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Next-generation human genetics for organism-level systems biology

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Systems-biological approaches, such as comprehensive identification and analysis of system components and networks, are necessary to understand design principles of human physiology and pathology. Although reverse genetics using mouse models have been used previously, it is a low throughput method because of the need for repetitive crossing to produce mice having all cells of the body with knock-out or knock-in mutations. Moreover, there are often issues from the interspecific gap between humans and mice. To overcome these problems, high-throughput methods for producing knock-out or knock-in mice are necessary. In this review, we describe 'next-generation' human genetics, which can be defined as high-throughput mammalian genetics without crossing to knock out human-mouse ortholog genes or to knock in genetically humanized mutations.

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Introduction

Systems-biological approaches are necessary to understand complex and dynamic biological phenomena, which occur by the interaction of multiple molecules and cells in mammalian organisms [1,2]. Systems biology consists of a multi-stage process beginning with (1) comprehensive identification and (2) quantitative analysis of individual system components and networks, which leads to the ability to (3) control existing systems toward a desired state, and (4) design new systems based on an understanding of the underlying structural and dynamic principles. Systems-biological approaches have been used in studies to understand phenomena at the molecular-to-cellular level in mammals [3]. However, the application of the same approach toward understanding of organism-level phenomena in mammals has just begun [4,5••], and approaches to understanding human physiology and pathology remain more difficult challenge.

Linkage analysis and genome-wide analysis, such as family-based linkage analysis, Trio linkage analysis, and Genome-Wide Association Study (GWAS), have been useful tools to find genetic variants that correlate with monogenic (or Mendelian) and complex diseases [6,7]. Moreover, in combination with next-generation sequencing techniques, it has been possible to find variants, such as single nucleotide polymorphisms (SNPs), structural variants (SVs), small insertions or deletions (indels), frequencies (common to rare), and regions (coding and non-coding) [6,8]. There are well-established correlations between genes and human diseases, but their causal relationships are still unclear.

Reverse genetics by gene knockout (KO) or knock-in (KI) is a powerful method to clarify the causal relationship between genetic variation and phenotype in organisms [9]. Reverse genetics in mice was established using embryonic stem cells (ESCs) [10–12]. However, the production of genetically modified mice is generally low-throughput and requires tremendous time, space, and effort to obtain mice of sufficient quality and quantity to use for experimental assays. One reason for the low-throughput generation of mutant mice is the low efficiency of homologous recombination. Another reason is the complicated crossing process, which can take several months to years, and repeated back-crosses are needed to obtain complete KO or KI homozygotes in the inbred genetic background. Using these conventional methods, it is difficult to comprehensively identify and analyze molecular properties, networks, and cellular circuits of complex and dynamic biological processes in an organism. For this reason, 'next-generation' mammalian genetics, which can be defined as mammalian genetics without crossing, using genome editing and developmental engineering is becoming popular [5••,13].

The mouse is a useful model animal for reverse genetics. However, in some cases, KO mice reproduce only a part of

the human disease phenotype, show a more severe effect than human cases, or have no phenotype at all [14]. Even in these situations, reverse genetics in humans is experimentally and ethically impossible, which makes mice experiments using established genetic techniques a logical alternative. Therefore, the development of genetically humanized mice that closely mimic the human condition [15] is needed. Technologies for the synthesis of genome-sized DNA [16] and artificial chromosome vectors [17] have been developed in recent years, and the production of genetically humanized mice is becoming easier. In this review, we discuss basic concepts and technologies for next-generation human genetics, which can be used to generate KO or KI mice without crossing.

