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REVIEW

Genetically modified mice as a tool for the study of human diseases

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Abstract

Modeling a human disease is an essential part of biomedical research. The recent advances in the field of molecular genetics made it possible to obtain genetically modified animals for the study of various diseases. Not only monogenic disorders but also chromosomal and multifactorial disorders can be mimicked in lab animals due to genetic modification. Even human infectious diseases can be studied in genetically modified animals. An animal model of a disease enables the tracking of its pathogenesis and, more importantly, to test new therapies. In the first part of this paper, we review the most common DNA modification technologies and provide key ideas on specific technology choices according to the task at hand. In the second part, we focus on the application of genetically modified mice in studying human diseases.

Keywords Transgenic animals · Genetic modification · Genome editing · Animal models · Disease models · CRISPR/Cas9 · Animal biotechnology

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
AAV	Adeno-associated virus
BMMC	Bone marrow mononuclear cell
Cu/Zn-SOD	Cu/Zn superoxide dismutase-1
DMD	Duchenne muscular dystrophy
DSB	Double-strand breaks
ESC	Embryonic stem cells
FUS	Fused in sarcoma
GM	Gene modified
GWAS	Genome-wide association studies
HBV	Hepatitis B virus
HDL	High-density lipoprotein
LDLR	Low-density lipoprotein receptor
HDR	Homology directed repair
HLA	Human leukocyte antigens
HPRT	Hypoxanthine phosphoribosyl transferase
HPV	Human papillomaviruses
LNS	Lesch–Nyhan syndrome
KI	Knock-in
KO	Knock-out
NHEJ	Non-homologous end joining
NOD	Non-obese diabetes
sgRNA	Single guide RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
ORFs	Open reading frames
PV	Poliovirus
PVR	Poliovirus receptor
RA	Rheumatoid arthritis

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SLE Systemic lupus erythematosus
SMA Spinal muscular atrophy

Introduction

Presently, many human diseases, both monogenic and multifactorial, are not amenable to treatment. Effective models of various diseases are needed for fundamental studies of their pathogenesis and the search for therapeutic approaches. The simulation of various pathological conditions in animals is gaining momentum in the scientific community. As technology develops, more and more new tools appear to solve these problems. In 2020 the Nobel Prize was awarded for the discovery of the CRISPR/Cas9 system, which has greatly simplified precise genome editing. This review includes a description of both classical and modern genome editing technologies and discusses their applicability in the most interesting animal models of human diseases.

Animals are used in the study of human diseases because of their genetic, anatomical, and often physiological similarities to humans [1]. Laboratory mice are easy to handle, their pregnancy lasts for 3 weeks, and they reach sexual maturity at 4–7 weeks old. In addition, the human and the mouse genomes have 80% homology, and some of their coding sequences are 99% identical. Despite big differences in body size and lifespan, together this makes the mouse the most suitable animal for studying human diseases [1].

In early biomedical studies, new mouse models were created by the process of selective breeding, aimed to produce offspring with the desired traits. Nowadays different genomic DNA modifications can be introduced into the animal genome using several technologies. The modifications, introduced into the germ line cells, can be transmitted to offspring and then to the following generations [2]. The term "genetically modified animal" is a broad term that refers to an animal with a deliberate modification of the genome in contrast to spontaneous mutations [3]. The term "genome-edited animal" is narrower and is usually applied to cases when precise editing tools (e.g., CRISPR/Cas9, TALEN, etc.) are used.

Currently, the most used DNA modification strategies are transgenesis (random insertion of linear DNA bearing an expression unit) and more precise approaches based on the CRISPR/Cas9 system: single-gene knockouts, precise editing when one or several nucleotides are altered, and knock-ins (site-specific insertions). The CRISPR/Cas system is now rapidly evolving and new Cas proteins with novel properties are being discovered and engineered. In this review, we are going to use the CRISPR/Cas9 term because the vast majority of genome-edited animals were obtained using this system.

Transcription activator-like effector nucleases (TALEN) system and zinc-finger nucleases (ZFNs) were used for precise genome editing in the recent past, but now they are almost completely superseded by the far more convenient CRISPR/Cas9 system. However, several valuable animal models were created using TALEN and ZFNs. Some models obtained long ago during random large-scale mutagenesis are still in use as well [4, 5].

The components of any of such modification systems can be introduced directly into zygotes or by means of embryonic stem cells (ESC). Direct delivery methods involve DNA/RNA/protein microinjections in the pronuclei or cytoplasm (I) [6, 7], electroporation (II) [8], and even viral delivery (III) (Fig. 1) [9]. Microinjections and electroporation require special equipment and the survival rate of embryos can be limited [8, 10]. There are two principal distinct approaches to embryo electroporation: ex-corporal and in-corporal. In the case of ex-corporal electroporation, embryos are retrieved from mice, placed in a specially constructed chamber that enables the modification of genes, and then electroporation occurs [8]. When in-corporal electroporation (called the i-Gonad method) is used, CRISPR reagents are delivered directly into the pregnant females' oviducts, then special electrodes are placed near the oviducts

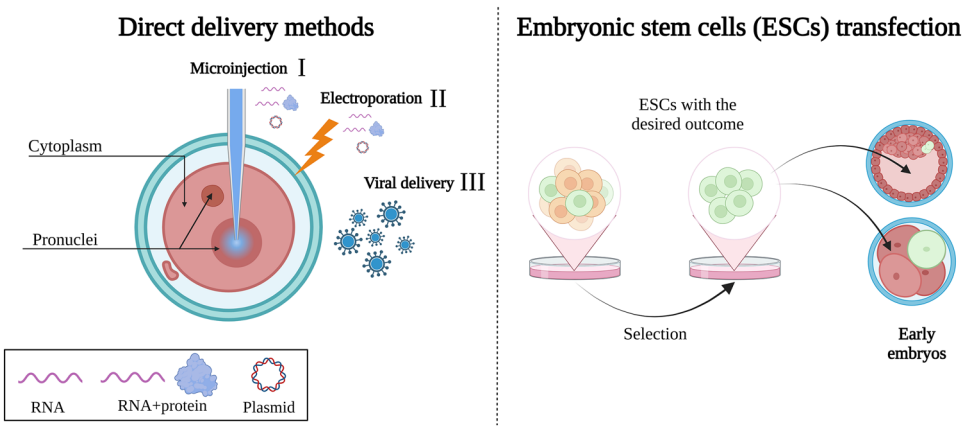

Fig. 1 Scheme of nucleic acid delivery to the mouse embryo

and electroporation occurs in vivo, in oviducts themselves [11–15]. Viral delivery requires additional steps to produce the viruses and to ensure a transient way of transgene expression to avoid off-targets. Manipulations with ESCs, on the other hand, have the advantage of preliminary selection and analysis of cells, after which the cells with the desired genotype can be transferred into the early embryos [16, 17]. Application of the ES cells makes it possible to detect and amplify rarer outcomes, however, direct methods are easier and cheaper.

It should be taken into account that the application of the ESC-transfer method or direct methods of delivery into the zygote as a rule leads to the birth of pups with different cells that have different genomes. Thus, in the case of direct methods of delivery, it is explained due to a phenomenon called genetic mosaicism. In ideal conditions, zygote genome editing should occur at the 2n2c stage, resulting in two alleles. However, DNA replication occurs soon after fertilization, before pronuclei fusion, transitioning to the 2n4c stage where genome editing can result in more than two alleles. To reduce the level of mosaicism, a microinjection of a genetic substance may be performed at the early zygote stage or directly into the oocyte before fertilization [18].

