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REVIEW

CRISPR/Cas-mediated genome editing in mice for the development of drug delivery mechanism

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Abstract

Background To manipulate particular locations in the bacterial genome, researchers have recently resorted to a group of unique sequences in bacterial genomes that are responsible for safeguarding bacteria against bacteriophages. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) are two such systems, each of which consists of an RNA component and an enzyme component.

Methods and results This review focuses primarily on how CRISPR/Cas9 technology can be used to make models to study human diseases in mice. Creating RNA molecules that direct endonucleases to a specific position in the genome are crucial for achieving a specific genetic modification. CRISPR/Cas9 technology has allowed scientists to edit the genome with greater precision than ever before. Researchers can use knock-in and knock-out methods to model human diseases such as Neurological, cardiovascular disease, and cancer.

Conclusions In terms of developing innovative methods to discover ailments for diseases/disorders, improved CRISPR/Cas9 technology will provide easier access to valuable novel animal models.

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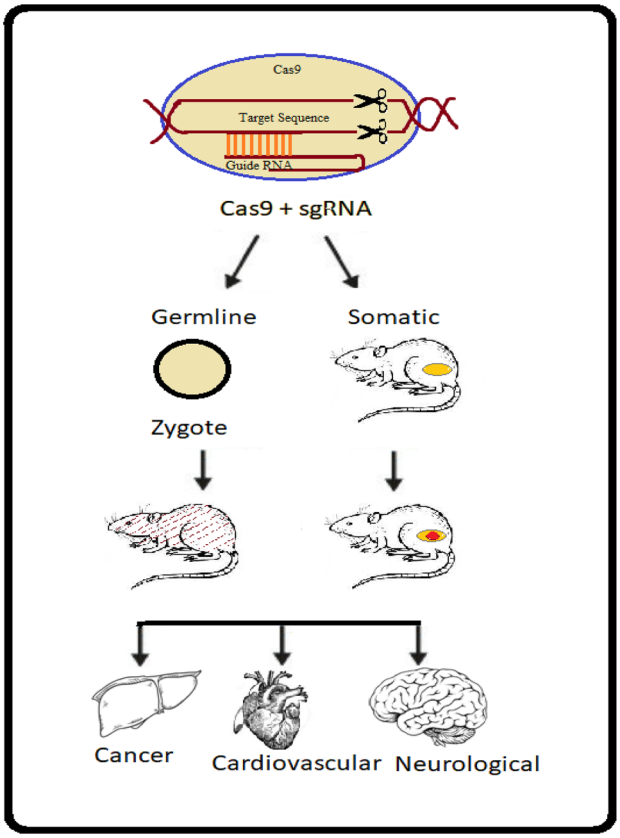
Graphical abstract



Keywords CRISPR/Cas9 · Germline · Somatic · Neurological · Cardiovascular · Cancer · Mice models

Introduction

Since the early 1960s, scientists have put in a lot of work in devising a precise, safe, and time-effective method of genome editing. Ranging from the infamous recombinant technology as a backbone for gene therapy to the zinc-finger nuclease (ZNF) technology and transcription activator-like effector nucleases (TALENs), all the methods had significant shortcomings which made them ineffective as a reliable genome editing tools [1]. This was until CRISPR technology came along as a genome editing tool in 2012. Initially discovered as a part of the bacterial adaptive immune system, Clustered Regularly Interspaced Short Palindromic Repeats, or CRISPRs, are short repetitive nucleotides found within the genome of prokaryotes (such as bacteria and archaea). In 1987, Atsuo Nakata et al. first reported the presence of repetitive sequences separated by non-repetitive (later this DNA arrangement was termed CRISPR) in the genome of *Escherichia coli* [2]. CRISPRs in bacteria confer them protection from attack by bacteriophages, viral DNA, and plasmids. Foreign DNA sequences called Spacers are found nestled between the palindromic repeats of bacterial origin. This arrangement accords a memory of the infection to the bacterial immune system. Mobile genetic elements, such as transposons and bacteriophages that have infected the bacterium at some point, give rise to these Spacers [3]. During infection, bacteria acquire a small piece of the foreign viral DNA and integrate it into the CRISPR locus to generate CRISPR arrays [4]. Transcription and associated modifications followed, giving rise to a CRISPR RNA (crRNA). An involvement of CRISPR-associated nuclease protein (Cas9) is thereafter established. Alongside, occur the molecules of trans-activating CRISPR RNA (tracrRNA), possessing sections complementary to and thereby can anneal to the palindromic repeats. Ribonucleases cleave the strands between the entire association of different RNA and protein molecules generating individual effector complexes of three components. When the effector complex encounters a section of viral DNA with complementary sequences to that of the CRISPR RNA (crRNA), nucleases coordinate with it. A unique viral genome sequence called the protospacer adjacent motif (PAM) acts as a binding signal for nuclease and

both domains of the latter cleave the two strands of DNA, just a few bases upstream from the PAM. Hereby, the viral genome is neutralized and infection is avoided.

