ZOOLOGICAL RESEARCH

Generation of genetically modified mice using CRISPR/Cas9 and haploid embryonic stem cell systems

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ABSTRACT

With the development of high-throughput sequencing technology in the post-genomic era, researchers have concentrated their efforts on elucidating the relationships between genes and their corresponding functions. Recently, important progress has been achieved in the generation of genetically modified mice based on CRISPR/Cas9 and haploid embryonic stem cell (haESC) approaches, which provide new platforms for gene function analysis, human disease modeling, and gene therapy. Here, we review the CRISPR/Cas9 and haESC technology for the generation of genetically modified mice and discuss the key challenges in the application of these approaches.

Keywords: CRISPR/Cas9; Haploid embryonic stem cells; Mouse; Genetic modification

INTRODUCTION

With the development of high-throughput sequencing and biological technology, a growing number of genes related to human disease and development have been mapped. It is therefore important to rapidly and efficiently develop animal models with which to evaluate the functions of these genes. Traditional transgenic animal generation is dependent on embryonic stem cells (ESCs) and homologous recombination techniques (Capecchi, 1989), which yield a higher success efficiency compared to other strategies, including direct viral infection (Jaenisch & Mintz, 1974), pronuclear microinjection of DNA (Palmiter et al., 1982), sperm vector (Maione et al., 1998), and somatic cell nuclear transfer (Schnieke et al., 1997). However, disadvantages, such as low efficiency, time consumption and species limitations, exist in ESC-mediated transgenic animal generation. The emergence of site-specific nucleases has opened new windows for rapid generation of transgenic models via nucleases, including zinc finger nucleases (ZFNs) (Kim et al., 1996; Porteus & Baltimore, 2003), transcription activator-like effector nucleases (TALENs) (Boch et al., 2009; Miller et al., 2011), and clustered regularly interspaced short palindromic repeat (CRISPR) systems (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). Compared with ZFNs and TALENs, CRISPR-mediated genome engineering is easy, efficient, and multiplexable. Moreover, CRISPR can be applied to functionally inactivate and activate genes in cells (Gilbert et al., 2014; Kiani et al., 2015). Importantly, direct injection of the CRISPR system into zygotes is an efficient method for producing genetically modified animals (Hai et al., 2014; Hwang et al., 2013; Li et al., 2013a; Niu et al., 2014; Wang et al., 2013). However, this method can be limited due to mosaic Founder 0 (F0) mice with unexpected genotypes, requiring crossbreeding and genetic transmission for the production of mutant mice with expected genotypes. Recently, haploid ESCs have been successfully generated from mice (Leeb & Wutz, 2011; Yang et al., 2012), rats (Li et al., 2014), monkeys (Yang et al., 2013a), and humans (Sagi et al., 2016; Zhong et al., 2016b) and demonstrated to sustain the ability to generate germline-modified animals via intracytoplasmic haploid androgenetic/parthenogenetic ESC injection into oocytes (Li et al., 2012, 2014; Yang et al., 2012; Zhong et al., 2015; Zhong et al., 2016a). Haploid ESC technology provides a new, convenient and ideal gene transfer vector for generating transgenic animal models because of two reasons.

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First, haploid ESCs possess one copy of each gene, making gene editing easy; second, haploid ESCs can be used as substitutes for sperm to produce healthy mice by injection into oocytes (Bai et al., 2016). In addition, the genetic backgrounds of haploid ESC-mediated transgene animals are evident, thus avoiding the need to crossbreed for the production of mutant mice.

This review describes the progress of germline-modified-animal generation based on CRISPR/Cas9 and haploid ESC systems. We also provide a perspective on future developments of the technology and its applications in biomedical research and clinical studies.

