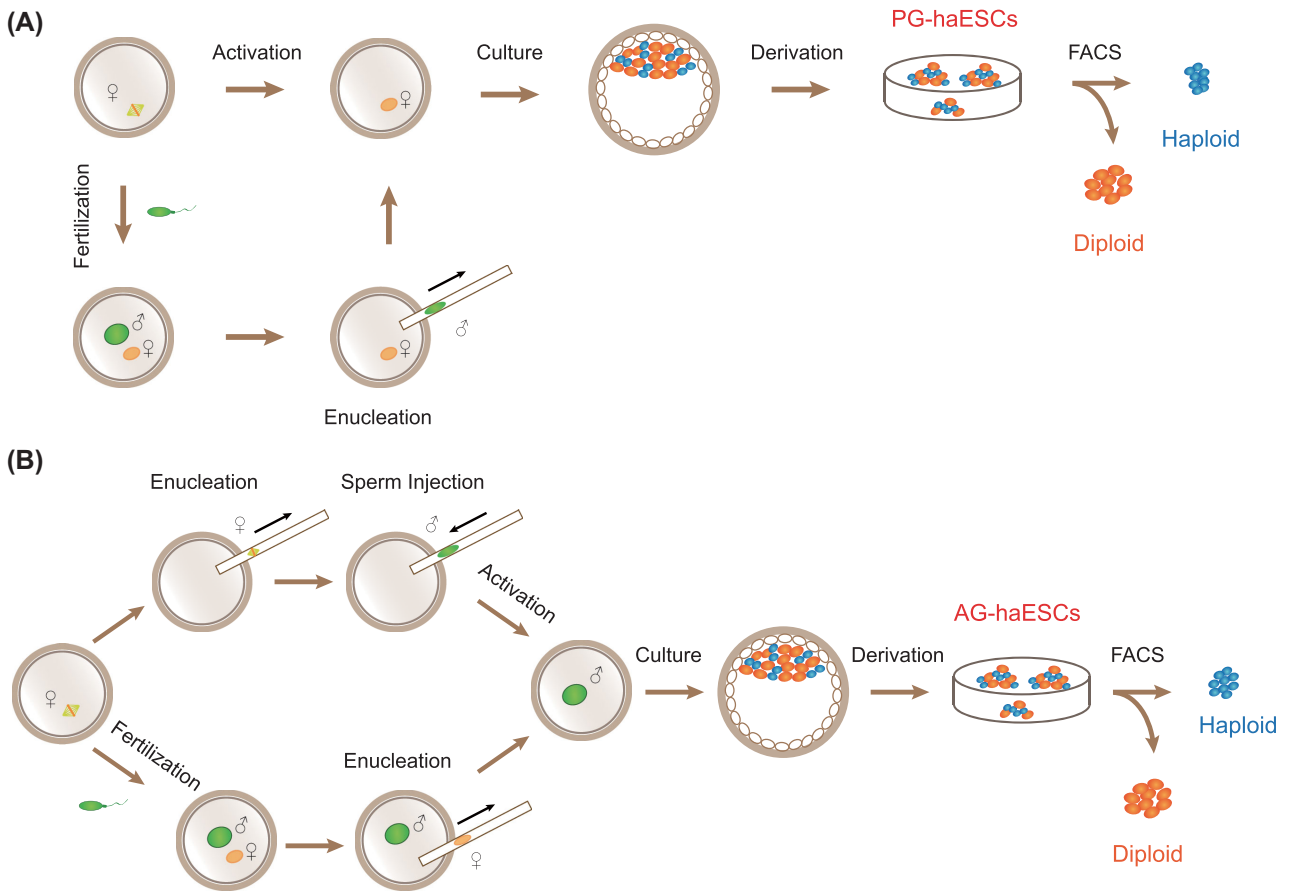

Figure 1. Derivation of haploid embryonic stem cell (haESC) lines from parthenogenetic and androgenetic embryos. (A) Artificial activation of oocytes without fertilization or by removing the paternal pronucleus from the zygote result in parthenogenetic embryos, from which parthenogenetic haploid ES cells (PG-haESCs) can be derived with the aid of fluorescence-activated cell sorting (FACS). (B) Injection of sperm into enucleated oocytes or removal of the maternal pronucleus from the zygote result in androgenetic embryos, from which androgenetic haploid ES cells (AG-haESCs) can be derived with the aid of FACS.