This entire mechanism within the bacteria formed the basis for CRISPR-Cas9 being proposed as a method of genome editing in modern applications. Dr. Jennifer Doudna and Dr. Emmanuelle Charpentier received Nobel Prize in Chemistry (2020) for their work propounded that the bacterial CRISPR-Cas 9 could be used as a programmable toolkit for site-specific genome editing in humans and other animal species [5]. A breakthrough was achieved by in vitro joining of crRNA and tracrRNA thus generating a single-guide RNA (sgRNA) [6]. The association of Cas9 protein to sgRNA forms a two-component functional effector complex that is as competent as the bacterial three-component system. From the studies of Carroll [7], it is understood that, with just the generation and insertion of an appropriate sgRNA with accurate complementary sequence and Cas9 sourced from *Streptococcus pyogenes*, it is possible to determine any 20 base pairs target sequence for editing along the PAM sequence. Nuclease forms an incision at the two target DNA strands and thereby a natural DNA repair mechanism occurs via either of the two routes: homology-directed repair (HDR) or non-homologous end joining (NHEJ). NHEJ, common in a eukaryotic domain, does not require a homologous template DNA and is error-prone due to the creation of indels which are DNA strands with either insertion or deletion nucleotide sequences [8]. While the complex and uniform HDR pathway, common in bacteria and archaea, uses a DNA template with homology to the adjacent sequences surrounding the site of cleavage to incorporate new DNA fragments.

CRISPR-Cas systems are classified into two major classes including six types and are further divided into sub-types [9]. Class I CRISPR systems possess multiple subunit effector molecules and includes DNA targeting Type I (seven subtypes; carry Cas3 loci), DNA and RNA-targeting Type III (four subtypes; carry Cas 10 loci), and a putative Type IV. Class II possesses single large proteins and include Type II, Type V and Type VI each with three subtypes carrying loci for Cas9, Cas12, and Cas13, respectively.

The raft of CRISPR applications has only expanded ever since the work of Doudna and Charpentier was published. CRISPR becomes a vital tool in genetic screening to identify genes such as in cancer immunotherapy [10], therapeutic management of acquired immunodeficiency syndrome (AIDS) [11], and an assay of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [12]. CRISPR carries with it the promise of curing allergies [13] and preventing certain gene-linked diseases [14]. Gootenberg et al. [15] have harnessed the knowledge of Cas13 to generate a CRISPR diagnostic kit-SHERLOCK which has successfully shown the detection of both the Zika virus and certain strains of dengue fever. Major work has already been done in targeted epigenome modification by the alteration of Cas9 [16]. This site-specific genome-modifying tool also finds several applications in conferring disease resistance along with the improvement of phenotypes, quality, and crop yield in agriculture.

As per Miano et al. [17] studies, traditional methods of generation of target clones, their incorporation into the blastocyst of animals and further breeding and validation of these animals to produce knock-in or knockout models have been a complicated maneuver. With the rise of CRISPR technology, it is now possible to generate new mouse models with high specificity and efficiency in shorter time frames by disrupting the gene sequence [18].

Components of CRISPR genome editing

In bacteria, CRISPR is made up of three essential elements: a trans-activating crRNA(tracrRNA), a CRISPR RNA (crRNA), and a CRISPR-linked endonuclease (Cas9). Through a straightforward base pairing, the tracrRNA binds to Cas9 and the crRNA attaches to the tracrRNA. The complex is subsequently bound to DNA at the desired location, where Cas9 carries out its cleaving action [19].

In a CRISPR system in which a designed, tracrRNA and crRNA are joined to create a single-guide RNA (sgRNA). Because of this, the CRISPR system is made up of just two parts: a Cas9 protein and a single-guide RNA. The CRISPR-Cas system is now a more flexible and practical tool for site-directed gene editing as a result of this simplification. The gRNA and Cas9 are joined to form a nucleoprotein complex in CRISPR investigations [20]. To allow an endonuclease to cleave the target DNA, the Cas9-gRNA complex recognizes the protospacer adjacent motif (PAM) region and forms a Watson–Crick base pairing with the 20 nucleotide target DNA [21].

Based on the type of protein that cleaves the target nucleic acid and the structure of the CRISPR-Cas locus, the CRISPR-Cas system is split into two classes and six types [9]. Type I, III, and IV CRISPR-Cas systems make up class 1. The first type is known as CRISPR-associated complex for antiviral defense (CASCADE); it consists of numerous Cas proteins and crRNA. It consists of Cas3, which degrades the target by containing helicase and DNase domains. Cas10 is a component of type III. They use crRNA complementarity to break transcriptionally active RNA. While Cas 10 cleaves ssDNA, Cas 7 cleaves RNA. Type IV is present in plasmid-like areas and may be necessary for plasmid maintenance [22].

Types II, V, and VI of the CRISPR-Cas system are categorized as Class 2. The Cas9, Cas1, Cas2, and Cas4 molecules make up Type II. Gene therapy employs them. With the aid of the endonucleases HNH and RuvC, Cas9 breaks the target DNA into dsDNA segments. They need tracrRNA,

a non-coding RNA, in addition to crRNA to cleave DNA. Adaptation involves Cas1 and Cas2. The Cas12 protein found in Type V uses the RuvC domain to cut DNA [22]. Additionally, it has the cpf1 endonuclease, which can identify the PAM 5'-TTN that is widely found in the human genome [21]. The complementary RNA target is located by the Cas13 protein of type VI by binding to crRNA. Due to its high efficiency and simplicity when compared to other tools and its capacity to combine with multiple single-guide RNAs to achieve effective genome editing in cells, the Cas9 from type II CRISPR system is one of these that is frequently used to facilitate genetic manipulation in organisms and various cell types [23, 24].