When the ESC-transfer method is used, typically chimerism is observed. Pups developed from the embryos that were injected with ES cells [19] or which were aggregated with ES cells [20] contain two subpopulations of cells, those that evolved from ES cells and those that evolved from initial embryos' cells. To reduce the level of chimerism injection of ES cells could be performed into the tetraploid blastocyst, which is typically obtained by electrofusion of the embryo at the 2-cell stage. Tetraploid cells of the blastocyst predominantly develop into the trophectoderm cells which take place in implantation but not in embryonic tissue formation, and injected diploid ES cells are mainly involved in the formation of embryonic tissues [21].

It should be taken into account that due to mosaicism or chimerism transmission of target mutation in the germline of F0 mouse is a probabilistic process, therefore multiple crossings may be required to obtain a completely transgenic F1 animal. In the worst case when the desired mutation did not present in germline due to mosaicism or chimerism it is required to obtain F0 *de novo* (see Table 1)

Genome alteration techniques for certain experiments must be chosen based on the final goal to strike a balance between the efficiency and the precision and the experiment's costs.

In the first part of this review, we are going to briefly discuss molecular biological approaches to genetically modified (GM) animal manufacturing, and in the second part, we will focus on the limitations of genetically modified animals' usage.

Approaches to genome editing

Historically, the first genome modification approach was random mutagenesis using physical or chemical mutagens [30]. Radiation is usually used to create large-scale mutations, translocations, and multiple deletions [5, 30], whereas chemical compounds (e.g., triethylenemelamine, *N*-ethyl-*N*-nitrosourea (ENU)) drive point mutations [4, 31].

Although more precise methods have become popular since that time, random mutagenesis still retains one advantage: it can be used for studying human diseases in screening "from phenotype to genotype" research. Screening of animals exposed to mutagens can be used for identification of those with mutations, presumably associated with certain diseases. For example, large-scale mutagenesis and phenotype screening were applied to identify mutant mice with alterations in the nervous system and behavior [32].

Statistically random mutations (alterations, insertions, and deletions), even small and point mutations, in ORFs (open reading frames) quite often result in the frameshifts. Insertions and deletions can cause frameshifts if the number of inserted/deleted nucleotides is not multiple of three or when they affect splicing sites. Alterations can interrupt splicing sites and lead to the frameshifts this way.

Table 1 Methods of nucleic acid delivery into the embryo

Method	Features	Examples
DNA/RNA microinjections into the zygote	Special equipment and qualified personnel are required. Widely used method	[10, 22, 23]
Ex corporal electroporation	Special equipment is required. Embryo manipulation skills are needed	[8, 24, 25]
iGonad (in corporal electroporation)	Special equipment is required. Personnel with skills in microsurgical procedures and operations on animals are required	[11–13, 15]
Viral delivery	No special equipment or highly qualified personnel are required for NA delivery into the embryo. Additional preparatory steps are required to produce the viruses	[9, 26, 27]
ESC injection into the blastocyst	Special equipment and qualified personnel are required. Additional steps for the preparation and maintenance of ESCs are needed; this method is expensive	[19, 28, 29]
ESC aggregation with embryo	Special equipment and qualified personnel are required. Additional steps for the preparation and maintenance of ESCs are needed; this method is expensive	[20]

Consequently, such mutations most often lead to the loss of gene functions, but sometimes translocations and small mutations can lead to the gain of certain gene functions.

Regardless of the method of mutagenesis, as a first step of classification, all genetically modified animals can be divided into two major groups: animals with the loss of a certain gene function and animals with the gain of a certain gene function.

Loss of function

International Knockout Mouse Consortium (IKMC) comprises the Knockout Mouse Project (KOMP), the European Conditional Mouse Mutagenesis Program (EUCOMM), and several others. Their goal includes producing and subsequent phenotype assessment of several thousands of constitutive and conditional knockout mouse strains [33]. Development of IKMC lasted for several decades and currently, the most popular method for knockout animal generating is the ES-mediated CRISPR/Cas9 system. IMPC (International Mouse Phenotyping Consortium) also provides phenotypic data of knockout mouse strains.

The CRISPR/Cas9 system originates from one of the mechanisms of the prokaryotic adaptive immune system, which was adopted by researchers for gene editing purposes. The topic is currently receiving a great deal of attention in the scientific community [34]. New slightly different variants are being developed to enable more thorough coverage of a wider range of tasks, including epigenome editing [35]. The basic principle is that an exogenous nuclease Cas9 cuts the genome at specific sites indicated by sgRNAs (single guide RNA) to generate a double-strand break [36]. After the cut, the resulting double-strand break is then repaired by one of three general DNA repair pathways: the non-homologous end joining (NHEJ) pathway, the homology-directed repair (HDR) pathway or microhomology-mediated end joining (MMEJ). The NHEJ pathway often leads to several nucleotide insertions or deletions leading to frameshifts and thus can be used for knockout generation. MMEJ can be employed to obtain bigger deletions, however are more often used for precise KI [37] (Fig. 2).

As S. pyogenes Cas9 making blunt-ended double-strand breaks is by a wide margin the most harnessed variant, novel variants can be used where SpCas9 drawbacks become critical. First, mutant variants and variants from another species can have different PAMs, which makes it easier to target a specific locus [38, 39]. Another drawback of SpCas9 is its relatively big size (more than 4000 bp and 1300aa). *S. aureus* Cas9 [39] and Cas12 (Cpf1) are about one-third smaller (about 1000aa), and this makes a difference, for example, for AAV packaging. Cas12 also has a feature important for GE animal manufacturing—it produces "sticky" ends, which facilitate homology-directed repair instead of non-homologous end joining [40]. Another interesting variant, CasRx, an RNA instead of DNA targeting system, was used to improve HDR efficiency [41].

The efficacy of CRISPR/Cas mediated KO approach is very high and depends mostly on sgRNA selection. The components of the CRISPR/Cas9 system can be injected directly into the zygotes in the RNA/protein form or even as a plasmid intended for transient expression. Also, viral delivery methods can be used. Another interesting approach for gene editing is to execute transgene mice expressing Cas9 [42] and then deliver to the embryos only sgRNA [43].