Conventional gene-targeted mouse production

To produce KO or KI mice by conventional methods (Figure 1a), a mutation is first introduced into ESCs using spontaneous homologous recombination, which usually has a relatively low frequency. Next, the KO-ESCs or KI-ESCs are injected into wild-type blastocysts to produce chimeric mice (F0) containing some cells derived from the introduced KO-ESCs or KI-ESCs. If the introduced KO-ESCs or KI-ESCs contribute to the germline of the chimeric mice, heterozygous mutant mice can be produced in the next generation progeny (F1). Therefore, in principle, homozygous mutant mice can be found in the F2 generation obtained about nine months after ESC injection. However, in practice, because of the low efficiency of homologous recombination, a small contribution rate of ESCs to the germline, and a low efficiency of mating with inbred strains or among F1 and F2 mice, there are difficulties and delays in obtaining the desired genetic background for reliable analysis of the phenotype. Therefore, the conventional method requires an extended period of time (several months to years), large space, and huge labor. International efforts, such as the International Knockout Mouse Consortium and International Mouse Expression Consortium, perform part of this labor for genome-wide production of mutant mice [18]. However, for organism-level systems biology, the establishment of next-generation mouse genetics without crossing, which can reduce the labor to a scale that can be executed by a single laboratory or even an individual researcher, is necessary.

Production of KO mice of a human-mouse ortholog gene without crossing