The Cas9 protein, also known as a genetic scissor, is a multi-domain, multifunctional DNA endonuclease enzyme that cleaves the genome at certain locations to create a double-strand break [4]. It is an RNA-guided DNA endonuclease that is non-specific. The specific DNA location where Cas9 breaks the double strands of DNA is directed by the sgRNA. Cas9 remains in an inactive state when sgRNA is not present. SpCas-9, the most widely used Cas9 nuclease, and the first Cas9 nuclease to be utilized for genome editing is one of several Cas nucleases that have been identified from bacteria [21].

The recognition (REC) lobe and the nuclease (NUC) lobe are the two lobes that make up the Cas9 protein. The REC lobe, which is made up of the REC 1 and REC 2 domains, is in charge of binding gRNA. The biggest domain, REC 1, is in charge of binding sgRNA. RuvC and HNH-like nuclease domains are the two endonuclease domains that make up the NUC lobe. The complementary strand is cut by the HNH domain, while the RuvC domain cuts the second non-complementary strand [4, 19].

Other Cas9 variations have been created, including nickase Cas9 (nCas9) and dead Cas9 (dCas9). Any one of the nuclease domains (HNH or RuvC) will be rendered inactive in the case of nCas9, whereas both domains will be rendered inactive in the event of dCas9. One strand of the DNA is cut when either one of these domains is inactive or when both domains are inactive, the endonuclease still can connect to DNA but is unable to limit DNA. Dead Cas9 is a DNA-binding protein that serves as a site-specific vehicle and can be applied in experimental research. A pair of nCas9, which produce paired nicks rather than double-stranded breaks, can lessen off-target cleavage [21].

The guide RNA (gRNA) is a particular RNA that directs the CRISPR system to the precise editing spot. For Cas9 to bind, a short synthetic RNA that has already been designed is required. The sgRNA is made up of a scaffold that binds the endonuclease and a spacer that contains 20 nucleotides that are intended to target particular genomic locations [21]. One tetraloop and two or three stem-loops make up the T-shaped strand of RNA that makes up gRNA. It consists of two RNAs that direct Cas9 to the intended site: (1) The CRISPR RNA (crRNA), which pairs with the target sequence to specify the target DNA, and (2) The trans-activating RNA (tracrRNA), which acts as a scaffold for Cas9 nuclease interaction. A linker connects the crRNA and tracrRNA [4].

The spacer sequence that guides the complex to the target DNA and a region that binds to the tracrRNA are the two main components of crRNA. It is made up of two domains, the first of which is at the 3′ end and is joined to the 5′ terminal area of the tracrRNA by Watson–Crick pairing. The second domain, which is target-specific and can be designed to base pair with the target DNA, is situated at the 5′ end [20].

Each species' tracrRNA is distinct and attaches to the host-specific Cas9 as part of the host immune system. The maturation of crRNA from precrRNAs is facilitated by the tracrRNA, which connects crRNA to Cas9. A functional gRNA is created when tracrRNA base pairs with crRNA [24].

The PAM sequence, a brief sequence found on the target DNA strand, precedes the gRNA. It is required for Cas9 to function as an endonuclease. Another crucial element of the CRISPR system is PAM. It is recognized by the Cas nucleases and resists cleaving in the absence of a PAM. They ensure that CRISPR arrays, not foreign viral DNA, are cleaved. Each Cas9 enzyme uses a different set of these sequences. Depending on the type of bacterium from which Cas9 was produced, the PAM sequence differs. The PAM sequence is NGG and is found on the 3′ end of the gRNA sequence in the most widely used type II CRISPR system, which is generated from *Streptococcus pyogenes* [19].

The Cas9 is localized to the target genomic sequence by the gRNA/cas9 interaction, and the Cas9 cleaves both strands of DNA to create a double-strand break (DSB) [23]. Cas9 removes DNA 3–4 nucleotides upstream of the PAM region, and DNA repair that follows uses one of the following mechanisms:

1. Non-homologous end joining (NHEJ): This method involves random base pair insertions or deletions at the cleaved site, and it is more common in most cell types. As a result, it is prone to errors and frequently causes frameshift mutations that result in premature stop codons or non-functional peptides.
2. Homology-directed repair (HDR): An error-free mechanism. The right sequence of a repair template is utilized to rectify mutations that cause disease [4].

The exploitation of CRISPR for genomic engineering

Oncogenesis is characterized by multiple aspects such as multiple gene interactions, systemic signals, and cell types, all of which can be interrogated only at the organizational

level [25]. Although the Genetically engineered mouse models (GEMM) of human cancer have been effective in interrogating cancer biology the protocol is very laborious, and lengthy and the transgenic generation of GEMM and targeting of a gene is very expensive [26]. These setbacks open up new avenues to gene editing in mammalian cells by employing the CRISPR/Cas9 adaptive immune system [27].

Methodology of gene editing

Double-stranded breaks are introduced in the genomic loci of the desired genome by the endonuclease Cas9 directed by the programmable sgRNA [28]. DNA repair by homology-directed repair (HDR) or non-homologous end joining (NHEJ) is explored for gene editing. Deletions (indel) or small insertions are left by NHEJ at the site of repair leading to mostly targeted reading frame disruption [25]. The usage of multiple sgRNAs (CRISPR multiplexing) can be employed for engineering alterations of complex genomes like structural aberrations, and large deletions such as translocations and inversions [26]. CRISPR in addition to Gene editing uses nuclease deficient Cas9 (dfCas9) dual with transactivation complexes for gene activation (CRISPRa) for direct regulation of gene expression or a Kruppel-associated box domain for gene repression [27]. Epigenetic Modifiers like histone-modifying enzymes or DNA methyl transferases linked with dcas9 enable epigenome engineering.