CRISPR/CAS9 SYSTEM

CRISPR is a segment of DNA containing short (20-50 bp) repetitive sequences. It was discovered in 1987 (Ishino et al., 1987) and was redefined in 2002 (Jansen et al., 2002). CRISPR loci are present in the genomes of more than 40% of bacteria and 90% of Archaea. A set of genes named *cas* or CRISPR-associated genes was recently found to be associated with CRISPR (Makarova et al., 2015). The *cas* genes encode putative nuclease or helicase proteins, which are enzymes that can unwind and cut DNA. CRISPR/Cas is currently divided into two major classes and six (I-VI) types, of which type II, consisting of three components, target-specific CRISPR-derived RNA (crRNA), target-independent trans-activating RNA (tracrRNA), and Cas9 nuclease, is the most widely used for genome-engineering applications (Mougiakos et al., 2016). CrRNA guides the cas9 complex to the target sequence, and tracrRNA binds to crRNA and forms a ribonucleoprotein complex with Cas9 nuclease. Cas9 then cleaves chromosomal DNA in a targeted manner, producing site-specific DNA double-strand breaks (DSBs). These DSBs are efficiently repaired in cells by endogenous DNA repair systems known as homologous recombination (HR) or non-homologous end joining (NHEJ) (Rouet et al., 1994; Smih et al., 1995). The former leads to precise gene correction or replacement, whereas the latter leads to insertion/deletion (indel) mutations in targeted sites that frequently result in frame shifts and gene disruption. Essential portions of crRNA and tracrRNA can be linked to form a single-chain guide RNA (sgRNA). The sgRNA base pairs with the DNA target and can be easily programmed to target an 18-25 bp sequence of interest (Mali et al., 2013) (Figure 1). The only constraint is that sgRNA binding sites must be adjacent to a short DNA motif, termed the protospacer adjacent motif (PAM) (Jiang et al., 2013). The PAM sequence is NGG, which can be found, on average, every 8 bp in the human genome. One Cas protein, *Streptococcus pyogenes* Cas9, is widely used in genome editing, including gene mutation, transcriptional regulation, and epigenetic regulation (Sander & Joung, 2014; Wiles et al., 2015).

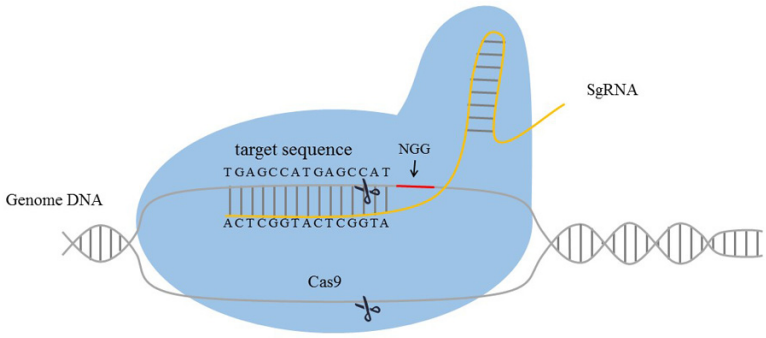

Figure 1 Structure and mechanism of sgRNA/Cas9

SgRNA guides the sgRNA/Cas9 complex to the target DNA. Cas9 then cleaves chromosomal DNA, resulting in site-specific DNA double-strand breaks (DSBs). Specificity is determined by an sgRNA-DNA hybrid and protospacer adjacent motif (PAM).

GERMLINE-MODIFIED MICE GENERATED BY CRISPR/CAS9 SYSTEMS

Gene editing in embryos

CRISPR/Cas9 technology enables easy editing of embryonic genomes and involves three major steps (Figure 2): (1) isolation of zygotes from super-ovulated females, (2) delivery of sgRNA and Cas9 mRNA into the zygote, and (3) subsequent embryo transfer into pseudo-pregnant animals to produce a viable F0 generation. The concentrations of sgRNA and Cas9 are an important factor to consider. High concentrations of sgRNA and Cas9 are toxic to embryos, whereas low concentrations result in low targeting efficiency (Horii & Hatada, 2015). Normally, Cas9 mRNA and sgRNA are used at concentrations of 10-100 ng/μL and 5-50 ng/μL, respectively. In 2013, the first gene knock-out mouse using CRISPR/Cas9 was created in Jaenisch's lab (Wang et al., 2013). The authors injected *Tet1* and *Tet2* sgRNA with Cas9 mRNA into zygotes, resulting in the production of mice that carried up to 80% mutations in both genes (Wang et al., 2013). Shortly after that, another group reported the similar results in mice (Shen et al., 2013). These studies indicate that the CRISPR/Cas9 system is a rapid, convenient, and efficient approach for one-step production of knockout mice, providing a new platform for the generation of transgenic animals. To date, this system has been successfully employed to generate mutant alleles in a variety of organisms, including rats (Li et al., 2013a, b), pigs (Hai et al., 2014), goats

