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Teaser This review provides insights into in vitro and in vivo use of CRISPR/Cas9 system for drug target identification and validation.

Use of CRISPR/*Cas9* gene-editing tools for developing models in drug discovery

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Clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/*Cas9*) enables targeted genome engineering. The simplicity of this system, its facile engineering, and amenability to multiplex genes make it the system of choice for many applications. This system has revolutionized our ability to carry out gene editing, transcription regulation, genome imaging, and epigenetic modification. In this review, we discuss the discovery of CRISPR/*Cas9*, its mechanism of action, its application in medicine and animal model development, and its delivery. We also highlight how the CRISPR/*Cas9* system can affect the next generation of drugs by accelerating the identification and validation of high-value targets. The generation of precision disease models through this system will provide a rapid avenue for functional drug screening.

Introduction

Genome engineering has been used in drug discovery to identify genes that are responsible for a particular disease. The role of the identified gene is then validated in physiologically relevant preclinical animal models. Both forward and reverse genetic screen approaches have successfully identified some mutations that are responsible for a disease, such as mutations in proprotein convertase subtilisin/kexin type 9 (*PCSK9*) in cardiovascular disease; mutations in *BRCA1* in breast cancer; and mutations in fusion of breakpoint cluster region-Abelson tyrosine kinase 1 (*BCR-ABL1*) in chronic myeloid leukemia (CML) [1].

Despite the progress made in developing genome-editing tools, they have some disadvantages, such as being time-consuming, laborious, and having suboptimal precision, low efficiency, and poor scalability. With the discovery of zinc-finger nucleases (ZFNs), endogenous manipulation was undertaking that later led to the discovery of transcription activator-like effector (TALE) nucleases (TALENs) in 2010 [2]. ZFNs comprise DNA-binding domains and zinc finger proteins (ZFPs) that are fused to FokI, a DNA cleavage domain. Given that the ZFP region interacts with nucleotide (nt) triplets, different combinations of ZFPs can be designed to recognize a DNA sequence of interest. ZFNs are specific in inducing double-standard breaks (DSBs) and this specificity results from two binding events, during which FokI first dimerizes and then cleaves

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DNA. Despite the specificity of ZFNs, they require highly skilled experts to engineer them. By contrast, proteins used by TALENs are derived from repeated domains of a highly conserved bacterial TALE, which targets a single nt and is fused to a FokI nuclease. TALENs are easier to engineer compared with ZFNs and are highly efficient for genome editing. The disadvantages of TALENs are their cytotoxicity, prokaryotic origin, larger size compared with ZFNs, and their repetitive sequences, which complicate not only their incorporation into delivery systems, but also their construction. In short, both of these genome-engineering systems have drawbacks that highlight the need for a more-practical system of genome engineering. A recently discovered genome-editing system, CRISPRs, has excited the scientific community with its more-simplified genome-editing approach and has been adopted as a novel targeted genome-editing system [3]. This technology has been used effectively in various species to generate model organisms. Table 1 provides a comparison of CRISPR with other genomic tools, highlighting its characteristics that make it the method of choice for genome editing. During the past three decades, CRISPR has evolved from 'peculiar sequences of unknown biological function' into a successful genome-editing tool.

Discovery of CRISPR/Cas9

In 1987, Ishino and coworkers discovered a group of 29-nt repeats in *Escherichia coli* that were divided by nonrepetitive short sequences [4]. In 2000, another research group discovered similar repeats in other types of bacteria and in some Archaea. [5]. Two years later, short regularly spaced repeats (SRSR) were renamed 'CRISPR'. Subsequently, other scientists discovered a conserved set of genes that link with CRISPR repeats. The researchers called them CRISPR-associated (Cas) genes, which encode proteins such as DNA helicases (Cas3) and exonucleases (Cas4). At the same time, Bolotin *et al*. [6] discovered a Cas gene, which is now known as *Cas9*. They showed that *Cas9* gene encodes a large protein anticipated to have nuclease activity. Following this discovery, the same group also found the protospacer adjacent motif (PAM). With the advent of all the required components of the CRISPR machinery, many technical details relating to CRISPR technology were resolved [6].

In 2012, a major observation was published that *Cas9*–CRISPR (cr)RNA complexes of *Streptococcus pyogenes* and *Streptococcus thermophilus* might work as RNA-guided endonucleases *in vitro* [7]. These studies led to the discovery that the *Cas9*-crRNA complex is a powerful genome-editing tool that can create specific DSBs. With the discovery of its endonuclease activity, the use of CRISPR technology is expanding daily (Fig. 1a) for different genome-editing activities (Fig. 1b).

CRISPR/Cas9 mechanism of action

Depending on the type of Cas protein involved in the CRISPR machinery, there are three types of CRISPR mechanism, with the CRISPR type II system the most well studied. This system comprises *trans*-activating (tra)crRNA, pre-crRNA, and *Cas9* proteins (Fig. 2a,b). *Cas9* has two domains that are responsible for its