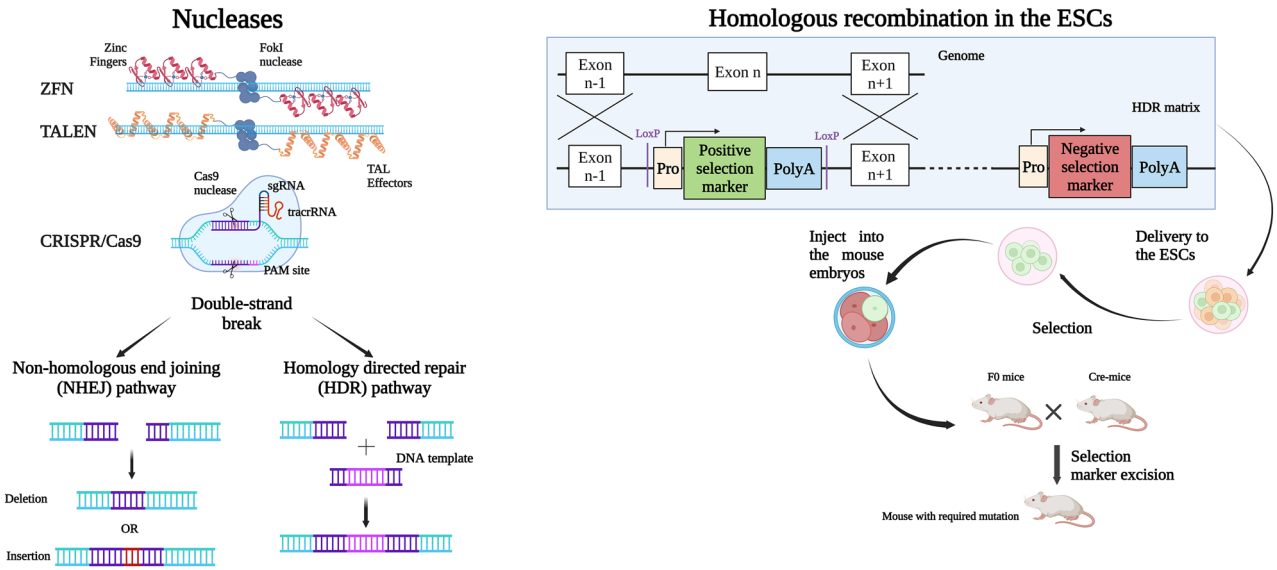

Fig. 2 Loss of function scheme

Before the advance of the CRISPR/Cas9 era, knockouts were often generated in the ESC by gene targeting—an approach based on naturally occurring homologous recombination. There could be some variations, but the general scheme is as follows (see Fig. 2). A genetic structure is made containing two parts of the target locus as homology arms and a selection marker flanked by *LoxP* sites (or *FRT* sites) inserted between them instead of the middle part. The structure was delivered to the ES cells and the cells were subjected to chosen selection. To ensure that the insertion is not random, negative selection can be performed as well. Expression cassettes encoding negative selection markers such as DT-A (diphtheria toxin fragment A) or HSV-TK which is toxic in the presence of ganciclovir can be placed outside the homology arms. The surviving cells then are injected into the mouse embryos or used for aggregation chimeras manufacturing, then embryos are transferred to the foster mothers, resulting in the birth of pups. Obtained mice (F0) are propagated and then crossed with Cre or Flp recombinase-expressing strains. After the selection marker excision by the recombinase, a pure line is bred as homozygous if possible, or as heterozygous if not. However, sometimes crossing with deleter strains is omitted. This approach takes much time and is more expensive than CRISPR/Cas9, but many models in use today were made that way [44, 45].

Although schemes implying homologous recombination are now redundant for simple knockout generation, they are still widely used for so-called conditional knockouts. Knockouts of certain genes can be lethal either at the embryonic stages or even in adult animals. In conditional knockout animals, one of the critical exons is flanked by *LoxP* sites. The exon is considered critical if its length is not divisible by 3 and all the protein isoforms contain this exon [46]. In this case, an inducible and/or tissue-specific Cre-recombinase can produce mouse strains with inducible and/or tissue-specific knockouts. The addition of the CRISPR/Cas9-induced double-strand breaks (DSB) enhances the efficiency of the homologous recombination because these breaks activate DNA damage response pathways, one of which is HDR. In some papers the combination of the CRISPR/Cas9 DSB with the homologous recombination has been shown to enable the production of animals with conditional knockout manipulating zygotes directly, avoiding the need for ES cells [47, 48].

The use of RNA interference may be considered if knockout of a gene is completely impossible even in adult mice or fine-tuning of the gene expression level is required. This mechanism regulates gene expression at the posttranscriptional level and requires short RNA expression. Such expression can be obtained by the random insertion or knock-in of the linear double-stranded DNA expression cassette, containing an RNA promoter, shRNA coding sequence, and a terminator. shRNA (small hairpin RNA) expression can be made inducible with the use of, for example, the TetOn system [49, 50]. The operation of the TetOn/TetOff systems is shown in more detail in Fig. 3.

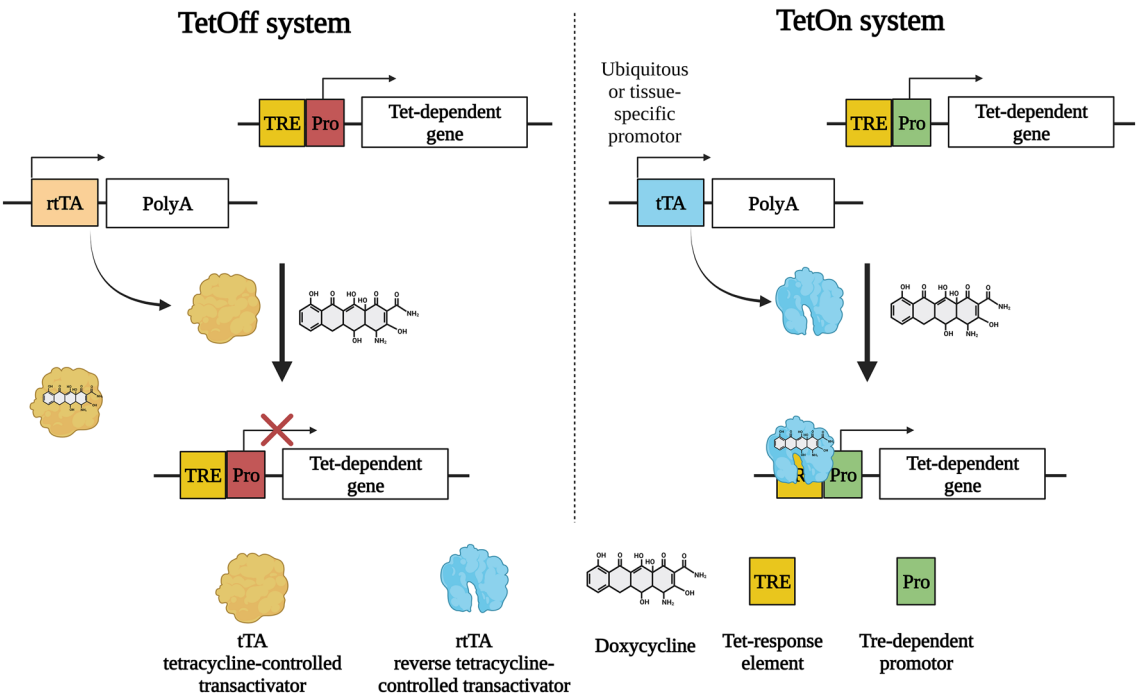

Fig. 3 The operation of the TetOn/TetOff systems

Gain of function

The most obvious example of the gain-of-function genome modification is a case when the whole ORF of the gene of interest with some regulatory elements is randomly inserted into the genome as a linear double-stranded DNA. This modification is often referred to as transgenesis (Fig. 4). The regulatory elements can provide inducible, tissue-specific, or inducible tissue-specific expression [6]. The addition of the insulators, sequences that isolate the insertion from its genomic environment, can help to avoid position effects, i.e., the mutual influence of the transgene and the sequences at the place of its insertion [51]. The addition of the homology arms to the structure and the CRISPR/Cas9 directed cuts in the genome allows insertion of the structure precisely into the selected locus, though it makes the process less effective. Precise transgenesis is often referred to as knock-in (Fig. 4). Surprisingly, there are a few limitations regarding the size of the KI sequence. Sequences up to 200 kb were successfully knocked in using single-strand adaptors [52].