(Ni et al., 2014), rabbits (Honda et al., 2015), dogs (Zou et al., 2015), monkeys (Chen et al., 2015; Kang et al., 2015; Niu et al., 2014), and human embryos (Liang et al., 2015). Usually, Cas9/sgRNA-directed gene knock-out is unexpected, in some cases resulting in non-functional mutations. Specific and precise genome editing, including knock-out, knock-in, and gene repair, can be achieved by co-injection of Cas9 mRNA and sgRNA into zygotes in the presence of a single-strand oligonucleotide template. For example, Yang et al. (2013b) created mice carrying a tag or fluorescent reporter construct in the *nanog*, *sox2*, and *oct4* genes by co-injection into zygotes of Cas9, sgRNA, and corresponding gene DNA vectors.

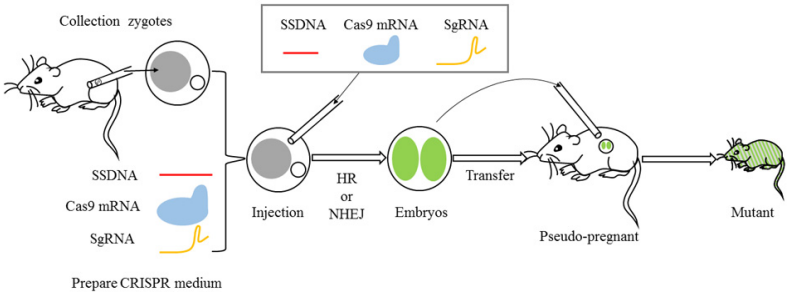

Figure 2 Germline-modified mouse from embryo editing using the CRISPR/Cas9 system

In vitro prepared Cas9 mRNA, sgRNA, and targeting template are microinjected into zygotes from super-ovulated females. The CRISPR/Cas9 system changes targeted DNA in embryos by HR or NHEJ. Developed embryos derived from injected zygotes are implanted into pseudo-pregnant mice to produce a mutant F0 generation.

Structural variations, such as insertion, deletion, duplication, inversion, and translocation of DNA segments, are associated with genetic diseases, including autism, epilepsy, and pancreatitis (Stankiewicz & Lupski, 2010). However, the major problem for genomic editing that involves large DNA fragment insertion, deletion, or inversion is low efficiency, because the larger the fragment, the lower the recombination efficiency (Canver et al., 2014). The Cas9 system, with two sgRNAs targeting different loci in the same chromosome, has been employed to manipulate large genomic DNA fragments with high efficiency. For example, Fujii et al. (2013) generated large-scale (approximately 10 kb) genome-modified mice by injecting two sgRNAs targeting different loci in the same chromosome into zygotes; they achieved 33% deletion efficiency. Furthermore, Zhang et al. (2015) showed that large DNA fragment deletions (21% efficiency) and insertions (11% efficiency), such as entire 65 kb *Dip2a* gene deletions and 5 kb *lacZ* reporter gene insertions, were achieved by the co-injection of two circular plasmids into zygotes using the Cas9 system. Using CRISPR-mediated genome editing of ESCs to generate chimeric mice, two research groups described a distinct mouse model for human limb malformations by using deletions, inversions, and duplications to alter the structure of the *DelB*, *Dbf*, or *Laf4* genes (Kraft et al., 2015; Lupiáñez et al., 2015). Thus, the CRISPR/Cas9 system, together with the use of two sgRNAs, is an easy and efficient approach for large genomic DNA fragment manipulation and can help accelerate the generation of animal modeling to study structural variations in disease and therapy.