CRISPR-engineered cellular transplantation mouse models

Transplantation assays commonly use CRISPR-based cancer cell lines to study solid tumors (Fig. 1a, Table 1). The ease of propagation and manipulation of cancer cell lines makes them advantageous for multiplexed applications [28]. Recent advances are made in developing *Myc*-driven B cell lymphoma and acute myeloid leukemia mouse models deploying haemopoietic stem cells having CRISPR-engineered single or combinatorial gene knockouts [29]. The cellular-derived mouse models are proven to be advantageous over human cells as it has the ease to perform transplantation experiments in syngeneic recipients that are immunocompetent which allows the investigation of complex interactions in cancer cells with the immune microenvironment [30].

Germline engineering by CRISPR

The exploration of CRISPR has gained tremendous importance for the genetic engineering of mouse germline in embryonic stem cells (ESCs or ES cells) and zygotes (Fig. 1b). The manipulation of ES cells is considered a very effective approach as it is not time-consuming and laborious whereas usually, the disadvantages pose ES cell targeting, construction of vector, and germline transmission will take several months to years [31]. The gene-targeting for both knock-in and knockout mouse generation can be accelerated using CRISPR to rising the efficiency of homologous recombination in ES mouse cells [25]. An alternative method in ES cells driven by CRISPR makes use of expression Cassettes for sgRNAs and tetracycline (Tet)-inducible Cas9 at the Col1a1 (Collagen, type I, alpha 1) locus. CRISPR-driven Biallelic inactivation of target genes can be induced by Doxycycline treatment of mice. The spatial and temporal over CRISPR editing can be supported by the use of this methodology [26].

Today with the use of CRISPR technology, the zygotes of a mouse have been favorable to highly efficient and accurate gene editing [32]. The use of CRISPR in mouse zygotes allows the inception of

(a) By the introduction of two Loxp cassettes flanking a critical exon for Conditional knockout mice and
(b) By NHEJ for whole-body knock-out mouse lines.
(c) Using HDR for Knock in mouse lines [28].

It is reported that the efficiency of combinatorial or simple knockout generation is more in comparison to the engineering of knock-in or conditional knock-outs [26]. Over 1000 CRISPR-edited zygotes (with the use of two ssRNAs and ssDNA templates) are required for the generation of one mouse progeny having a desired floxed conditional allele as shown in a meta-analysis [28]. Recently using long ssDNA template improvisations which were promising were reported where 50 zygotes were required for the generation of a correctly targeted animal, ex: Efficient additions with ssDNA inserts (Easi)-CRISPR, and without using vector backbone sequences, linearized in vitro dsDNA templates were used for targeting, ex: targeted integration with linearized dsDNA (Tild)-CRISPR [25]. These studies give us a new dimension though their application is still under trial. CRISPR has overcome the impossibility of integrating chromosome engineering directly into zygotes. The high reported rate of mosaicism is one of the major disadvantages of germline editing using CRISPR delivery [27]. By the use of techniques like electroporation-mediated delivery of sgRNA/Cas9 instead of RNA or expression plasmids into early pronuclear zygotes, the development of mosaic mutants can be increased. This helps in genome editing before replication due to the rapid availability of CRISPR components. CRISPR is widely considered for undesired editing on target loci and potential off-target activity.