Reproduction of SNPs (single nucleotide polymorphism) and other small mutations is a very important type of animal model used in the study of human diseases [53]. They are often found during Genome-Wide Association Studies (GWAS), but without direct experiments, their role in the etiology and the pathogenesis of certain diseases remains unproven and unclear. Small mutations can be reproduced using the CRISPR/Cas9 system and homologous recombination. This approach is most effective when targeted nucleotides lie very close to the selected PAM site (the element in the sequence required for CRISPR/Cas9 targeting). The desired outcome can be obtained by the microinjections of a synthetic single-stranded oligodeoxynucleotide (ssODN) as an HDR matrix and a plasmid structure transiently expressing Cas9 protein and sgRNA into the zygotes, bypassing the need for ESC. However, for a direct approach, it is important to choose genes which knockout is not lethal, because HDR is less effective than NHEJ [54, 55], and in the majority of the embryo's cells NHEJ would cause different frameshifts and only a small part would contain target mutation. (Fig. 4).

As HDR efficiency in mouse embryos (as well as in other species) is rarely higher than 5% [56], there is a branch of research focused on improving the efficiency of precise KIs. One possible approach lies in the plane of manipulation with DSB repair pathways inhibiting, most often chemically, NHEJ and promoting HDR [57]. Besides chemical inhibition, as RNA can be easily delivered to the embryo along with the components of the editing system, RNA interference-based methods are suitable for gene suppression in embryos (for example, KU70 and KU80 NHEJ proteins). Also, mRNA of the protein promoting HDR (e.g. Rad50 and Rad51) can be delivered this way. These approaches are thoroughly reviewed in [55] and [58]. Interestingly, RNA targeting Cas variant, CasRx was efficiently used to destroy mRNA (*Rad52, Ku70, and Polq*) coding proteins involved in undesired repair pathways to promote HDR [41].

Another approach involves improved design of the template for recombination. For example, it was shown that chemical modifications of ssODN [59], chemical prevention of template concatemerization [60], or biotin-streptavidin linking of Cas protein and DNA template [61] can increase HDR efficiency. Different template designs can

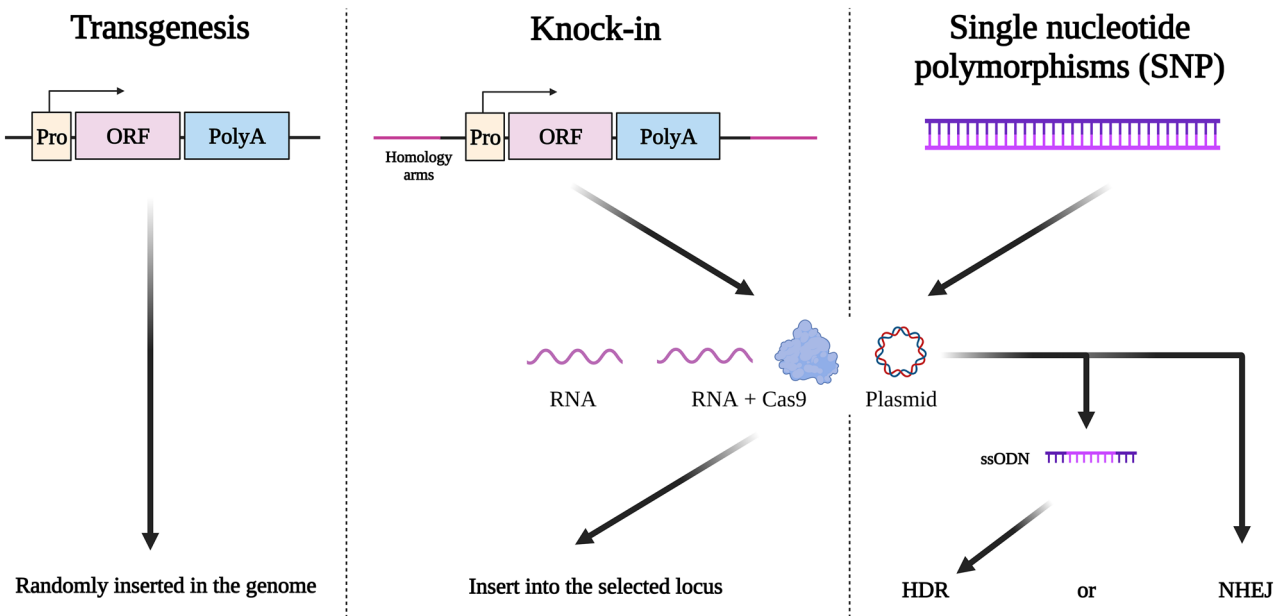

Fig. 4 Gain of function scheme

also be related to employing different DNA repair pathways. MMEJ—a less studied DSB repair pathway, which requires much shorter homology arms (about 20 bp against 800 bp for HDR) (Fig. 5), not only makes template cloning much easier but also increases KI rate in mouse embryos and other clinically relevant cell types [56]. Its characteristic feature is also the design of the template plasmid which includes sgRNA recognition sites at the 5' and 3' ends of homology arms so that circular plasmid is processed to the linear template already in the embryo (Fig. 5) [62]. Homology-mediated end joining (HMEJ) technique combining HDR long homology arms and MMEJ template design appeared to be even more efficient (Fig. 5) [56]. Combi-CRISPR strategy successfully combined HDR and NHEJ options by adding an adjacent intronic sgRNA both to the template and to the editing mix (Fig. 5) [63].

Another potentially important aspect of HDR efficiency is the cell cycle stage and corresponding chromatin state. As HDR is restricted to late S/G2 stages, it was proposed that postponing microinjections from the standard pronucleus stage to the 2-cell stage can favor HDR. This hypothesis was successfully confirmed by Gu et al. [64]

Several diseases are shown to be caused by chromosomal rearrangement. Besides inborn genetic disorders, chromosomal rearrangements play an important role in tumor formation (for example, Philadelphia translocation in leukemias) [65]. These cases can also be regarded as "gain of function" examples. The targeted rearrangement of chromosome regions can be achieved by *LoxP* site insertion in specific positions. In this case, rearrangement can occur upon induction of Cre recombinase [66]. Depending on the *LoxP* orientation, deletions, duplications, and inversions may be produced (reviewed in [67]) (Fig. 6).

Similarly, chromosome rearrangements can be generated using the CRISPR/Cas9 system. Simultaneously cleaved chromosomes can be repaired incorrectly, which leads to the formation of chimeric chromosomes and gene fusions. This approach enabled the reproduction of CD74-ROS1, EML4-ALK, and KIF5B-RET rearrangements occurring in lung cancer in mouse models [68, 69].

Viral delivery

Viral delivery of the components of the genome-editing systems represents an attractive though understudied topic in the field of genome-modified animals. Of all the viral vectors used in laboratories, adeno-associated viral (AAV) vectors seem to be the most promising for the transduction of zygotes. Likely because of their small size (18–26 nm against 80–130 nm for lenti- and retro-viruses, and 70–105 nm for adenoviruses [70]), AAVs, unlike other viral types [71] can penetrate freely through zona pellucida (Fig. 7). The genome of AAVs consists of a single strand DNA molecule, which is probably the best way to deliver the HDR matrix. AAVs do not integrate into the genome and sustain only transient transgene expression, which