One of the most promising applications of CRISPR-mediated genome editing is the correction of genetic mutations associated with hereditary disease. The first example of gene repair using the CRISPR/Cas9 system was reported by Wu et al. (2013). They chose a mouse model of a dominant cataract disorder caused by a *Crygc* gene mutation with a 1 bp deletion in exon 3 that leads to a stop codon at the 76th amino acid and the production of truncated γC-crystallin, resulting in cataracts in both homozygous and heterozygous mice. To correct the mutation of the *Crygc* gene, the authors co-injected Cas9 mRNA and sgRNAs targeting the mutant allele into zygotes, with correction occurring via HR based on the endogenous wild-type allele or exogenously supplied templates (Wu et al., 2013). Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by progressive muscle weakness and shortened life span; no effective treatment is currently available. The molecular basis of DMD is a *C* to *T* point mutation in exon 23 of the dystrophin gene, which leads to the complete lack of skeletal muscle protein. Similarly, the point mutation of DMD can be corrected based on HR repair in the germ line of mdx mice via co-injection of Cas9 mRNA, sgRNA-DMD, and exogenous single-stranded oligonucleotide template into zygotes (Long et al., 2014). These exciting advances indicate that we are not far from the final application of CRISPR/Cas9 to human gene therapy; however, whether Cas9/sgRNA can be used in the therapy of multiple-gene disorders or chromosome structure variations requires further study.

Gene editing in spermatogonial stem cells (SSCs)

Genetic manipulation of spermatogonial stem cells (SSCs) by CRISPR/Cas9 is another approach for efficient generation of germline-modified animals. Wu et al. (2015) successfully used this approach in correcting mouse cataract disease caused by *Crygc* gene mutation. They applied the CRISPR/Cas9 system to repair the endogenous *Crygc* gene in SSCs *in vitro*. The SSCs carrying corrected genes were differentiated into round spermatids after transplantation into mouse testes. The round spermatids were then injected into mature oocytes, and the offspring generated by these preselected SSCs were cataract-free. Genomic manipulation in SSCs with CRISPR/Cas9 has also

been demonstrated in rats (Chapman et al., 2015). These studies demonstrate that the CRISPR/Cas9 system can be successfully applied in SSCs to generate germline-modified animals.

Limitations of CRISPR/Cas9-mediated animal modeling

CRISPR/Cas9 opens a new era for the generation of transgenic animal models and expedites biological research. However, this system brings additional considerations. One challenge with transgenic animal models generated by CRISPR reagents is the production of mosaic F0 mice with unusable genotypes, requiring the screening of a large number of individuals, which is both time-consuming and costly. In addition, there is no guarantee that positive mice will be identified from the first CRISPR microinjection. Off-target effects, resulting in genome instability and gene functional disorder, are another concern. The recent development of haploid ESCs from mice, especially the generation of semi-cloned mice based on haploid ESC-mediated intracytoplasmic injection, provides a more time- and cost-saving approach for the generation of germline-modified mice.