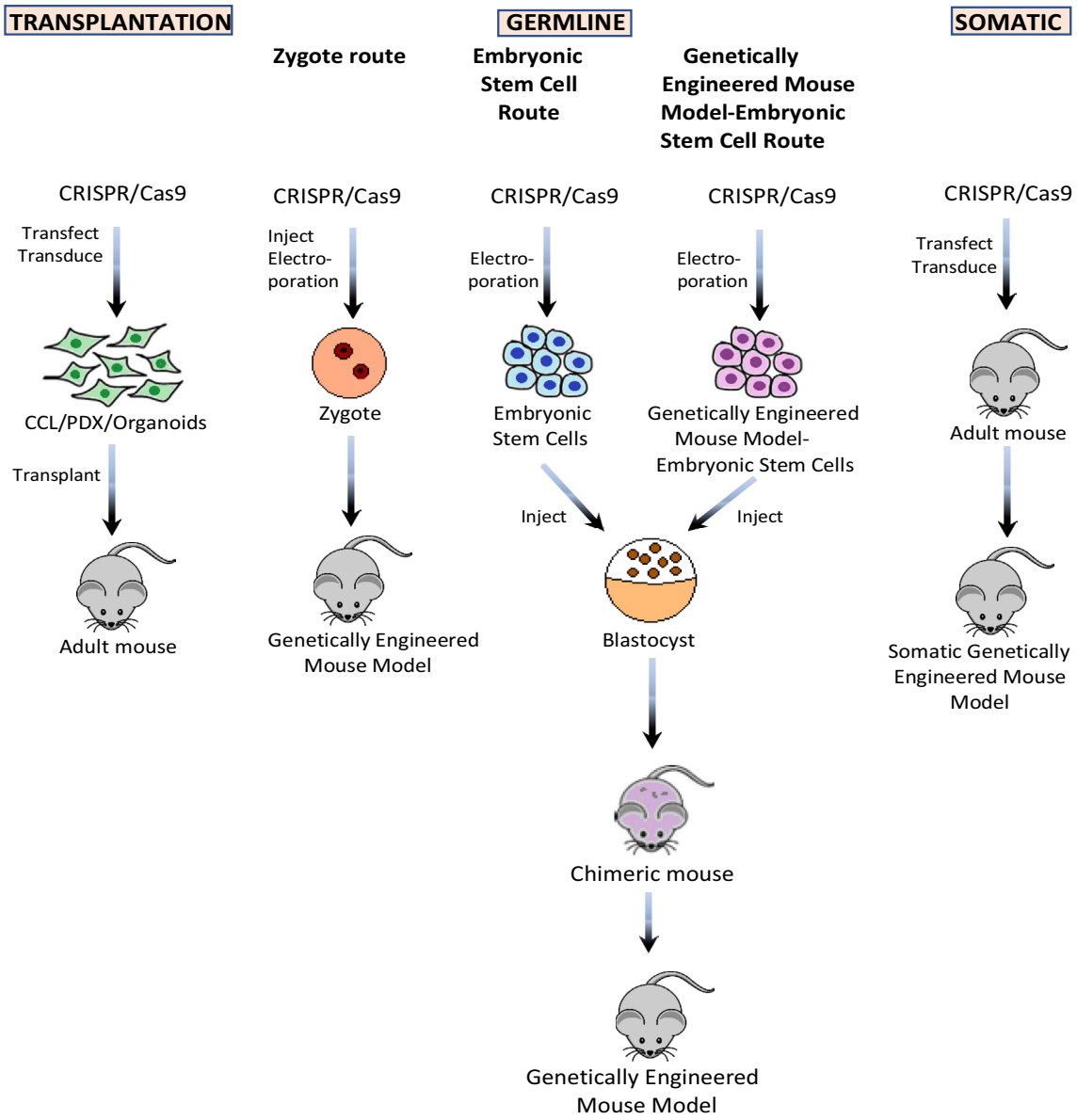


Fig. 1 Engineering approaches for mouse models (*a* Transplantation, *b* Germline, *c* Somatic)

Somatic engineering by CRISPR

In vivo editing of somatic cells to develop Somatically Engineered Mouse Models (SEMMs) (Fig. 1c) has proven to be advantageous over germline Engineering. The delivery of the CRISPR components to the targeted cells are organs is one of the major challenges [26]. Easy methods for efficient targeting can be employed for some organs such as the lungs and liver while organs like the intestine cannot be targeted easily by systemic methods and the need arises for invasive protocols [27].

DNA transfection can be used as one of the possible for delivering CRISPR components as multiple CRISPR plasmids can be delivered to individual cells. There is usually a transient expression of the transfected DNA, thereby mobilization of transposons from plasmid to genome carrying CRISPR components can be achieved by Genomic CRISPR integration which allows tagging of cells mainly useful for screening [28]. Currently, the viral vectors are dominating CRISPR vehicles as Transfection-based delivery having naked DNA is efficient on only a few organs [25]. Adeno viruses which belong to non-integrating viruses have proven to be very effective in infecting only selected organs while integrating viruses are advantageous in tagging cells which is very useful for CRISPR Screening applications. But the limitation poses in the restriction

Table 1 CRISPR mouse model characteristics

	Germline cells	Somatic cells	Transplantation
Quality of model used for investigating oncogenesis	Good	Exemplary	Poor quality cancer cell lines; good quality stem cells and organoids
Phenotypic strength	Exemplary	High for the genes with strong effect and model dependent for genes with mild effects	High with varying rates of engraftment
Genetic screening potential	Not possible	Possible to some extent	Possible
Productive CRISPR editing	Generation of knockout alleles: high; conditional allele generation: low	Often low (variable)	High: cancer cells; low: precursor cells and organoids
CRISPR versatility	Low for zygotes while high in case of embryonic stem cells	Fair	High; modeling a wide range of alteration types are possible
Pace	High in case of zygotes/genetically engineered mouse models; relatively low for embryonic stem cells	Extremely high	Extremely high
Costs	Expensive	Cost-effective	Cost-effective
Advantages	Manageable and robust models	Scalable and rapid; appropriate for complex research-based applications; akin to human oncogenesis	Scalable and rapid; large-scale genetic screening feasible
Disadvantages	Time-intensive; difficult conditional model generation	Difficult delivery; low efficiency of editing	Non-autochthonous model—inappropriate for studying cancer prevention; orthotopic way of transplantation is difficult

of the Cas9 packaging efficacy due to the low viral cargo capacity [28].

To overcome this disadvantage the Cas9 knock-in mouse was developed which supports tissue-specific nuclease expression and the immune response against Cas9 expression can be prevented which usually has the drawback of deleting Cas9-expressing cells and causing inflammation [26].

The use of nanotechnology-based approaches is currently a diversified research area due to the tremendous applicability of the nanoparticles and their unique physiochemical properties and biocompatibility which makes their integrated approaches eco-friendly and pose no toxicity and deeper penetration and targeted delivery due to their nano sizes [28]. Nano bioconjugates can be efficiently used as vehicles for CRISPR delivery systems such as lipid, gold, and polyethylene glycol (PEG)-based nanoparticles. It was reported that 80% editing efficacy was achieved by the systemic injection of Lipid nanoparticle carriers having Cas9 [25]. mRNA along with modified sgRNAs in the mouse liver have shown a reduction in the susceptibility of nuclease degradation.