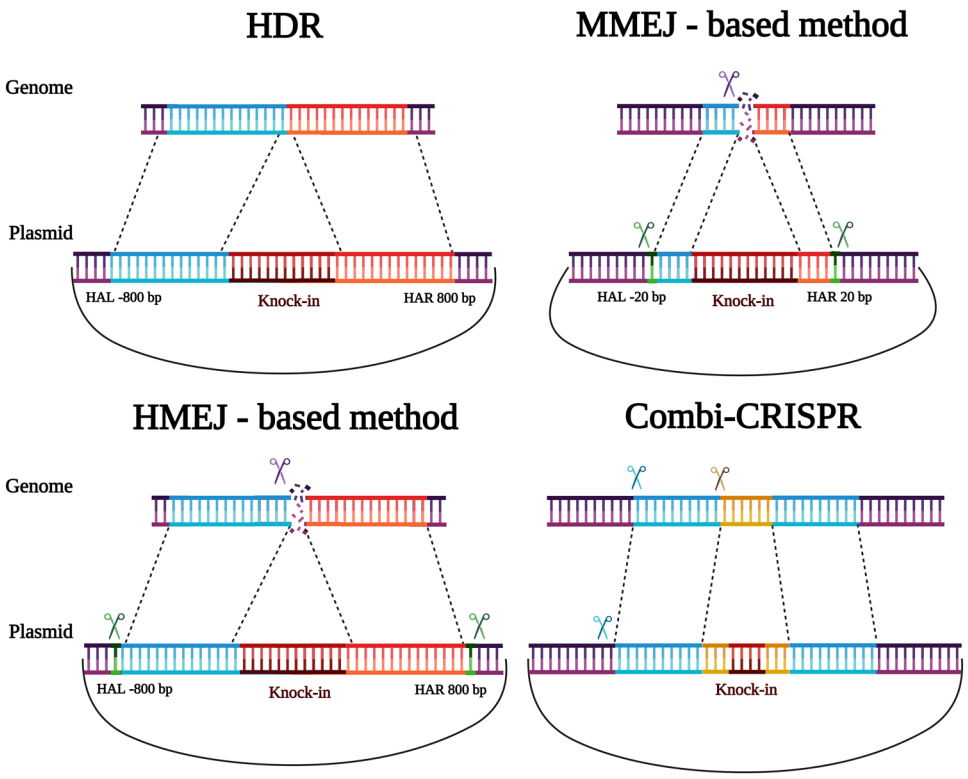

Fig. 5 Advanced KI approaches

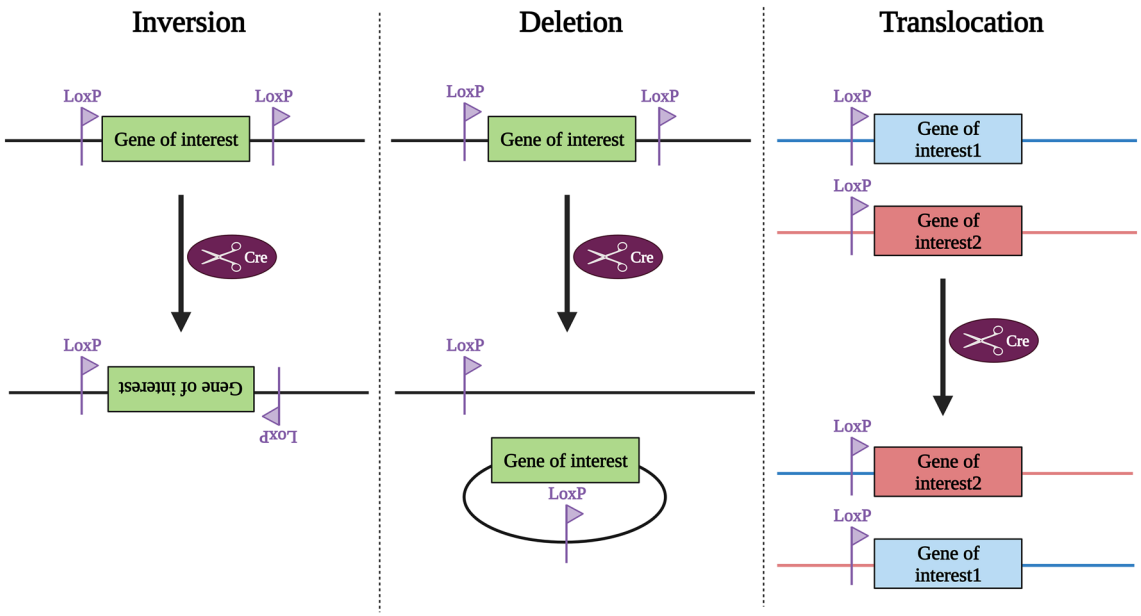

Fig. 6 Changes in the gene of interest that occur depending on the location of the *LoxP*

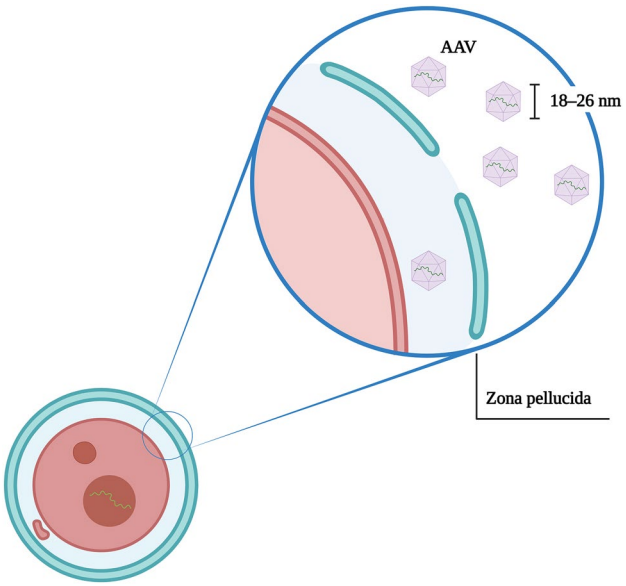

Fig. 7 Viral delivery of nucleic acids using AAV

enables the avoidance of off-target effects and leads to better biosafety characteristics [70].

AAVs were used to deliver HDR matrix into the murine and rat zygotes [27] and successfully transduced bovine zygotes [26, 27]. The main drawback of AAVs—their limited packaging capacity (< 4.5 kb)—makes it difficult to deliver the most common Cas variant—SpCas9—ORF along with the sgRNA expression unit. Nevertheless, newly emerged Cas variants with shorter ORFs solve this problem [70]. Alternatively, genome editing effectors that induce double-strand breaks could be delivered by microinjections or electroporation.

Selection of the optimal way of transgenesis depends on many factors: available equipment, personnel, and the type of required transgene. Thus, getting the knockouts by NHEJ can be achieved easily by the injection of a genetic substance into the cytoplasm or pronuclei, while insertion of long site-specific structures requiring HDR may be difficult; the better way in this case may be production, selection, and further injection of transgene ESC into the blastocysts to obtain chimeras.

Disease models

An animal model of a disease enables the study of its pathogenesis and, more importantly, in some cases, to test new therapies. Several types of disorders, not only monogenic but also chromosomal and multifactorial disorders can be mimicked in lab animals by genetic modification. Even human-specific infectious diseases can be studied in genetically modified animals.

Monogenic disorders are the most natural field for the transgenic animal models application as they are relatively easy to reproduce and the causal relationship between the genotype and the phenotype is obvious. The models representing amyotrophic lateral sclerosis, Duchenne muscular dystrophy, and Lesch-Nyhan disease are of great interest.

Multifactorial disorders such as cancer, cardiovascular diseases, obesity and diabetes, autoimmune diseases, etc. rarely demonstrate Mendelian inheritance but have genetic

components that may be revealed, for example, using GWAS. In this case, animal models can serve as a tool to prove the role of certain genes in the disease's etiology and pathogenesis.