again and successfully generated the first mouse parthenogenetic haploid ESCs (PG-haESCs) [55, 56] by employing fluorescence-activated cell sorting (FACS) to enrich for haploid cells (Figure 1A). Consistent with previous observations [53], mouse haploid cells spontaneously converted into diploid cells during cell proliferation, so haploidy was maintained through FACS-based isolation of haploids from cell mixtures according to differences in DNA content [55, 56]. Shortly following the generation of PG-haESCs, also with the aid of FACS technique, mouse androgenetic haploid ESCs (AG-haESCs) were generated from haploid androgenetic embryos, obtained either by injecting sperm into enucleated oocytes or removing the maternal pronucleus from the zygote (Figure 1B) [12, 13]. Similar strategies have been used to successfully generate haESCs from parthenogenetic or androgenetic embryos of other mammalian species, including rat [57], monkey [58], and human [59, 60].

HaESCs exhibit typical diploid ESC features, including stable genomic integrity, infinite self-renewal and developmental pluripotency [61–64]. Moreover, haESCs possess only one allele for each gene (so allele disruption would likely cause a loss-of-function phenotype) and offer a yeast-like platform to identify novel genes involved in X-chromosome inactivation, DNA mismatch repair, ESC self-renewal and toxin resistance in mammals [55, 56, 58, 59, 65–68]. Interestingly, differentiation of haESCs into haploid epiblast stem cells (haEpiSCs) and somatic cells [59, 69–72] enables large-scale mutagenesis in different cell lineages [69, 70]. Strikingly, the reversible mutagenesis strategy mediated by conditional insertional mutagenesis vectors was successfully introduced into haESCs, leading to a repairable mutant Haplobank of 16 970 genes that permits high-throughput, genome-scale gene functional annotations directly in sister cells [70].

Similar to diploid ESCs carrying genetic modifications that can be extended to the organismal level through blastocyst injection, both PG-haESCs and AG-haESCs are germline competent [12, 57, 73], suggesting that they are suitable for being vehicles for the transmission of genetic modifications from chimeras to descendants in a conventional way. However, germline transmission of chimeras needs additional mating with wildtype mouse, a time-consuming and somehow unpredictable process. Sperm-originated AG-haESCs generally maintain parental genomic imprints and can be injected into oocytes to produce live and fertile SC mice through ICAHCI, a procedure that is similar to ICSI (Figure 2). Thus, ICAHCI is a novel approach for the one-step transmission of genetic modifications in AG-haESCs to animals. In this approach, genetic manipulations (introduced into AG-haESCs either by homologous recombination-mediated gene-targeting [12] or electroporation-mediated transgene