HAPLOID ESCs AND THEIR SCREENING APPLICATION

In diploid cells, heterozygous mutations often result in no phenotypic change because a functional allele on a second chromosome set can mask the effects of disruption of the same allele on the first chromosome set. Thus, genetic analysis in diploid cells is complex. Compared with diploid cells, it is much easier to use haploid cells to produce homozygous mutants for the study of recessive traits (Shi et al., 2012; Bai et al., 2016). However, haploidy is normally restricted to the post-meiotic stages of germ cells and represents the end point of cell proliferation in mammals. The first example of haploid ESCs was established in Medaka fish, which not only maintained haploidy during cell culture *in vitro* but also showed pluripotency like that of conventional ESCs (Yi et al., 2009). In 2011, two groups independently reported on the generation of haploid ESCs from parthenogenetic haploid embryos by means of fluorescence-activated cell sorting (FACS) based on DNA content (Elling et al., 2011; Leeb & Wutz, 2011). Subsequently, androgenetic haploid ESC lines were generated by the removal of the maternal pronucleus from zygotes or the introduction of sperm into enucleated oocytes (Li et al., 2012; Yang et al., 2012). Similar approaches for the generation of haploid ESCs in mice have been extended to rat (Li et al., 2014) and monkey (Yang et al., 2013a) cell model systems. These haploid ESCs from different species contain only one set of chromosomes, show pluripotency as well as self-renewal capabilities. More recently, Sagi et al. (2016) and Zhong et al. (2016b) reported the derivation of human parthenogenetic ESC lines from haploid oocytes. These human haploid ESCs exhibited typical pluripotent stem cell properties as well as usefulness in loss-of-function genetic screening. Furthermore, the human haploid ESCs displayed distinct properties, including differential regulation of X chromosome inactivation and of genes involved in oxidative phosphorylation. Generation of haploid ESCs from mice to humans provides new possibilities in mammalian genetics and a valuable tool for genome exploration.

Haploid ESCs have broad applications in functional genomic studies, such as cell-based reverse and forward genetic screening on the whole-genome scale. Initial reports of haploid mouse ESCs have suggested their usefulness for genetic screening in ricin-mediated toxicity (Elling et al., 2011). In this study, the bioweapon ricin was used to challenge virus-mediated mutagenesis in haploid ESCs at a lethal dose, with the emergence of ricin-resistant ESC colonies from mutagenized haploid cells, indicating that the genes mutated in these colonies were resistant to ricin toxicity. These clones were further pooled and sequenced to determine the integration sites and mutant genes, resulting in the discovery of Gpr107 as a novel molecule essential for ricin-induced cell death. Such research opens the possibility of combining the power of a haploid genome with the pluripotency of ESCs to uncover fundamental biological endpoints, including cell fate signals. In a second study, haploid ESCs clearly exhibited the power of recessive genetic screening. A haploid ESC line expressing GFP under the control of the endogenous promoter for Rex1, a known marker of pluripotency, was used to monitor the state of self-renewal. The *piggyback* (PB)-mediated mutated haploid ESCs were then cultured under high differentiation-permissive conditions, and GFP-positive cells were selected for sequencing, resulting in the discovery of new differentiation factors, including the small zinc finger protein Zfp 706 and the RNA-binding protein Pum (Leeb et al., 2014). More recently, a new interspecific hybrid mouse-rat allodiploid ESC created by the fusion of haploid ESCs from two species was employed to screen X inactivation-escaping genes based on allodiploids exhibiting X chromosome properties. The results showed that the mouse X chromosome was specifically inactivated in the mouse-rat allodiploid-derived somatic cells after *in vivo* and *in vitro* differentiation. After systematic analysis of RNA-seq data from different allodiploid ESC-differentiated somatic cells, more than 163 new genes related to X inactivation-escaping genes were screened, and five genes were selected to validate their roles during X inactivation in hybrid mice models carrying SNPs. In addition, allodiploid ESCs were used to screen for genes regulating species-specific pluripotency maintenance, and two regulators, Wnt3a and BMP4, contributing to rat ESC differentiation were identified (Li et al., 2016a).

INTRODUCTION OF GENETICALLY MODIFIED MICE WITH HAPLOID ESCS

Similar to diploid ESCs, haploid ESCs have robust germline competence, enabling the production of transgenic mouse strains from genetically modified haploid ESCs (Leeb et al., 2012). In addition, haploid ESCs have been derived from sperm and oocytes, raising interest in their potential use as gametes supporting embryonic development. Yang et al. (2012) demonstrated this utility by applying intracytoplasmic androgenetic haploid ESC injection technology, similar to artificial fertilization based on intracytoplasmic sperm injection, in which sperm is injected into oocytes. After injection of androgenetic haploid ESCs into metaphase II oocytes and $SrCl_2$ stimulus activation, full-term pups (referred to as semi-