Moreover, although GM models do not ideally mimic corresponding human multifactorial disorders, they can be helpful in preclinical studies of potential therapies.

The most significant models of human diseases performed on mice are given in Table 2.

Concerns

Although many human diseases can be easily reproduced in mice, the application of mice as a model has certain limitations. As has been mentioned above, the human and mouse genomes have 80% homology but 20% are not identical, resulting in different lifespan and body structure. Thus, many age-related human diseases are not observed in mice because the mice's lifespan usually is limited to 1–2 years. Other potential concerns about the application of mice as a models will be considered below.

Genetic background

When planning an experiment for modeling human pathological conditions in mice, it should be considered that the genetic background of the model can affect its phenotypic manifestations [95]. This is especially important for the manifestation of various neurodegenerative diseases' symptoms. It is known that different inbred mice lines are prone to certain pathologies. For example, DBA/2 J(D2) mice are prone to diseases related to nerve cell death, in particular, to glaucoma [96] and hearing loss [97]. As a result, DBA/2 J. APPswePSEN1de9 model of Alzheimer's disease with overexpression of human *PSEN1* and *APP* genes with Alzheimer-related mutations on the DBA/2 genetic background has more pronounced phenotypic manifestations than the same model on the C57BL/6 genetic background [83].

It has also been shown that mice with knockout of the *Cln3* gene encoding a lysosomal protein, a model of the neurodegenerative juvenile Batten disease, have different manifestations on two genetic backgrounds: 129S6/SvEv and C57BL/6 J. It was shown that $Cln3^{-/-}$ has more pronounced symptoms on the 129S6/SvEv genetic background, which makes these mice the most suitable for the development of therapeutic approaches [98].

A similar effect is observed in the myodystrophy mouse model (e.g. MDX): in MDX mice, in which a mutation in the *Dmd* gene results in the premature stop-codon in exon 23, symptoms appear more clearly on the DBA/2 J genetic background than in the same model on the C57Bl/6 line [99].

Mouse models of SMA (spinal muscular atrophy), also demonstrate that the severity of symptoms depends on the genetic background. Mice carrying a mutation in the splicing region of exon 7 of the mouse *Smn* gene ($Smn^{2B/2B}$) were created and then transferred to the C57BL/6(BL6) and FVB genetic backgrounds and crossed with $Smn^{-/-}$. It was shown that $Smn^{2B/-}$ mice on the FVB background have a shorter lifespan than the same model on the C57BL/6 background (median lifespan was 19 days and 25 days, respectively). No difference was found in the level of *Smn* gene expression between these strains [100].

In a model of familial amyotrophic lateral sclerosis (fALS) with the G93A mutation in the *hSOD1* gene, the most severe symptoms develop in ALR, NOD.Rag1KO, SJL, and C3H mice. Less severe phenotypic manifestations are observed in hybrid B6xSJL mice and, finally, the mildest manifestations were observed in the inbred mouse strains B6, B10, BALB/c, and DBA [101].

Comparative analysis of genetic pathologies and their manifestations in different lines of animals will allow us not only to obtain an animal model that is closest in phenotype to human pathology but also to find concomitant genes that affect the severity of the phenotypic manifestation with the same mutation, which will enable full exploration of the pathogenesis and possible therapeutic approaches [102].

Atherosclerosis

Another type of diseases that are difficult to model by creating genetically modified animals is atherosclerosis. Atherosclerosis is a condition when the wall of the artery develops abnormalities, called lesions. These lesions may lead to blood vessel narrowing due to the buildup of atheromatous plaque.

Wild-type mice do not develop atherosclerosis due to their very short lifespan compared to humans. Several genetically modified atherosclerosis mouse strains are used to study the fundamental aspects of atherosclerosis development, but their use as models for the development of therapeutic approaches is not always possible due to the peculiarities of the development of atherosclerotic lesions. The most well-known model of atherosclerosis is the apolipoprotein E gene knockout mouse, $ApoE^{-/-}$ [79, 80], which has a significant increase in plasma cholesterol levels compared to wild-type animals. The formation of atherosclerotic vascular lesions in these mice occurs even on a standard diet. However, it must be considered that the APOE protein is multifunctional and is involved in many processes, such as inflammation, proliferation, and migration of smooth muscle cells. Therefore, *ApoE* knockout can lead to the formation of atheromas unrelated to the lipid profile of blood plasma [103].

Table 2 Basic disease models on mice

Disease	Model mice	Features	References
Amyotrophic lateral sclerosis	SOD1G93A (Gly93- > Ala substitution in Cu/Zn superoxide dismutase-1 (Cu/Zn SOD) gene)	ALS symptoms develop	[72, 73]
	Knockout of Cu/Zn superoxide dismutase-1 (Cu/Zn SOD) gene	No ALS symptoms	[72, 73]
	Humanized FUS, FUS protein is overexpressed in the nervous system after the random insertion	Severe symptoms specific to ALS	[74]
Duchenne muscular dystrophy	C57BL/10ScSn-Dmdmdx/J(BL10-mdx), a nonsense mutation in exon 23 of the *Dmd* gene, which leads to the premature termination of dystrophin protein synthesis	The severity of the ongoing disease is much lower than in humans	[75]
	DMDdel8-34, exons 8–34 deletion in *Dmd* gene	Elevated levels of creatine kinase in serum, decrease in the number of centralized nuclei, decrease in the average Feret diameter, and decrease in the cross-sectional area of myofibrils in muscles. Decreased muscle strength and endurance	[76]
	DMD mouse model lacking exon 51 (ΔEx51 mice)	The absence of dystrophin, replacement of degenerative muscle fibers with inflammatory cells and fibrotic and fatty tissue, increased percentage of centralized nuclei in myofibers	[77, 78]
Atherosclerosis	Apolipoprotein E gene knockout mouse, $ApoE^{-/-}$	Significant increase of cholesterol level in plasma, formation of atherosclerotic vascular lesions even on a standard diet	[79, 80]
	Low-density lipoprotein receptor gene knockout, $Ldlr^{-/-}$	Cholesterol level in the blood plasma is not reduced as effectively as in humans, a characteristic for animals that do not have human-specific complications of atherosclerosis, atherosclerotic plaque rupture, and thrombus formation	[81, 82]
Alzheimer disease	DBA/2 J. APPswePSEN1de9, overexpression of human *PSEN1* and *APP* genes with Alzheimer-related mutations	Premature lethality, plaque deposition	[83]
Diabetes	Disrupting the mRNA splicing in the leptin receptor was shown to cause a diabetes-like condition in mice (db/db mice)		[84, 85]
Cardiovascular diseases	So-called blotchy mice have spontaneous mutation at the *Atp7a* locus which leads to abnormal intestinal copper absorption	Due to impaired interaction between elastin and collagen, they have weak elastic tissue and tend to develop aortic aneurysms	[86]
Immunodeficiency	Scid mice (Severe combined immunodeficiency), a mutation in the *Prkdc* gene	*Prkdc* gene encoding a DNA-dependent protein kinase involved in V (D) J-recombination, and, consequently, maturation of B- and T- cells	[87, 88]
	Nude mice, *Foxn1* gene mutation	Absence of hair, athymic	[89, 90]
Autoimmune diseases	SKG mice expressing a mutant (W163C) variant of the *Zap70* gene, the product of which is normally involved in the T-cell receptor signaling (BALB/c-ZAP-70^{W163C})	Can be used as a model of autoimmune arthritis, spondylitis, and psoriasis, however, not all the human symptoms are observed in these animals	[91]
	K/BxN strain, created based on the KRN strain with a transgenic T-cell receptor recognizing RNase 41–61 peptide from the bovine pancreas in the context of the MHC Ak molecule	When transferred to the NOD genetic background the transgenic T-cell receptor is specific to the autoantigen—glucose-6-phosphate isomerase, which leads to the development of autoimmune pathologies in these animals	[92, 93]
Cancer	Transgenic mice that carried HPV16 (human papillomaviruses) E6 and E7 open reading frames under the control of the human β-actin promoter	The incorporation of two HPV oncogenes leads to the development of several types of cancers in mice (anaplastic neuroepithelial tumor, choroid plexus carcinomas, and pituitary carcinomas)	[94]

Another extremely popular atherosclerosis model is the *Ldlr* gene knockout, which encodes the low-density lipoprotein receptor. LDLR is a glycoprotein on the cell surface of hepatocytes, which plays a key role in the endocytosis of low-density lipoproteins; patients with mutations in this gene develop hereditary hypercholesterolemia with an increased level of low-density lipoproteins in blood plasma and the development of atherosclerotic vascular damage [104].