Modeling of complex structural rearrangements

CRISPR poses another application other than gene inactivation by allowing the modeling of complex structural rearrangements at the chromosomal level (Fig. 2). Fusion of the echinoderm microtubule-associated protein-like 4 (EML4) gene- anaplastic lymphoma kinase (ALK) oncogenic gene is one of the reported most common chromosomal rearrangements in human solid cancer found in 7% of Non-Small Cell

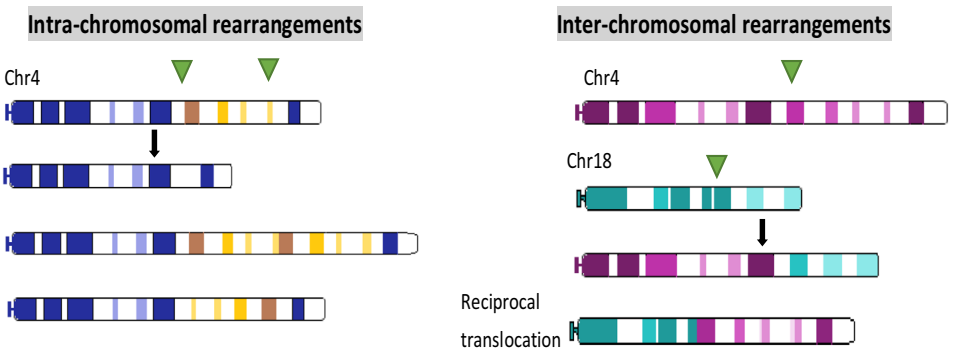


Fig. 2 Chromosome engineering by CRISPR

Lung Cancer (NSCLC) cases [27]. The NSLCs sensitive were developed in animals to anaplastic lymphoma kinase (ALK) inhibitor treatment exhibiting their relevance as models for preclinical and basic research.

CRISPR-induced kilo-mega base scale deletions were reported in the pancreas, brain tumor, and liver in addition to intra-chromosomal inversions. In one of the studies, large chromosomal alterations were reported which were induced by Combinatorial CRISPR editing apart from focal deletions [26]. In a study employing in vivo CRISPR multiplexing, inter-chromosomal translocations are observed other than rearrangements affecting one chromosome which exhibits the ambidexterity of CRISPR in engineering the complex genomic arrangements in mouse models [28].

Mice models and CRISPR technology

The pathophysiological study of various conditions relies heavily on genetically engineered animal models that simulate human diseases. When compared to traditional gene-targeting methods using embryonic stem cells, the development of the clustered regularly interspaced short palindromic repeats (CRISPR) system has enabled a faster and cheaper production of animal models. Genome editing tools based on the CRISPR-Cas9 system are game changers because they allow for the precise introduction of mutations at specific DNA sequences. This accelerated the development of animal models, which greatly aided research that relied on them [33]. Previously, only a few labs were able to master the sophisticated vector design and onerous methods required to target the mouse genome for the addition, deletion, or substitution of physiologically important sequences. As a result of CRISPR's ease of use and low cost, almost any laboratory can swiftly put together reagents for the development of new mouse models for research on various diseases or disorders [34]. CRISPR-associated (Cas)9 genome editing has revolutionized the generation of mutant animals by making null alleles in virtually any organism simple to create [35].

Creating CRISPR/Cas9 models in mice/rats

The recent development of CRISPR/Cas9-mediated genome editing has sparked widespread enthusiasm among the scientific research community due to its promise of straightforward and efficient genomic manipulation of virtually any cell type. CRISPR is rapidly replacing other methods of genetic engineering because of its incredible potential as a platform for studying gene function in vivo [36]. Since the beginning of genetic engineering, the mouse has played a central role as a model organism, so it should come as no surprise that studies involving nuclease-directed genome editing have been conducted primarily on mice. As a result, CRISPR has been rapidly adopted by the mouse modeling community because of the increased flexibility it provides in genomic manipulation. Using CRISPR/Cas9 genome editing to create rat and mouse models of human disease has transformed the profession, saving time, perhaps reducing the use of animals, and being more cost-effective. However, the possibility for off-target effects, mosaicism, and mutations, as well as prospective downsides for constructing more sophisticated genome changes, must be taken into account while creating personalized CRISPR in mice and rats. CRISPR/Cas9 is a powerful and simple tool with a short learning curve. The multiplex capacity of this technology is particularly useful because it allows for the simultaneous editing of multiple genes [37]. Through the use of a single transfection of embryonic stem cells (ESCs), lee Jaenisch et al. showed for the first time how quickly novel animal models can be generated using CRISPR. They also showed that direct injection of Cas9 mRNA and sgRNAs into fertilized zygotes resulted in surprisingly high efficiency for producing single (95%) or double mutant (70–80%) mice [38].