Production of KO mice of a human-mouse ortholog gene is the most straightforward approach in reverse genetics to investigate the function of a human gene. Genome editing using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a widely used technology for gene KO [19]. Introduction of Cas9 mRNA and one sgRNA into a zygote can quickly and efficiently generate mosaic mice containing KO cells. Microinjection or electroporation of ribonucleoprotein (RNP: a complex of synthesized crRNA, tracrRNA, and recombinant Cas9 protein) into the zygote, especially at the early pronucleus stage considerably increases the proportion of non-mosaic variants [20,21]. Thus, a more efficient KO method is needed to obtain whole-body bi-allelic KO mice without crossing.

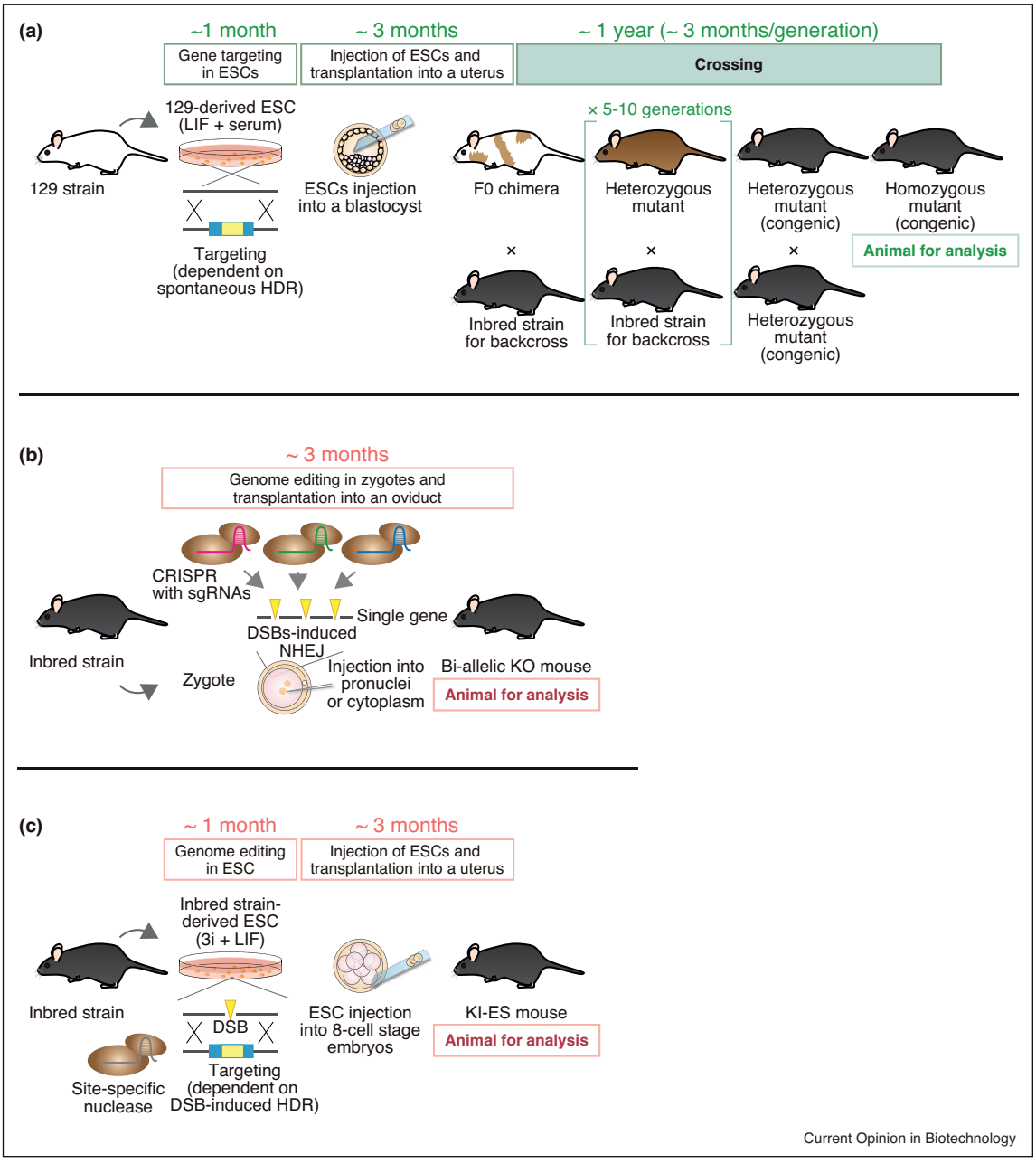
A recently developed triple-target CRISPR (Triple-CRISPR) method [22] using three kinds of sgRNA simultaneously targeting one gene, can generate whole-body bi-allelic KO mice with almost 100% probability (Figures 1b, 2b, and 2d). In the case of tyrosinase gene KO, efficiency of obtaining complete bi-allelic KO was >95% by using three sgRNAs simultaneously, compared with the low efficiency of ~50% using single sgRNA. Moreover, the high efficiency was reproducible by using other sets of three sgRNAs [22]. Exome sequencing results for both tyrosinase and Nr3a KO animals revealed that there is no apparent sign of off-target cleavages [22], possibly because sgRNAs are designed to minimize the off-target effect and Cas9 cleavage is only transiently active around one to four cell stage due to the usage of Cas9 mRNA. Furthermore, by utilizing two sets of independent sgRNA trios for KO animal phenoscreening, the possibility of off-target effects was eliminated [22]. The efficient identification of critical genes for NREM sleep [22–24] and essential genes for REM sleep [25] by the Triple-CRISPR system demonstrated the efficacy of this method. An open database of the set of triple-sgRNA targets (http://crispr.riken.jp/) covering approximately 80% of all the genes in the mouse is a useful tool for comprehensive identification and analysis of components of a system.