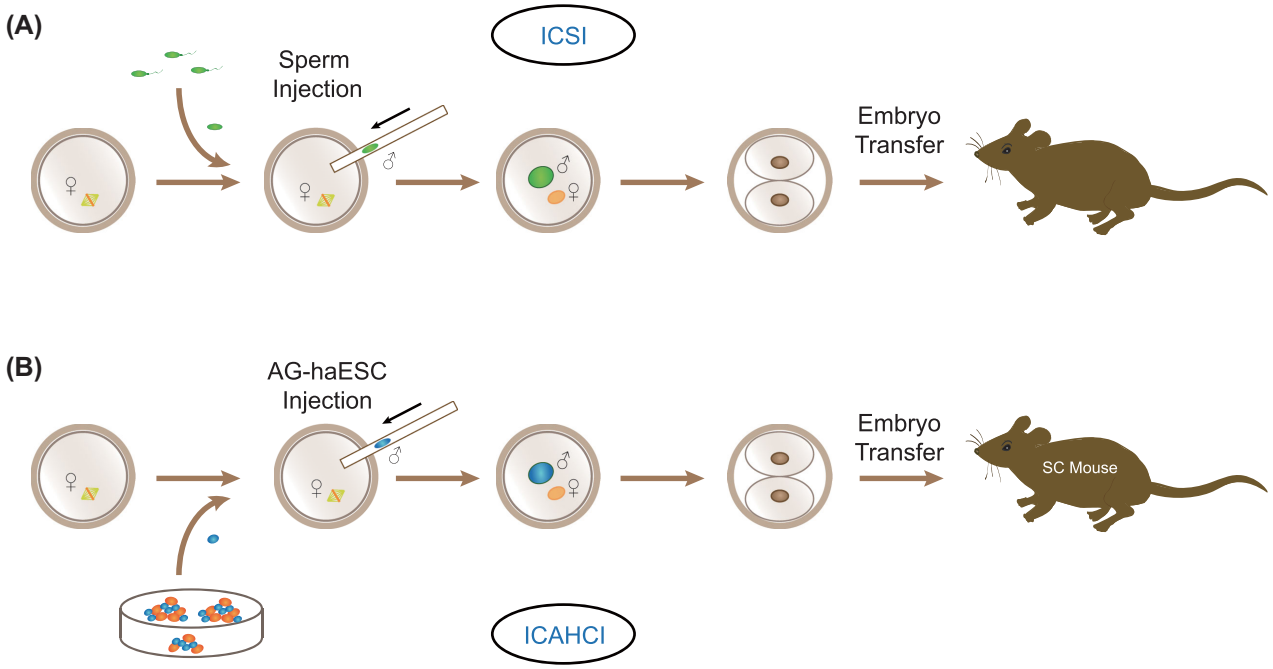

Figure 2. Schematic diagram of mouse generation via ICSI and ICAHCI.

integration [13]) can be transmitted to all the descendants in a controllable and non-randomized manner through 'fertilization'. In general, AG-haESCs act as a genetically tractable fertilization agent for the production of animal models [12].

Generation of 'Artificial Spermatids'

The generation of mouse AG-haESCs provided a sperm replacement that could be genetically manipulated in vitro and used to produce animal models with desired genetic traits in a single step through oocyte injection; however, the birth rate of healthy SC mice using this technique was extremely low (~2% of transferred embryos) [12]. Another approximately 2% of transferred embryos developed into growth-retarded pups, which died shortly after birth. Live birth rates were worse still with higher passage number or after genetic manipulation that required more culture time in vitro [12, 13], greatly limiting the application of AG-haESCs to generate gene-modified mouse models. Further analyses revealed an aberrant epigenetic state in two paternally imprinted genes, *H19* and *Gtl2*, which are not expressed in sperm but are overexpressed in both AG-haESCs and growth-retarded pups. Consistently, DNA methylation of *H19*-DMR (differentially methylated regions) and *IG*-DMR, which control the expression of *H19* and *Gtl2* respectively, was reduced in AG-haESCs and lost in growth-retarded pups [12, 74]. Surprisingly, removal of *H19*-DMR and *IG*-DMR from AG-haESCs markedly improved the birth rate of SC pups to approximately 20% of transferred embryos (Figure 3A) [14, 75]. More importantly, AG-haESCs with both genes deleted (DKO-AG-haESCs) could stably support the SC pup generation after multiple rounds of gene editing, even with high passage numbers. For example, two gene families (*Tet1/Tet2/Tet3* and *p53/p63/p73*) were successfully mutated in DKO-AG-haESCs and led to stable cell lines, which were employed as fertilization agents for efficient, one-step generation of mice carrying the corresponding heterozygous triple mutations [14]. Meanwhile, DKO-AG-haESCs with *Tet1-EGFP/Tet2-mCherry/Tet3-ECFP* knock-in alleles were established in vitro and used for reproducibly production of live SC pups through ICAHCI [14]. In summary, the genetic deletions in *H19* and *IG* DMRs improve the 'fertilization' capability of AG-haESCs, making it an efficient tool for production of genetically modified animals. We, therefore, refer to DKO-AG-haESCs as 'artificial spermatids'.