Cardiovascular mice models

In the developed world, cardiovascular disease is still the major cause of death and disability. Several studies have been carried out in the last few decades to define the molecular and pathophysiological characteristics of the diseased heart and vasculature. Using mouse models has been especially helpful in elucidating the multicellular interactions, genetic and epigenetic regulatory circuits, and signaling pathways that underlie cardiovascular disease. With the recent invention of the CRISPR-associated (Cas)9 system, the technique of genome editing has been substantially simplified in the development of mouse models with cardiovascular diseases [39]. Carroll et al. [40] have developed a transgenic mouse strain that expresses Cas9 only in heart muscle cells. They have also genomic insertions and deletions in the heart that can be induced quickly utilizing this approach, which delivers single-guide RNA (sgRNA) via Adeno-associated virus 9. As a proof of concept, the cardiac-specific Cas9 mouse developed cardiomyopathy and heart failure after receiving sgRNA directed against the Myh6 gene and the transgenic mouse model is a useful tool for cardiovascular research since it is a simple technique to modify heart-related genes of interest. Confirming that the H530R mutation is directly connected to PRKAG2 cardiac syndrome, Xie et al. [41] generated H530R PRKAG2 transgenic and knock-in mice models which mirrored human symptoms, including cardiac hypertrophy and glycogen accumulation. They used a combination of the CRISPR/Cas9 gene-editing system and adeno-associated virus-9 (AAV9) to silence the H530R-encoding mutant PRKAG2 allele without damaging the adjacent wild-type allele. H530R PRKAG2 transgenic and knock-in mice showed significant improvement in heart

architecture and function after receiving a single systemic injection of AAV9-Cas9/sgRNA on a postnatal day 4 or day 42.

Neurological mice models

Many neurological disorders such as Alzheimer's, amyotrophic lateral sclerosis, epilepsy, Huntington's, and Parkinson's lack disease-modifying treatments, making them one of the biggest public health challenges. There is a demanding need for model systems that provide experimental access to the underlying biology, especially in light of the recent discovery of multiple new genetic causes of neurological disorders. Recent functional genomics approaches based on CRISPR can help researchers learn more about the underlying causes of neurological disorders and potential treatment options. CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) are two mechanisms used by the bacterial CRISPR system to edit genomes and regulate gene expression levels, respectively, in experimental disease models (CRISPRa) [42]. As a result of CRISPR-Cas9 technology, which enables precise and effective gene editing in nearly every cell type and organism, the pace of basic biological research has increased dramatically. A growing number of research institutions are committed to elucidating the molecular mechanisms of neurological disorders and using the CRISPR-Cas9 system to develop novel therapies. By correcting or knocking out mutant genes and editing other related genes, CRISPR-Cas9 technology is being used to treat neurological disorders. Examining the onset and progression of diseases in whole organisms is best accomplished through the use of animal models rather than cellular-level studies [43].

Zhou et al. [44] recently developed in vivo viral delivery of an RNA-targeting CRISPR system, CasRx, which led to highly efficient conversion of Müller glia into retinal ganglion cells (RGCs), alleviating disease symptoms associated with RGC loss. This was accomplished by downregulating a single RNA-binding protein, polypyrimidine tract-binding protein 1 (Ptbp1). Dopaminergic neurons were induced in the striatum, and motor defects in a mouse model of Parkinson's disease were reduced as a result of this strategy. Therefore, Cas13d family RNA editing (CasRx)-mediated polypyrimidine tract-binding protein 1 (PTBP1) knockdown in glia is a potentially useful in vivo genetic approach for treating a wide range of disorders characterized by neuronal loss. Shah et al. [45] showed that the Lund Human Mesencephalic (LUHMES) female human neuronal cell line can be efficiently manipulated genetically to generate multiple lines harboring point mutations that cause neurological diseases. They have shown that LUHMES cells could prove to be an invaluable resource for uncovering the molecular basis of neurogenetic disorders, which in turn could pave the way for future advances in drug development and therapeutic approaches.

Cancer and mice models

Point mutations, translocations, and chromosome gains and losses per tumor contribute to the complexity of the cancer genome. Accurate models are required to comprehend the consequences of such changes. The standard methods for creating mouse models are lengthy and complicated, requiring the manipulation of embryonic stem cells and several stages. Recent advances in genome editing technology, the CRISPR-Cas9 system, are revolutionizing the process of creating mice models [46]. Rapid and expandable in vivo methodologies based on CRISPR open the door for a new era of functional cancer genomics. Improvements in the CRISPR/Cas9 system producing more accurate mouse models and in the humanized mice xenograft models simulating the intricate interactions between the tumor and its environment may be one of the successful approaches to precisely tailored cancer therapy, resulting in enhanced cancer patient survival and quality of life.

To directly target the tumor suppressor genes Pten5 and p536 in the liver, Xue et al. [47] have used hydrodynamic injection to transport a CRISPR plasmid DNA expressing Cas9 and single-guide RNAs (sgRNAs). Phenocopying the effects of gene deletion via causes recombination (Cre)—a locus of crossover (x) (LoxP) known as (Cre/loxP) technology, CRISPR-mediated Phosphatase, and tensin homolog (PTEN or Pten) mutation increased Akt phosphorylation and lipid accumulation in hepatocytes. Liver tumors were induced by simultaneous targeting of Pten and p53, just as they were by Cre-LoxP-mediated deletion of Pten and p53. Tumor suppressor gene insertion and deletion (indel) mutations, including bi-allelic Pten and p53 mutations, were discovered by sequencing DNA from tumor and liver tissue. Hepatocytes with nuclear localization of -Catenin were generated by co-injecting Cas9 plasmids encoding sgRNAs targeting the -Catenin gene (Ctnnb1) and a single-stranded DNA (ssDNA) oligonucleotide donor carrying activating point mutations. Annunziato et al. [48] have reported the generation and evaluation of knock-in mice that express a cytidine base editor under the control of the Cre recombinase, intending to facilitate targeted somatic engineering of missense mutations in important cancer drivers. They have shown point mutations in one or more endogenous genes could be efficiently installed in situ after intraductal delivery of sgRNA-encoding vectors, allowing to an evaluation of the impact of defined allelic variants on mammary tumorigenesis. Table 2 represents the genes that are targeted and the vectors for the delivery of genes in mice models.