Unlike the Triple-CRISPR method to introduce the CRISPR/Cas9 system into a zygote, an *in vivo* (in oviduct) electroporation (i-GONAD) method [26••] does not require isolation of zygotes from donor mice and transplantation into recipient mice. Therefore, a combination of the Triple-CRISPR and i-GONAD methods may be able to further reduce labor and the number of mice needed for the production of the desired KO mice.

Production of KI mice of a human-mouse ortholog gene without crossing

To reproduce human SNPs and SVs in a mouse, various genome-editing methods have been used on the zygote. For the introduction of one SNP, the use of base editors [27–29] may be useful because direct editing of the base without a DNA double-strand break avoids generation of unintended indels. Base editing in mouse embryos [30] and human embryos [31] has been successful. However, for reproducing several SNPs within a narrow range of several tens of bases, a KI method using single stranded oligodeoxynucleotides (ssODNs) as donor DNA in genome-editing tools, such as CRISPR/Cas9, TALEN,

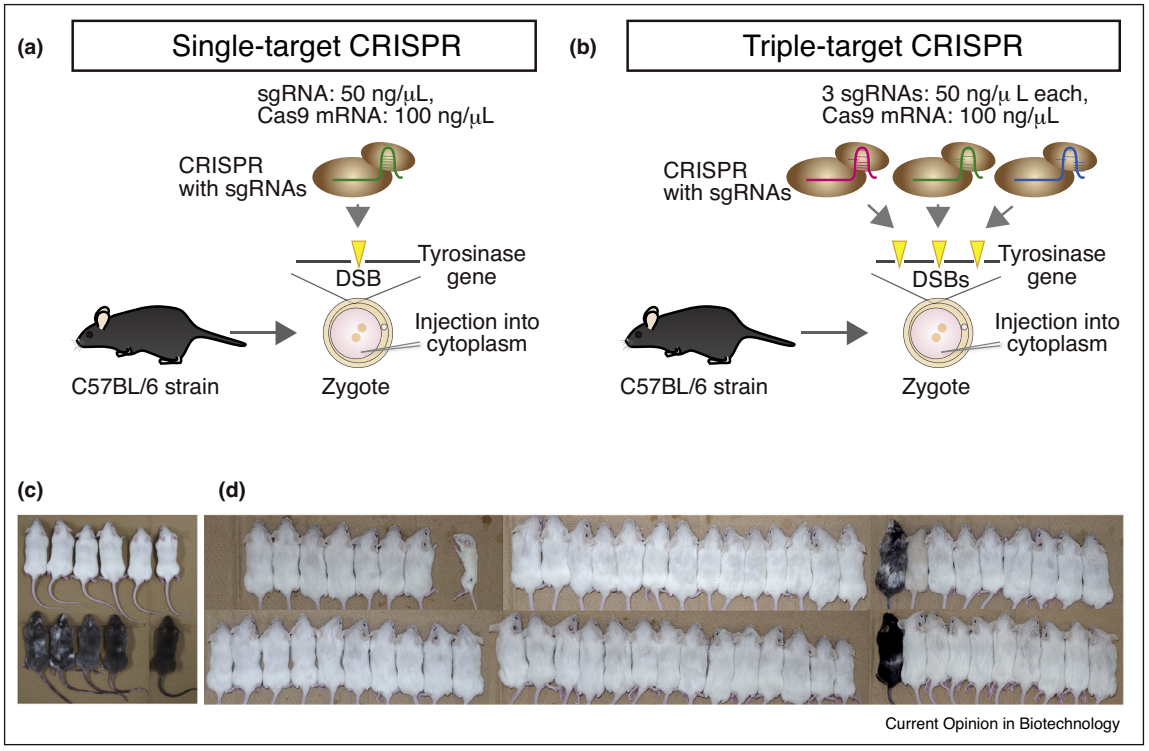
Figure 1



Conventional and next-generation methods for mutant mouse production.
(a) A typical procedure for conventional targeted KO or KI mouse production. An inbred strain such as C57BL/6 is widely used for analysis. However, hybrid or other inbred strains are used in the production stages for practical reasons. Therefore, in many cases, backcrossing is repeated to generate congenic mice. In addition, inefficient gene targeting depending on spontaneous homologous recombination in ESCs increases time and labor. **(b)** Next-generation KO mouse production and **(c)** KI mouse production. The use of inbred strain-derived zygotes or ESCs, efficient genome editing in zygotes, and one-step production of whole body KI-ES mouse by 8-cell-stage embryo injection eliminate all difficulties associated with crossing procedures. These F0 animals can be used in subsequent phenotyping experiments. In addition, efficient gene targeting using site-specific endonucleases in ESCs eliminates unnecessary labor.

and zinc-finger nuclease (ZFN), has been the most used option [32]. To introduce multiple SNPs into a broad range of the genome or reproduce SV, insertion of a larger DNA fragment is necessary. Therefore, methods using long ssODN [32], double-stranded DNA [33], or both simultaneously [34] as donor DNA have been used. However, the efficiency of the precise introduction of large fragments by homologous recombination is still not

Figure 2



One-step generation of KO mice.
We designed a tyr gene KO experiment by single-target CRISPR **(a)** and Triple-CRISPR **(b)** in the C57BL/6 strain. Tyr is a gene coding for tyrosinase, which is an enzyme responsible for black coat color. A typical result of the experiment by single-target CRISPR **(c)** and Triple-CRISPR **(d)**. The Triple-CRISPR method efficiently produces 100% bi-allelic KO littermates with white coat color. All animal experiments were approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch, and all animal care was in accordance with the Institutional Guidelines.

high enough to produce KI mouse without crossing. In contrast to the low KI efficiency in the zygote (up to 6.5%), the introduction of DNA into the embryo at the two-cell stage has high KI efficiency (up to 35%) [35]. However, first generation mice obtained by these methods are often mosaic. Thus, applying these methods to the zygote or embryo is not the best solution to produce KI mice without crossing at present.