One obstacle in haESC application is the inclination to spontaneously convert into diploid cells during cell passages. Although regular FACS-based enrichment of haploid cells can be used to stably maintain the haploidy, this method is complicated, expensive, and requires large equipment; stabilization of the haploid state by optimizing culture conditions or establishing a simple method to enrich haploid cells is preferable. Recent studies have shown that diploidization in culture is largely due to endoreduplication [73] or prolonged metaphase in haESCs compared to that of diploid ESCs [76], and chemically induced acceleration of S-G2/M phases has been shown to stabilize haploidy for several weeks longer [77–79]; we and others have established physical filtration methods to enrich haploid cells based on differences in cell diameter (Figure 3B) [80, 81].

Recent application of the CRISPR-Cas9 system in early-stage embryos enables one-step generation of mice carrying knock-out/knock-in modifications at one or multiple sites [82–85]; however, the genotypes were varied in founders generated through direct injection of CRISPR-Cas9 into zygotes. Furthermore, a portion of the founders exhibited somatic mosaicism and allele complexity, making it difficult to analyze founder phenotype [86]; some had additional mutations in germ cells and generate offspring with different genotypes [87]. Thus, the approach of zygote injection is simple, but subsequent analysis of founders is complicated. In contrast, the strategy of 'artificial spermatid'-mediated gene editing, although it requires maintenance of haploid ESC in culture, can produce gene modifications in cultured cells in vitro and select haploid cells with the

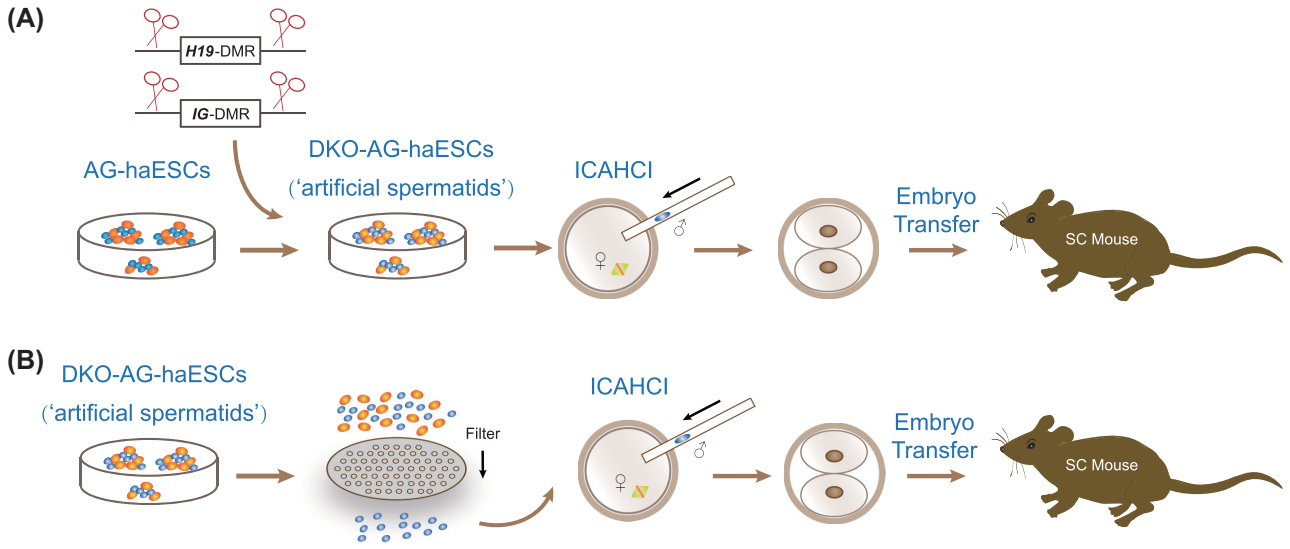

Figure 3. Refinements of haESCs. (A) *H19*-DMR and *IG*-DMR deletions in AG-haESCs markedly improve the birth rate of SC pups to approximately 20%. (B) Physical filtration using commercialized membranes with pore size of 5 or 8 μm enriches haploid cells, and provides a convenient and harmless way to maintain haploidy as an alternative to FACS.