During the testing of drugs intended for the treatment of atherosclerosis and its consequences on $Ldlr^{-/-}$ mice, it turned out that the level of cholesterol in the blood plasma in these animals is not reduced as effectively as in humans [82], moreover, these animals do not have a human-specific complication of atherosclerosis, atherosclerotic plaque rupture, and thrombus formation [81].

The reasons why mouse models of atherosclerosis do not fully represent the phenotype typical for humans are not well understood. Potentially, it depends on the life span, blood flow, or structure of blood vessels.

Two published studies [105, 106] aimed to evaluate the effects of the human apolipoprotein AI overexpression in db/db mice (db/db mice carry mutation-producing type 2 diabetes [84, 85]). Elevating the APO-AI level or mimicking it with other peptides is one of the modern therapeutic approaches supposed to improve HDL (high-density lipoprotein) atheroprotective functions. The researchers bred transgenic APO-AI mice [107] with the overexpression of the human apolipoprotein *A-I (APOA-I)* gene with db/db mice. Mendez-Lara et al. [105] demonstrated in this research that the overexpression of *APO-AI* in db/db mice enhanced the anti-atherogenic properties of high-density lipoprotein (HDL). However, the overexpression of *APO-AI* also exacerbated weight gain and the fatty liver phenotype in mice; due to these side effects the use of APO-AI-mediated or HDL-based therapies is not recommended in humans suffering from obesity. This study illustrates how experiments with GM animals can help to predict adverse side effects of certain therapies.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a long-term autoimmune disorder that primarily affects joints, typically resulting in warm, swollen, and painful joints.

In the case of rheumatoid arthritis, genetically modified mouse models elucidated different aspects of the pathogenesis such as the roles of CD4 + T− and B-cells, proinflammatory cytokines, and autoantigens. The main problem of such models is that due to the relatively low homology level between animal and human immune targets, most of the potentially effective therapeutics tested in model animals are not effective in humans. To overcome this problem, humanized animal models were proposed (reviewed in [108]). Humanized animal models imply transgenic expression of human molecules, such as HLA class II, RA-associated synovial autoantigens, and/or an autoantigen-specific T cell receptor, in immunocompetent mice. Being a step forward, these models partially share their main drawback with classical models: inflammation is driven by the murine immune system.

Cancer

There are two main distinct purposes for using genetically modified mouse models in cancer research. The first is to identify the genes with sequence alterations or expression level changes between tumor and normal tissue which are responsible for tumor formation, growth, and metastasis. The second is to produce models for preclinical studies of newly emerging therapies.

Knockouts and knock-ins of specific genes are often used to determine their role in cancer. However, constitutive knockouts and knock-ins are often unsuitable because they may be lethal or change the phenotype too much or their effect may be even neutralized by the developmental compensation [109].

The use of highly specific promoters can partially solve this problem. For example, a pancreatic cancer model was obtained by overexpression of the SV40 T antigen under the control of the insulin promoter [110]. But Cre and Tet systems offer a more precise level of control over transgene expression. The APC mice that spontaneously develop colorectal adenomas were obtained by infection of mice harboring floxed 14th exon of the *APC (Adenomatous polyposis coli)* gene with Cre-expressing adenovirus with gastrointestinal tropism [111]. Expression of the *c-Myc* gene under TetOff control demonstrated that activation of the expression leads to the lymphoma formation and the expression switching off leads to its regression [112].

Chromosome disorders

Although homology levels in murine and human genomes are high, multiple genetic differences do not allow extrapolation of data obtained from mice to humans. Thus, the application of mouse models in the investigation of human chromosome aneuploidies is limited because of the divergence between human and mouse genomes. For example, mouse homologs of the human genes from the part of the human chromosome 21 responsible for Down syndrome are divided between mouse chromosomes 10, 16, and 17. The Down syndrome mouse models obtained through microcell-mediated chromosome transfer and similar techniques in embryonic stem cells include *Tc1* (with part of human chromosome 21) [113], T65Dn and Ts1Cje (with the part of mouse

chromosome 16 containing about 75% of the involved genes) and Dp(10)1Yey/+, Dp(16)1Yey/+, Dp(17)1Yey/+(with the parts of mouse chromosomes 10, 16 and 17) (reviewed in [114]). All these models had phenotypes similar but not identical to Down syndrome. At the same time, they made a significant contribution to the study of its pathogenesis and helped to prove that it is the increased gene copy number that causes the phenotype. These models were also used in the works that demonstrated the possibility of the therapy of cognitive disorders in patients with Down syndrome. It is worth noting separately a study [115] that revealed that the *Ets2* gene from chromosome 21 contributed the most to the antitumor protection described for patients with Down syndrome.

In contrast with aneuploidy, mouse models of large deletions (0.5–10 megabase) closely mimic the effects of similar mutations in the human genome. These models greatly contributed to the molecular understanding of such disorders as Prader–Willi syndrome, Angelman syndrome, DiGeorge syndrome, Williams–Beuren syndrome, etc. They helped to identify specific genes responsible for the phenotype and to distinguish between the effects of the copy number alteration and the changes in the genomic environment.

Infectious diseases

Despite significant healthcare progress, viruses remain one of the greatest threats to public health. On the one hand, many viruses like HIV are still lethal and incurable despite being well studied; and on the other hand, novel viruses appear periodically, like the SARS-CoV-2, Zika virus, H1N1 swine flu, etc. Surprisingly, mouse models can be relatively easily generated even for infectious diseases that do not normally affect mice. The cause of such different susceptibility to viruses often lies in small distinctions in surface molecules (receptors), variations in immune response, and other processes important for viral cell cycle progression. This difference can be amended by humanization—transgenesis aimed at the expression of selected human genes. Ideally, this expression must be inducible (not to affect embryonic development) and tissue-specific (to mimic natural viral tropism more accurately) (reviewed in [116]).

Coronaviruses

The global SARS-CoV-2 pandemic that broke out at the end of 2019 challenged the scientific community. The first animal models suitable for SARS-CoV-2 studies had already been developed due to the SARS-CoV-1 outbreak in the early 2000s [117]. In this model, the human ACE2 receptor required for SARS-CoV entry into the cells was expressed under the control of the constitutive CMV promoter [118] or tissue-specific promoters: mouse *Ace2* promoter [119], the *Krt18* promoter [117], *Hfh4/FoxJ1* lung ciliated epithelial promoter [120], or even under the control of an inducible and tissue-specific system [121]. All these mice developed symptoms similar but not identical to COVID-19 in humans. Some of these strains developed encephalitis [122–124]. The symptoms differed much in severity from 100% lethality [123] to slight transient body weight loss [125]. In one of the studies [126], K18hACE2 mice developed such COVID-19 conditions distinctive for humans as anosmia and thrombosis.