cloned mice) were generated after transferring the embryos to the uterus of pseudo-pregnant mice. This experiment revealed that haploid ESCs with robust proliferation capacity can be used as a substitute for gametes to produce healthy mice. Based on this result, the authors further knocked out "von Willebrand factor C and EGF domain (Vwce)" genes in androgenetic haploid ESCs by homologous recombination, and after medicine selection and PCR genotyping, Vwce-targeted androgenetic haploid ES cells were generated. After injection of the genetically modified haploid ESCs into oocytes, Vwce knock-out mice were obtained (Yang et al., 2012). In another study, Li et al. (2012) demonstrated that androgenetic haploid ESCs can be used as gene delivery vectors to generate GFP transgene mice using a similar strategy. This strategy was extended to rats for the generation of RFP transgenic animals via injection of rat androgenetic haploid ESCs into oocytes (Li et al., 2014). These results demonstrate that androgenetic haploid ESCs are an ideal gene transfer vector for the generation of transgenic animals, providing a more efficient and simple platform for the production of genetically modified animals. One advantage of haploid ESC-mediated transgenic animals is that the genetic background of F0 animals is clear and can be directly used without crossbreeding, thereby saving time, cost, and animals. Another advantage is that haploid ESCs are an ideal tool for studying haploinsufficiency-associated diseases, such as Mendelian susceptibility to mycobacterial disease (MSMD), Ehlers-Danlos syndrome, and genetic diseases with mutations in multiple genes.

Abnormal epigenetic expression patterns in cultured haploid ESCs result in very low birth rates (4.5%) of semi-cloned pups (Yang et al., 2012), which limits the practical use of haploid ESC-mediated transgenic animals. To improve the birth rate, differentially methylated regions (DMRs) controlling two paternally repressed imprinted genes, *H19* and *Gtl2*, were removed from androgenetic haploid ESCs, and the birth efficiency of semi-cloned mice increased significantly, from 4.5% to 20% (Zhong et al., 2015). The successful increase in the birth rate with genetically modified androgenetic haploid ESCs further raises the question as to whether similar results can be achieved in parthenogenetic haploid ESCs. Surprisingly, by removal of DMRs of *H19* and *Gtl2*, parthenogenetic haploid ESCs can efficiently produce the transgenic semi-cloned pups, although wild-type parthenogenetic haploid ESCs fail to support embryonic development (Li et al., 2016b; Zhong et al., 2016a). These findings help to accelerate the generation of genetically modified mice based on the haploid ESC approach.

CRISPR/CAS9 AND HAPLOID ESCS TO GENERATE MULTIPLE GENETICALLY MODIFIED MICE

The major advantage of using haploid cells is the ease of genetic editing. Recently, CRISPR/Cas9 was applied in haploid ESCs to generate multiple knockouts and large deletions with high efficiency. For example, co-transfection of haploid ESCs with vectors expressing Cas9 nuclease and sgRNAs targeting the *Tet1*, *Tet2*, and *Tet3* genes resulted in simultaneous disruption of all three genes and corresponding loss-of-function at high frequency (50%) (Horii et al., 2013). Similar efficiency of CRISPR/Cas9-mediated *Tet* genes mutation was achieved in rat haploid ESCs (Li et al., 2013b). In contrast, only 20.8% of triple targeting efficiency (*Tet1*, *Tet2*, and *Tet3*) was achieved in diploid ESCs using the CRISPR/Cas9 system (Wang et al., 2013). Site-specific knock-in haploid ESC lines carrying specific reporters can be produced via CRISPR/Cas9 (Kimura et al., 2015). In addition to knock-in or loss of function, CRISPR/Cas9 has been used to create chromosomal structure variation, including deletion and inversion, after co-transfection of cells with vectors expressing Cas9 and two sgRNAs targeting two loci on the same chromosome (Horii et al., 2013). Initially, PB transposon was used for larger-scale insertional mutagenesis in haploid ESCs (Leeb & Wutz, 2011; Leeb et al., 2014); however, it is very difficult to identify the causal mutations and eliminating unrelated background mutation (Wutz, 2015). Background mutations can arise spontaneously in culture and are not marked by a gene trap insertion. CRISPR/Cas9 is an alternative method to PB in genome-scale mutations in haploid ESCs. To date, genome-scale sgRNA libraries of CRISPR/Cas9 systems for human and mouse cells have been constructed (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014). More recently, sgRNA libraries of CRIPSR-Cas9 were successfully applied to gene mutations at the genome scale (Zhong et al., 2015). Therefore, the use of CRISPR/Cas9 genome engineering of haploid ESCs has great potential for applications that extend beyond the previous mutation strategies.