expected genetic traits for ICAHCI and produce SC founders with homogenous genotypes at lower cost than zygote injection. This is particularly true for animals with multiple modifications at DNA loci: genotyping in haploid cells before making animals is easier and relatively lower in cost than sorting through founders derived from zygote injection.

Applications of 'Artificial Spermatids'

Investigating the function of imprinting in embryonic development

Imprinting plays critical roles in biparental embryonic development in mammals through inhibition of uniparental embryonic development [88–90]. Fifteen years ago, the bimaternal mouse was successfully produced by genetically removing *H19*-DMR from a non-growing oocyte before maternal-specific imprinting had been established, followed by fusion with a fully grown oocyte to form diploid embryos [91]. Although the birth rate was very low, this result suggests that genomic imprinting blocks mammalian parthenogenetic development. Consistent with this theory, after removal of *IG*-DMR, non-growing oocytes that lack both *H19*-DMR and *IG*-DMR efficiently supported bimaternal development following fusion with a mature oocyte, and generated live animals at a high rate [92, 93]. PG-haESCs, originally derived from oocytes, maintained the typical pattern of maternal imprinting of oocytes at early passages but gradually lost it during later cell passaging [75, 94–96]. Meanwhile, although PG-haESCs and AG-haESCs are derived from oocytes and sperm respectively, both exhibited a hypomethylation state and similar global expression profiles after long-term in vitro ESC culture [75]. Moreover, the removal of *H19*-DMR/*IG*-DMR in AG-haESCs or non-growing oocytes could result in high-efficiency generation of SC or bimaternal mice, respectively [14, 92]. Based on these observations, we and others proposed that deleting *H19*-DMR and *IG*-DMR in PG-haESCs may also lead to efficient production of bimaternal mice. As expected, PG-haESCs with *H19*-DMR and *IG*-DMR deletions gained the ability to efficiently support the full-term embryonic development after injection into oocytes, with a striking birth rate comparable to that of round sperm injection (Figure 4A) [75, 94]. Therefore, PG-haESCs with deletions in both *H19* and *IG* DMRs can also be used as 'artificial spermatids'.

Given that the deletion of two paternally imprinted regions (*H19*-DMR and *IG*-DMR) allows PG-haESCs to be a sperm replacement to support the generation of bimaternal mice, we hypothesized that it might also be possible to produce modified AG-haESCs with deletions in certain imprinting regions to replace the oocyte genome and support bipaternal development. To test this, seven maternally imprinted regions (*Nespas, Grb10, Igf2r, Snrpn, Kcnq1, Peg3* and *Gnas*) were deleted in AG-haESCs. AG-haESCs carrying seven mutant imprinted genes (7KO-AG-haESC) were subsequently used as maternal genome donors for successful production of bi-paternal pups through a two-step strategy: the androgenetic diploid ESCs were derived from diploid embryos reconstructed by co-injection of the 7KO-AG-haESC and sperm into enucleated oocytes, and then injected into tetraploid blastocysts to produce all-ESC-derived mice [95]. While this procedure resulted in the birth of two live pups, both died shortly after birth, suggesting that further optimization is required (Figure 4A).