Genome-editing methods have also been successfully applied to ESCs to generate KI ESCs with high efficiency (almost 90%) [13]. Advanced culture methods of ESCs using three kinds of inhibitors (3i: SU5402 for FGFR, PD184352 for ERK, and CHIR99 021 for GSK3) and leukemia inhibitory factor (LIF) was efficient for establishing and culturing germline-competent ESCs, even if the mouse supplying embryos was the inbred mouse such as BALB/C and C57BL/6 (hereafter denoted as B6) [36]. The stable establishment and maintenance of ESCs derived from B6 in ES mouse production are particularly critical for the next-generation mammalian genetics. Although B6 is the most standard strain in mouse genetics, B6-ESCs have limitations of less efficient chimera formation and germ-line transmission, difficult maintenance, and genomic instability in standard culture conditions. However, the injection of 3i-medium-cultivated KI ESCs into an 8-cell stage embryo was able to directly and efficiently produce fully ESC-derived mice (ES mice) with all the cells of the body carrying KI mutations (contamination of the host embryo cell was ~0.1% or less) even after many passages for the KI process [13,36,37•]. Live-birth rates of the F0 ES mice reached 11–29% (% of ES mice/embryos transferred). Therefore, 30 (the best case) to 100 host embryos were enough for generating a sufficient number (~10) of ES mice for phenotyping. These technologies can produce many KI mice with less labor and time and without the complexities of crossing [13,37•] (Figure 1c).

Compared with zygote and embryo-based methods, the ES-based production of KI mice may allow for more complex genetic engineering, such as introducing SNPs into multiple genes. Furthermore, ESCs allow for ease of preservation, re-examination, and selection of the desired sex. The current mainstream method to produce ES mice is an 8-cell-stage injection method requiring advanced

technology and expensive equipment. However, devices that can generate ES mice easily by aggregation with 8-cell stage embryos were also developed [38•]. These devices may further improve throughput in the future. Thus, KI ES-based production of KI mice without crossing is an effective method for organism-level systems biology to analyze human disease-related genes.

Next-generation mouse genetics with a genetically humanized mouse

Mice that are genetically engineered based on genetic variations in humans can be useful for various research studies, such as the elucidation of human disease mechanisms and the development of therapeutic methods. However, there are only few proteins in which the amino acid sequence is 100% conserved between human and mouse [39]. The differences in the sequences of most orthologs may lead to differences in the expected phenotypes. Indeed, the phenotype observed in KO mice is often not similar to that in humans [14]. Furthermore, some reports have suggested that a gene unique to humans is involved in human diseases, such as psychiatric disorders [40,41]. In these cases, reproducing the phenotype in mice may not be possible because no counterpart gene is present. Therefore, it may be crucial to make genetically humanized mice [15] in which the mouse genes or systems are replaced by the human genes or systems (Figure 3).

Because genetic variations in humans occur not only in the coding sequence but also in the non-coding sequence, the DNA fragments introduced into the mouse for humanization can be extremely long. Recently, de novo synthesis of chromosomal-level long DNA has become feasible [16]. Advanced synthetic biological technology may be useful for the preparation of long DNA that is responsible for a set of gene locus involved in any human type system or quantitative trait loci (QTL).

Classically, artificial vectors, such as YAC/BAC/PAC, have been used for preparing and transferring extremely long DNA fragments [42]. Random or site-specific insertion into the zygote of long-DNA fragments necessary for humanization may produce mice in a short period compared with insertion into ESCs. However, generation of mosaics may be a problem. Moreover, random integration of genes may occasionally destroy endogenous genes, which complicates phenotypic analysis.