Therefore, there are examples of mouse models when overexpression of a single gene is sufficient to make an animal susceptible to disease. However, there is a hypothesis that TMPRSS2 [127] expression is also required for proper SARS-CoV-2 cell entry and more precise modeling.

Poliovirus

In some cases, expression of the viral entry molecules in target tissues is not enough to make mice susceptible to the virus. Thus, an interesting example [128] of a two-step model is the mouse model for oral poliovirus infections. Transgenic mice expressing the human PVR (poliovirus receptor) gene could be infected by PV, but the virus could not replicate in the digestive tract, which is an important feature of the poliovirus. As it was shown that the mouse interferon system prevents replication, knock-out mice without alpha/beta interferon receptors (*Ifnar*) were obtained. In these mice, the virus replicated successfully, but the severity of the disease was higher than in immunocompetent humans. Likely the third step—introduction of the human *IFNAR* gene—is required to obtain a more precise model. However, these models made a valuable contribution to poliovirus research.

Non-translatable mice models

As mentioned above, sometimes it is not possible to create an adequate model of human pathology based on genetically modified mice. Usually, this is due to the difference in the structure of human and mouse genomes, the low homology of the corresponding proteins, the mechanisms of regulation that have changed in the course of evolution, and several physiological differences, such as, for example, lifespan. One of the most striking examples of the inability to adequately reproduce the symptoms of human disease in mice is the attempt to create a model of the Lesch–Nyhan syndrome (LNS) (repeated in numerous studies). This failure is particularly noteworthy because LNS is an inherited monogenic disease caused by mutations (including point mutations) in the *HPRT* gene.

Lesch–Nyhan disease

This hereditary disease is associated with a deficiency of the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and at the biochemical level is manifested in impaired purine metabolism, hyperuricemia, and hyperproduction of uric acid. This pathology is characterized by a spectrum of neurological manifestations, such as dystonia, choreoathetosis, impaired cognitive abilities, and aggressive behavior with self-harm. Lesch–Nyhan syndrome belongs to the group of orphan diseases, the prevalence is 1–9 cases per 1,000,000 people. The enzyme hypoxanthine phosphoribosyl transferase is responsible for the recirculation of purines and converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate in the presence of phosphoribosyl pyrophosphate [129]. The gene encoding hypoxanthine phosphoribosyl transferase, HPRT1, is located on the X chromosome and is inherited as a monogenic recessive trait. Simulation of the syndrome in animals is a promising approach to the development of treatment for this disease. The first attempt to create a mouse model for this pathology was made in 1987. Then mice with hypoxanthine phosphoribosyltransferase deficiency were generated, but they exhibited no phenotypic traits. To date, several models of Lesch–Nyhan syndrome have been created, both with gene knockout and with point mutations [129, 130]; however, they are all asymptomatic and are mainly used for studying the metabolism [131]. There is a hypothesis that the difference between mice and humans with HPRT1 mutations and knockouts is associated with inactivation of the *Prtfdc1* gene in mice (an *HPRT1* paralog with unknown functions). Overexpression of this gene in mice resulted in neurological symptoms similar to those in humans [132, 133].

Thus, the use of genetically modified animals as models of human diseases undoubtedly makes a significant contribution to understanding the pathogenesis of various conditions but has a limited scope of application that must be considered when planning experiments.

Alzheimer's disease

Currently, Alzheimer's disease (AD) is one of the most intensively studied diseases. At the same time, despite all the efforts, there's no effective cure, the exact causes of the disease onset are unknown, and causal relationships between different aspects of pathogenesis are still being discussed. Although in some cases AD is genetically determined and these cases are characterized by early onset, most often AD develops as a multifactorial disease among patients older than 60 years, when there is only partial genetic predisposition.

Since 1995, many genetically modified mouse strains have been created for the study of AD, and new ones continue to be created [134]. Specific and easily detectable features of AD pathogenesis are the formation of extracellular beta-amyloid plaques and intracellular neurofibrillary tangles, consisting of a hyperphosphorylated form of tau protein, in the brain. For a long time, the focus of AD research has been directed to the study of these processes. The first transgenic mice obtained for this purpose in 1995 were mice expressing the mutant APP (amyloid precursor protein) gene [135]. They were followed by strains with other variants of the *APP* gene carrying mutations specific for patients with early onset of genetically determined AD, as well as animals with overexpression of mutant forms of the *PSEN1* and *PSEN2* genes with the same specificity, which encode proteins included in the gamma-secretase complex, responsible for proteolysis of the APP protein. These mutant forms cause the predominant formation of the Aβ42 isoform, which is prone to transition to a pathological conformation, as opposed to other isoforms. Crossing these strains with each other made it possible to obtain mice with an extremely early onset of AD, which seemed convenient for research (reviewed in [136]). However, such models have a significant defect. Genetically determined cases of early AD onset make up a small percentage of all AD cases, and such animals are poorly suited for studying the etiology and pathogenesis of other cases [137].

In addition to the models based on the mutations that are directly associated with the occurrence of AD with the early onset, several models reproducing allelic variants associated with the increased risk of developing AD in old age have also been generated. Allelic variants of the *APOE* gene are associated with the risk of AD. The ε4 allelic variant significantly increases the risk, while the ε2 variant reduces it, and the ε3 variant is neutral. A similar relationship was observed in mice expressing human variants ε3 and ε4 [138, 139]. Another gene with allelic variants associated with the increased risk of AD is the *TREM2* gene (triggering receptor expressed on myeloid cells 2), which encodes a receptor that controls, among other processes, inflammation in the microglia. Mice expressing corresponding human TREM2 variants have also been generated and show similar phenotypes. To a certain extent, the last two classes of models can serve as models of AD with late-onset [137].

However, genetically modified animals as a model of AD also showed a more significant drawback. It turned out that drugs that lead to improvements (primarily the dissolution of amyloid plaques) in model mice are ineffective in humans [140].

Recent studies show that beta-amyloid plaques are sometimes found in asymptomatic people. It has been suggested that the immediate cause of symptoms development is not the formation of plaques and neurofibrillary tangles, but the death of cholinergic neurons [136, 141], which is not fully observed

in model animals, in contrast to the deposition of amyloid plaques and the formation of neurofibrillary tangles.

This case clearly illustrates that a certain level of etiology and pathogenesis understanding is necessary to create a model of a disease, and models that reproduce only some elements of pathogenesis may not be very useful.

Conclusion

As genetic engineering progresses, the number of molecular tools for genome editing increases, allowing it to modify and edit genes surgically, to knock-out and knock-in single or multiple genes.

Genetically modified animals, especially mice, can contribute to the research of human disease, as various animal strains can be developed relatively fast and easily to track the pathogenesis of disease and to evaluate the involvement of certain genes. Mouse models proved to be a useful tool for discovering targets for therapeutic drugs.

However, it is important to be realistic about the limitations of animal models. Sometimes due to the large differences between mice and humans, certain preclinical treatments in animal models cannot be translated to human clinical trials. The most problematic fields in this respect are behavior, immune system, and chromosomal disorders. On the other hand, mouse models can turn out to be useful in unexpected circumstances—for example, for research of viruses that do not affect wild-type mice.

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Declarations

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