Genetic screens at the organismal level

Genetic screening in mice has been hampered by technical bottlenecks including low-efficiency production of mutant mice and difficulty of large-scale gene targeting. Using the recently developed cutting-edge technologies of 'artificial spermatids' as fertilization agents for reproducible production of genetically modified mice and CRISPR-Cas9 for genome-wide mutagenesis in cells we have established three protocols that can be used to generate mice with different biallelic mutations in a single step (Figure 4B) [14]. In the first strategy (termed 'lenti-sgRNA + Cas9 injection'), lentivirus containing a single guide RNA (sgRNA) library [97] was first infected into 'artificial spermatids' to establish a cell line with constitutive expression of sgRNA, which was then injected into oocytes and followed by Cas9 mRNA injection to produce biallelic mutant mice

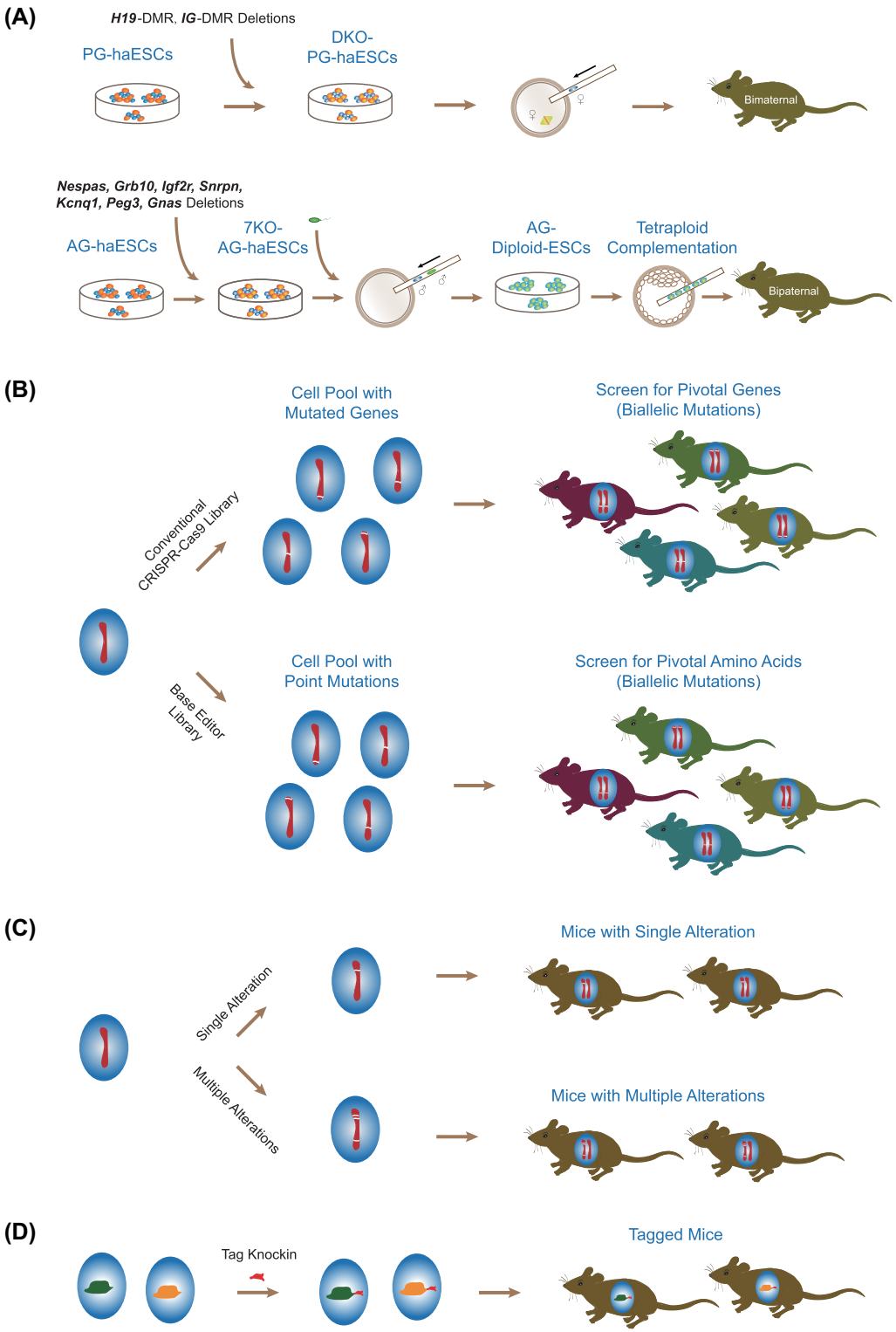

Figure 4. Applications of haESCs. (A) PG-haESCs with *H19*-DMR and *IG*-DMR deletions efficiently support the full-term embryonic development after injection into oocytes. AG-haESCs with *Nespas, Grb10, Igf2r, Snrpn, Kcnq1, Peg3,* and *Gnas* maternal imprinted regions deletions can be used as maternal genome donors for production of bipaternal pups through a two-step strategy: androgenetic diploid ESCs are derived from diploid embryos (reconstructed by co-injection of the 7KO-AG-haESC and sperm into enucleated oocytes) and are then injected into tetraploid blastocysts to produce all-ESC-derived mice. (B) Conventional CRISPR-Cas9 library and base editor library can be introduced into 'artificial spermatids' to obtain haploid cell pools carrying mutated genes and point mutations, respectively. After injection into oocytes, these modified 'artificial spermatids' give rise to collections of mice with different mutations, which can be screened for pivotal genes or amino acids in vivo. (C) Single or multiple genomic alterations can be installed into 'artificial spermatids'. After genotyping and expansion, these modified haploid cells could be injected into oocytes to obtain mouse models that can recapitulate major symptoms of human diseases. (D) CRISPR-Cas9-mediated tagging of protein-coding genes in 'artificial spermatids', followed by ICAHCI, gives rise to tagged mice, which can be used to depict protein-protein or protein-DNA interaction networks in vivo at a large scale using a standard tag antibody.