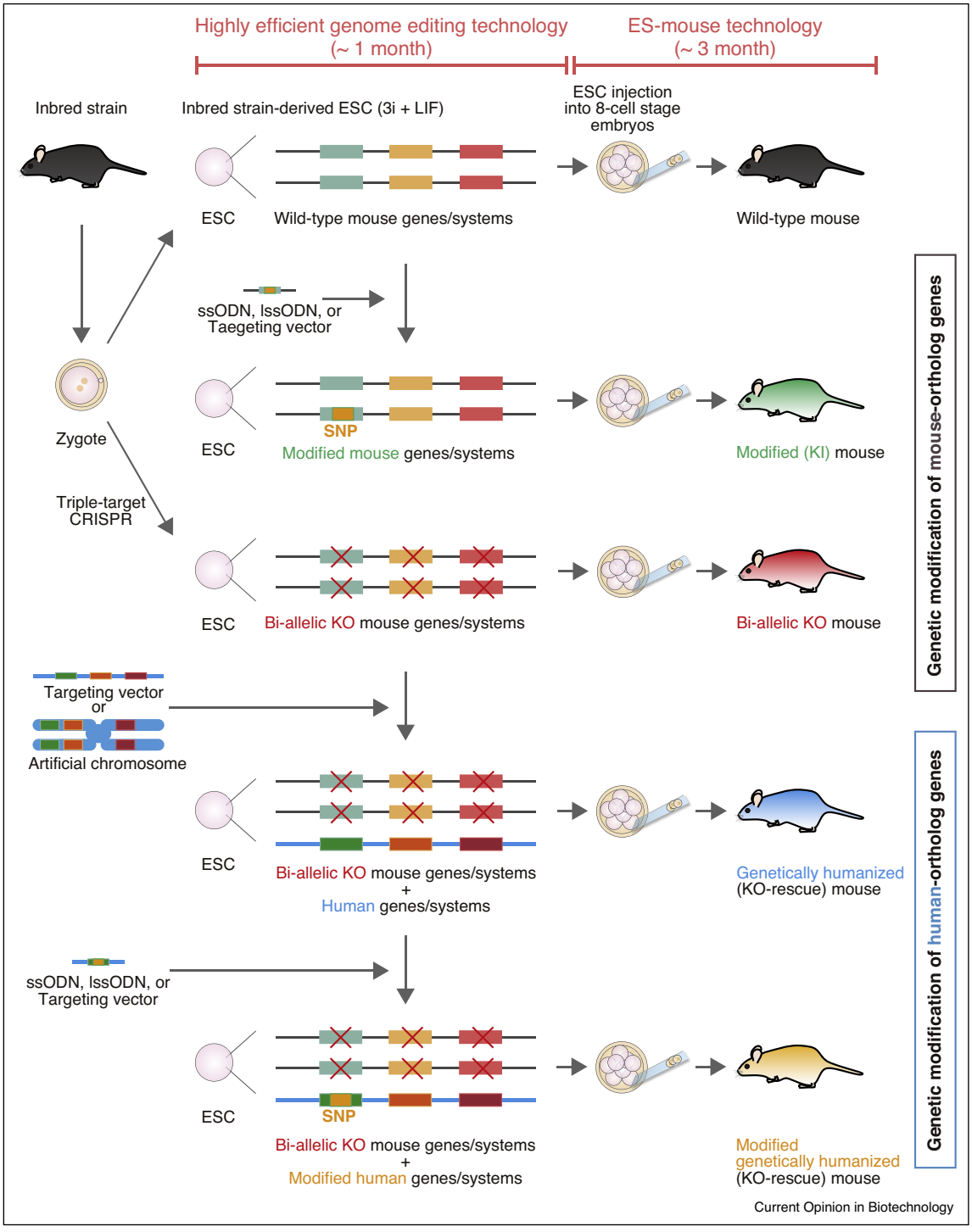
Another approach for humanization is the use of artificial chromosomes, such as HAC/MAC [17], which does not require insertion into a host chromosome. Humanized drug-metabolizing model mice were produced using artificial chromosome technology [43]. However, artificial chromosomes often fall out during the crossing process of chimeric mice produced by conventional methods. Thus, stable production of mice containing artificial chromosomes has been difficult. To overcome this problem, a method using germline stem cells (GSC) was developed [44••]. MAC-containing GSCs microinjected into the seminiferous tubules of infertile recipients successfully produced mice containing MAC in cells in the whole body without crossing. This method is suitable for the production of mice containing human-specific genes.