Table 2 The genes targeted and the vehicles used for the delivery of genes in mice models

Disease/Disorder	Animal	Target tissue	Targeted Gene	Delivery	Vehicle for delivery	Disease caused/prevented; utility	References
Cardiovascular	Mouse	Heart	Myh6 knockdown	Intraperitoneal delivery	Adeno-Associated Virus 9 (AAV9)	Extreme cardiac hypertrophy in cardioedited mice; Myh6's importance to cardiac function and its role in the onset and progression of congestive heart failure are confirmed by Cas9-mediated knockdown	[40]
Cardiovascular	Mouse	Liver	Pcsk9 gene disruption	Intravenous injection	Adenovirus	Lowers blood cholesterol levels in mice; medicinal value in preventing and treating cardiovascular disease in humans	[49]
Cardiovascular	Mouse	Heart	Jph2 deletion	Subcutaneous injection	Adeno-Associated Virus 9 (AAV9)	Heart failure is caused by severe cardiomyopathy	[50]
Neurological	Mouse	Brain	Bace1	Hippocampal injection	Cas9 and single-guide RNAs (sgRNAs) loaded into an amphiphilic nanocomplex	Alzheimer's disease; Cas9 nanocomplexes were more effective at preventing the expression of Bace1 than chemical Bace1 inhibitors. Alzheimer's disease-related phenotypes were reduced because there was a significant decrease in the buildup and release of A42 plaques and an improvement in spatial working memory; Studies have shown that in vivo Cas9 nanocomplex-mediated neuronal gene-targeting is possible and that it could be used to treat mice with Alzheimer's disease	[51]
Neurological	Mouse	Brain	Single gene target: Mecp2; Multiple gene target: Dnmt1, Dnmt3a and Dnmt3b	Stereotactic delivery	Adeno-Associated Virus-9 (AAV9)	Rett syndrome; Learning/memory	[52]

Table 2 (continued)

Disease/Disorder	Animal	Target tissue	Targeted Gene	Delivery	Vehicle for delivery	Disease caused/prevented; utility	References
Neurological	Mouse	Brain	HTT depletion	Stereotaxic injection	Adeno-Associated Virus 9 (AAV9)	Huntington disease; polyQ expansion-mediated neuronal toxicity in the mature brain can be effectively and irreversibly eradicated by depleting HTT via CRISPR/Cas9. Regardless of the allele that causes HD, a treatment option that involves removing the polyQ domain from the N-terminus of HTT is being explored	[53]
Cancer	Mouse	Breast	Knockout of LPP2	Tail vein injection	In vitro DNA transfection using Polyjet and Lipofectamine CRISPRMAX	Breast cancer; Reducing c-Myc signaling by inhibiting high LPP2 expression in cancer cells may be a potential approach to slowing tumor growth. Reducing c-Myc expression and tumor progression may be available through targeting LPP2	[54]
Cancer	Mouse	Pancreas	Expression of an oncogenic Kras G12D allele through homology-directed repair (HDR) and CRISPR-induced cooperating alleles (Trp53, Lkb1 and Arid1A) disruption	Direct intra-pancreatic injection	Adeno-Associated Virus 9 (AAV9)	Pancreatic ductal adenocarcinoma (PDAC); pancreatic intraepithelial neoplasia (PanIN) or intraductal papillary mucinous neoplasia (IPMN) in the genetically altered animal models that developed into PDAC	[55]

Table 2 (continued)

Disease/Disorder	Animal	Target tissue	Targeted Gene	Delivery	Vehicle for delivery	Disease caused/prevented; utility	References
Cancer	Mouse	Bone marrow and Blood cess	Knockout of *BEND3*	Subcutaneous injection	Lentivirus	Acute Myeloid Leukaemia (AML); Breast cancer resistance protein (BCRP; ABCG2), an ATP-binding cassette efflux transporter, was increased and intracellular TAK-243 levels were decreased after BEND3 knockdown. TAK-243 sensitivity associated with BCRP expression in various cancer cell lines. TAK-243 is a substrate for BCRP, and its expression is controlled by BEND3. In addition, BCRP expression might be used as a marker of TAK-243 responsiveness	[56]
Cancer	Mouse	Ovaries	Knockout of single (*Trp53*; T), double (*Trp53*/*Brca1*; TB), and triple (*Trp53*/*Brca1*/*Pten*; TBP or *Trp53*/*Brca1*/*Nf1*; TBN) gene	Subcutaneous and orthotopic injections	In vitro RNA transfection using Stemfect RNA Transfection Kit (Stemgent)	High-grade serous ovarian cancer (HG-SOC)	[57]

Conclusion

Without a doubt, CRISPR editing has revolutionized the field of biomedical research, and it promises to develop medicine for various diseases/disorders and also for rare genetic disorders. Since the first demonstrations of CRISPR-based mammalian gene editing in 2012, CRISPR technologies have proliferated, promising even more explosive expansion over the next decade, to the eventual benefit of scientists and patients globally.

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Declarations

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval We furthermore declare that there is no ethical issue in our experiments.

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