[14]. Genotyping of the SC pups born showed that 24% carried biallelic mutations. To increase efficiency, in the second strategy (termed 'lenti-sgRNA + pX330 + Cas9 injection') 'artificial spermatids' with constitutively expressed sgRNA were transiently transfected with pX330 plasmids [98] before ICAHCI, followed by Cas9 mRNA injection. With this protocol, 42% of the total born SC pups carried biallelic mutations. In the third strategy (termed 'lenti-Cas9 + lenti-sgRNA'), 'artificial spermatids' with constitutively expressed Cas9 and sgRNA were established and then used as donors for ICAHCI. Although the overall biallelic mutation rate of 30% of total born SC pups was lower than that of the second strategy, the one-step microinjection involved in this strategy could make it a more accessible tool for genetic screening in mice [14].

The recent development of a modified CRISPR-Cas9 technology called base editor (BE) that combines the application of cytidine deaminases and nickase Cas9 (nCas9) or dead Cas9 (dCas9) has enabled targeted point mutation installation or correction in genomic DNA without inducing double stranded DNA cleavage [99–106]. An intriguing application of 'artificial spermatids' combined with BE system is genetic screening for pivotal amino acids of a specific protein at the organismal level. To test this, we transfected 'artificial spermatids' with an enhanced third-generation base editor (BE3) containing two additional nuclear localization sequences and a library of 77 sgRNAs targeting *Dnd1* (essential for primordial germ cell development) to generate a cell line with expressed BE3 and sgRNAs. These cells were used as donors to produce SC pups carrying different point mutations in *Dnd1*. Using this strategy we quickly identified four essential amino acids for DND1 function in vivo: E59, V60, P76, and G82, whose mutation led to primordial germ cell developmental failure. We further performed in vitro structure and biochemistry analyses and found that these amino acids are essential for DND1 stability and protein–protein interactions [16]. This combined application of 'artificial spermatids' and the CRISPR-Cas9-mediated BE system allows for the protein functional analyses in vivo, and shows the potential for quick identification of single-nucleotide variations related to human diseases, especially developmental defects (Figure 4B) [107].

Taken together, 'artificial spermatids' together with CRISPR-Cas9 technologies, provide new options for functional screening at the organismal level in mice [14, 16]. In the future, the 'artificial spermatid'-based screening systems could be further strengthened with the development and involvement of new Cas9 variants to broaden the coverage of targeting sites, reduce off-targeted effects, and improve editing efficiency [107–111].