In contrast, to substitute some mouse genes or systems on the host chromosomes with human genes or systems on the artificial chromosome, a KO-rescue approach [37•] combined with a bi-allelic KO of the corresponding mouse gene is necessary (Figure 3). However, a GSC-based method alone cannot accomplish this. Furthermore, a single phenotype in humans can be the result of the interaction of multiple genes [45], which suggests that the simultaneous KO of multiple mouse genes may be necessary for humanization of the system. These highlight the many difficulties involved in replacing mouse genes or systems with that of a human at GSC and zygote-levels.

Multiple rounds of genome editing are possible in one ESC clone [46], such as numerous KOs of related mouse orthologs or further modification of introduced human genes [47•] after the introduction of an artificial chromosome carrying human genes or systems (Figure 3). In addition, using a method for mouse production without crossing, such as the ES-mouse method, there may be no need to worry about loss of the HAC or MAC during crossing. Thus, production of a genetically humanized mouse by combining chromosome-level DNA synthesis and manipulation technology, highly efficient genome editing techniques, and ES-mouse production without crossing is a useful technique for next-generation human genetics.

In contrast, researchers should carefully consider to what extent potential mosaicism (e.g. mutational variations in the triple-CRISPR method, or undetectable contamination of wild-type cells in the ES mouse method) would affect the final results of a scientific study. In our previous experiments, the phenotypic variations of F0 mice were comparable with suitable control animals, suggesting that mutational variation/unwanted contamination of wild-type cells do not seem problematic at lease in these cases [22,37•]. Even if an unfortunate unwanted mutational variation, including the very rare off-target effect, is transmitted to the germline, such variation would not significantly affect the phenotyping under the concept of 'without crossing.' To further exclude the possibility of artifact phenotypes due to mosaicism, we recommend the researchers to independently generate the KO/KI mice by using the second set of triple-CRISPR for the same gene, or by using an independent clone of ES cells. Realizing such stringent criteria is feasible in the next-generation genetics because it only takes a few months.

Figure 3



Next-generation genetics to generate genome-edited humanized mouse.
ES-mouse technology can generate KI mice with modified (SNP/CNV introduced) mouse genes without mating. Introduction of human-derived genes/systems into bi-allelic KO ESCs prepared using Triple-CRISPR technology by KI or artificial chromosome-mediated transfer can produce genetically humanized ESCs. Moreover, further modifications (introduction of SNP/CNV) in humanized ESCs are possible. ES-mouse technology can also make genetically humanized mice from humanized ESCs without mating. Different colors of mice indicate that each mouse is of a different genotype.

The next-generation genetics also contributes to 'Reduction' of 3R. In the next-generation methods, only the F0 littermates of non-KO or non-ES mice are sacrificed, and no further animal is needed. In the conventional method, dozens of littermates are produced and sacrificed during the crossing procedure to select mice with an expected genotype. The number exponentially increases when a more complicated genetic background (e.g. reproducing several SNPs) is needed in the conventional method, while the number of used animals is not dependent on the genetic complexity in the next-generation genetics. Thus, the next-generation techniques reduce the overall animal usage by the biomedical research community.

Conclusions

Comprehensive identification and analysis of system components and networks are necessary to understand the design principles of complex and dynamic biological phenomena in organisms. 'Next-generation' mammalian genetics for the direct production of KO or KI mice from zygotes or ESCs without crossing, such as Triple-CRISPR and ES-mouse methods, will facilitate the identification and the analysis of molecular networks and cellular circuits in organisms. Compared to the Triple-CRISPR method to produce whole-body KO mice from zygotes, the ES-mouse method has lower throughput. Therefore, additional development of technologies based on the concept of the next-generation genetics is needed to improve the throughput of the ES-mouse method of KI mice production.

Organism-level systems biology is coming to fruition with the development of next-generation methods for mouse production, genomics, and phenotype analysis. The discovery of disease-correlated genetic variants by GWAS and whole exome sequencing following the development of next-generation sequencing enables comprehensive identification of system components. In addition, improvements in whole-body clearing and imaging methods with single-cell resolution provide comprehensive and quantitative experimental data at cellular resolution on an organism scale [4,48,49]. Furthermore, the development of a high-throughput and non-invasive method for phenotyping [22] will also be an attractive direction. Organism-level systems biology based on these technologies will accelerate our understanding of complex and dynamic molecular and cellular circuits in humans.

Conflict of interest statement

Nothing declared.

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