An important aspect of mouse haploid ESCs is the potential to introduce mutations that have been identified in cell culture-based screens of mice. Zhong et al. (2015) demonstrated that androgenetic haploid ESCs with *H19* and *Gtl2* DMR deletion can be used in combination with CRISPR/Cas9 system to efficiently generate semi-cloned mice with multiple-gene modifications and to screen genes at organismal level (Figure 3). First, they showed that androgenetic haploid ESCs carrying mutant *Tet* family genes or *p53* family genes could efficiently produce semi-cloned mice with corresponding genetic traits. Second, they generated androgenetic haploid ESCs carrying *Tet1-EGFP*, *Tet2-mCherry* and *Tet3-ECFP* knockin alleles, followed by production of semi-cloned mice through injection of haploid cells into oocytes. Finally, they generated androgenetic haploid ESCs with a lentiviral sgRNA library containing 87 897 sgRNAs targeting 19 150 protein-coding genes and a lentiviral Cas9. By injection of haploid cells into oocytes, a total of 224 semi-cloned mice were produced, of which 83 carried biallelic mutations. These data demonstrate that haploid ESCs enable the efficient generation of mutant mice in one step; and together with an sgRNA library, haploid ESCs make it possible for gene function screening in mice.

CONCLUSIONS AND FUTURE DIRECTIONS

Although the first successful application in mammalian cells and animals was reported only three years ago, CRISPR/Cas9 has become a simple and convenient platform for the generation of germline-modified animals and holds considerable therapeutic potential. In addition, CRISPR/Cas9

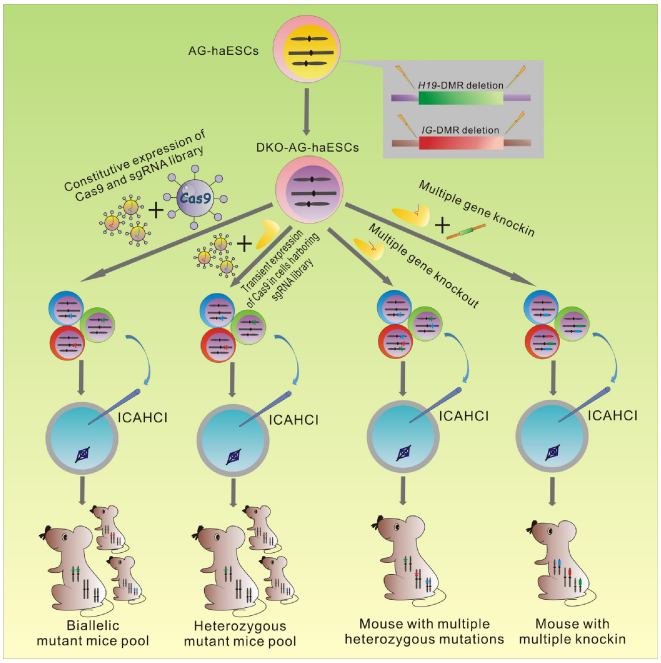

Figure 3 *H19* and *Gtl2* double-knockout androgenetic haploid ESCs (DKO-AG-haESCs) carrying multiple-gene modifications support the efficient generation of different mutant mice in one step (Zhong et al., 2015)