Modeling human genetic disorders in mice

Genomic alterations, including small indels of bases, copy number variants (CNVs), and structural rearrangements, are closely related to human genetic disorders [112–116]. The basis for all diagnoses (preimplantation, prenatal, or conformational) of genetic disorders is the establishment of a correlation between genotype and phenotype [117]. Recently, the development of high-throughput sequencing techniques has facilitated the discovery of genomic alterations underlying a patient cohort [118]; however, the large amount of data generated from these techniques makes it difficult to identify pathogenic alternations. The dilemma is solved by introducing genomic alterations into mammalian cells (e.g. induced pluripotent stem cells) or animals to test their biological effects at a cellular or organismal level, respectively [112, 119]. Previous studies have demonstrated that desired DNA variations, such as point mutations [15, 16], indels [14], large deletions [81], or DNA fragments [14], could be effectively introduced into 'artificial spermatids' and further transmitted to mouse individuals via ICAHCI. Therefore, it is possible to install genomic alterations discovered in patients into 'artificial spermatids' in a short period, either individually or as a library. Subsequently, the gene modified 'artificial spermatids' could be injected into oocytes to obtain large quantities of SC mice, enabling the evaluation of genomic alterations in mice on a large scale in a limited time. Furthermore, for diseases which are associated with lethality [120–122] or polygenic factors [123, 124], 'artificial spermatids' also have potential for one-step generation of mouse embryos or adults (without laborious breeding and regular genotyping) for disease modeling. 'Artificial spermatids' provide a platform to rapidly evaluate genomic alterations related to human diseases at the organismal level at a large scale and in complex conditions, and generate disease models for subsequent mechanistic investigations (Figure 4C).

In vivo tagging every protein

Illustration of protein in vivo dynamic localization and physical interactions is challenging when a reliable antibody is unavailable. High-throughput functional analyses of mammalian proteins are often cost- and time-prohibitive, even if appropriate antibodies are available. This problem has been solved in yeast by tagging nearly all protein-coding genes at their endogenous genome loci, allowing standard tag-based assays to be used for global protein analysis. To overcome the challenge in mammals, we have recently launched the 'Genome Tagging Project (GTP)' with the goal of labeling every endogenous protein in mice by introducing a tag (HA) into each protein-coding gene. In this way, all tagged proteins can be consistently detected by a standard tag antibody to depict the protein–protein or protein–DNA interaction networks in less and at a reduced cost compared to producing endogenous protein antibodies. To achieve this, a two-step workflow has been established: first an appropriate tag is introduced to a target gene in 'artificial spermatids' by CRISPR-Cas9, and a correctly targeted clone is selected and expanded; the clone is subsequently subjected to ICAHCI to produce a collection of identical descendants, allowing for protein analysis at the organismal level (Figure 4D).

Outlook

'Artificial spermatid'-mediated semi-cloned technology has demonstrated great potential in a wide range of applications in vivo including gene imprinting analyses, genetic screening, disease modeling and protein tagging. In the future, it is intriguing to apply this technology to more fields that include: combining CRISPR-Cas9 activation/repression systems with 'artificial spermatid' technology to activate or repress gene expression in vivo [125–127]; screening in non-coding regions for disease related DNA alterations at the organismal level [128–130]; validation of transgenerational epigenetic inheritance through adding epigenetic marks in 'artificial spermatids' to make mouse models with corresponding epigenetic traits or to test the potential mechanisms underlying epigenetic inheritance [131]; producing humanized SC mice by introducing human DNA fragments into SC mice to facilitate gene regulation study or drug testing [132–135]. It is worth noting that future technical advances in blocking the spontaneous diploidization of 'artificial spermatids', as well as reducing the degree of difficulty of the ICAHCI procedure, may further accelerate the applications of the 'artificial

spermatid'-mediated SC technology. In summary, combined applications of 'artificial spermatid' and CRISPR-Cas9 technologies enable complex gene modifications in mice in one step and greatly enhance functional analyses of genes in vivo.

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