DKO-AG-haESCs are derived from androgenetic haploid ESCs (AG-haESCs), in which differentially methylated regions (DMRs) of *H19* and *Gtl2* are deleted. DKO-AG-haESCs with multiple-gene modifications, including constitutive expression of Cas9 and sgRNA library, transient expression of Cas9 and sgRNA library, or multiple-gene knockout or knockin, generate semi-cloned mice with multiple-gene modifications for genetic screening using intracytoplasmic AG-haESC injection (ICAHCI) technology.

shows a broad-application potential in cancer modeling (Chiou et al., 2015; Matano et al., 2015; Platt et al., 2014; Xue et al., 2014; Zuckermann et al., 2015) and gene therapy (Yin et al., 2014). Cas9 has the capacity to cleave chromosomal DNA. To date, several variants of the Cas9 protein, including dead Cas9 (Gilbert et al., 2014; Konermann et al., 2013; Maeder et al., 2013), sgRNA scaffold (Zalatan et al., 2015), and RNA targeting Cas9 (O'Connell et al., 2014), have been generated after modification of the Cas9 domain. For example, dead Cas9, a mutation of the RuvC and HNH domains of Cas9, has been used both for mechanistic studies into Cas9 DNA interrogative binding and as a general programmable DNA-binding RNA-protein complex (Gilbert et al., 2014), providing a new approach for exploring the diversity of transcripts across complex genomes. The application of Cas9 variants to the production of transgenic animals should greatly expand our understanding of how the gene sets control cell function and fate. Although rapid progress has been achieved in CRISPR/Cas9 systems, some issues remain, including off-target effects and editing efficiency. Off-target genomic editing can cause genome instability and gene functional disorder. Several approaches have been applied to improve target specificity, such as using paired Cas9 nickases instead of Cas9. Paired Cas9 nickases generate paired nicks instead of DSB, which can markedly reduce off-target cleavage (Ran et al., 2013). Editing efficiency is another concern in the CRISPR/Cas9 system; however, improvements in delivery efficiency, target recognition affinity, and nuclease catalytic activity will help to increase editing efficiency (Maruyama et al., 2015; Ramakrishna et al., 2014).

To date, haploid ESCs have been derived from four mammalian species: mouse (Elling et al., 2011; Leeb & Wutz, 2011; Li et al., 2012; Yang et al., 2012), rat (Li et al., 2014), monkey (Yang et al., 2013a), and human (Sagi et al., 2016; Zhong et al., 2016b). Recent reports of transgenic mouse generation based on haploid ESCs have provided a simple and convenient way to analyze gene function and disease modeling. Combining CRISPR/Cas9 and sgRNA libraries, haploid ESCs have produced semi-cloned mice with different mutations, enabling screening to be extended from the cell to mouse in one step. In addition, haploid ESC-mediated semi-cloned mice are an ideal animal model for studying gene dosage effects and haploinsufficiency genetic diseases. This approach enables extrapolation from mouse models to other animal models. In particular, it will be interesting to see whether non-primate haploid ESCs can support the generation of semi-cloned monkeys. Other challenges lie ahead, however, such as the diploidization of haploid ESCs. Thus, methods that increase the stability of haploid genomes in cultured cells are an important future aim. In addition, interspecific hybrid ESCs between mice, rats, monkeys, and humans should accelerate haploid ESC studies for screening differentially expressed genes between species.

In conclusion, given the rapid progress in the past few years, haploid ESCs and the CRISPR/Cas9 systems will make

important contributions to our understanding of the function and regulation of genes through various specific animal models. Distinct advantages and limitations should to be considered, however. CRISPR/Cas9 can be used to easily edit genomes but can also result in genetically mosaic mice with unusable genotypes. The use of haploid ESCs improves this approach and has been successfully applied in genetic screening from cell to organism. Thus, combining haploid ESCs with the CRIPSR-Cas9 approach should accelerate progress in transgenic mouse generation.

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