TABLE 1

Comparison of different genome-editing tools

Feature	ZFNs	TALENs	CRISPR/*Cas9*
Introduced (Year)	1996	2010	2013
Design	Engineering of protein for each target	Engineering of protein for each target	A 20-nt sgRNA
Mechanism	DNA–protein interactions	DNA–protein interactions	DNA–RNA interactions
Cargo	Two ZFNs for each target sequence	Two TALENs for each target sequence	*Cas9* protein with sgRNA complementary to the target sequence
Predictability	Low	Low	High
Transformation for genome-wide and library construction	Challenging technically	Challenging technically	Feasible
Affordability and cost	Expensive and time consuming	Cheap but time consuming	Highly affordable
Determinant of specificity	ZF proteins	Transcription activator-like effectors	CRISPR RNA of sgRNA
Nucleases	*Fok*I	*Fok*I	*Cas9*
Off-target effects	Moderate	Low	Variable
Cytotoxicity	Variable–high	Low	Low
Mode of *in vivo* delivery	Usually viral vectors	Usually viral vectors	Usually viral vectors, nanoparticles, or PEI-mediated transfection
Advantages	Can be programmed with modular arrays; each module recognizes triplet bps	Can be programmed with modular arrays; each module recognizes a single bp	gRNA serves as recruiter; design and construction are simple; multiplex targeting possible
Disadvantages	Specificity is influenced by adjacent ZFs; need screening of ZF library to achieve the best on-target activity and specificity	Small differences between each TALE module, hence repetitive design and difficulty in cloning; large size	Off-targets

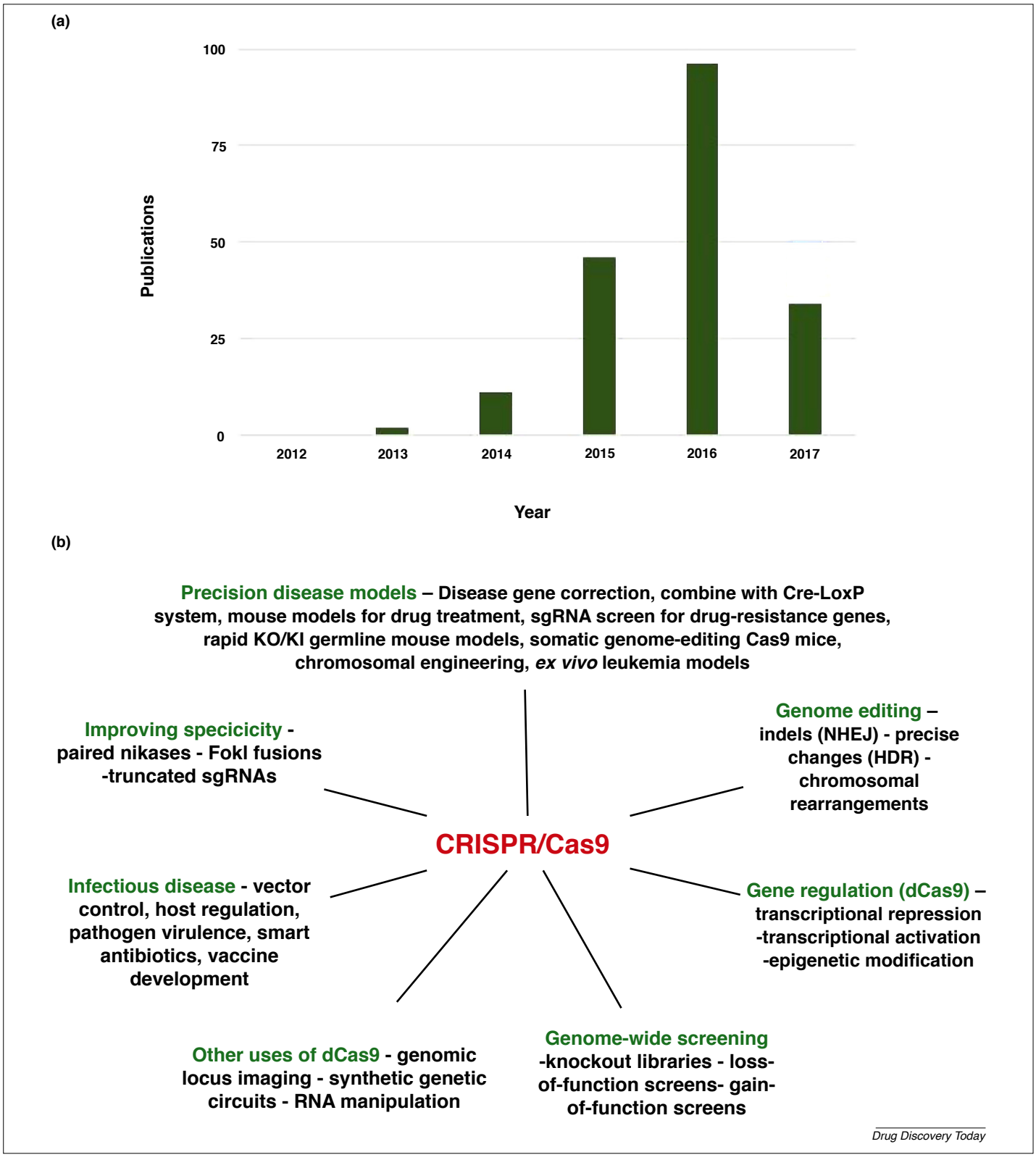

FIGURE 1

Rise in use of CRISPR/*Cas9* system in drug discovery. (a) PubMed citation hits for the words 'CRISPR/*Cas9* and drug' within the indicated years. The annual increase in the number of relevant publications from2013 until half way through 2017 indicates the interest in clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/*Cas9*) in drug discovery. (b) Application of CRISPR/*Cas9* in biomedical research: since the initial discovery and application of CRISPR/*Cas9* as a genome-editing tool, this system has found varied applications in medicine. Genetic mutations associated with altered biological functions or disease phenotype are precisely recapitulated in CRISPR/*Cas9*-mediated cellular or animal models. The CRISPR/*Cas9* system, along with its genome-editing role, can also be applied for gene regulation, genome-wide screening for drug discovery, and in infectious diseases for antibiotic or vaccine development. Abbreviations: KI, knock-in; KO, knock-out.

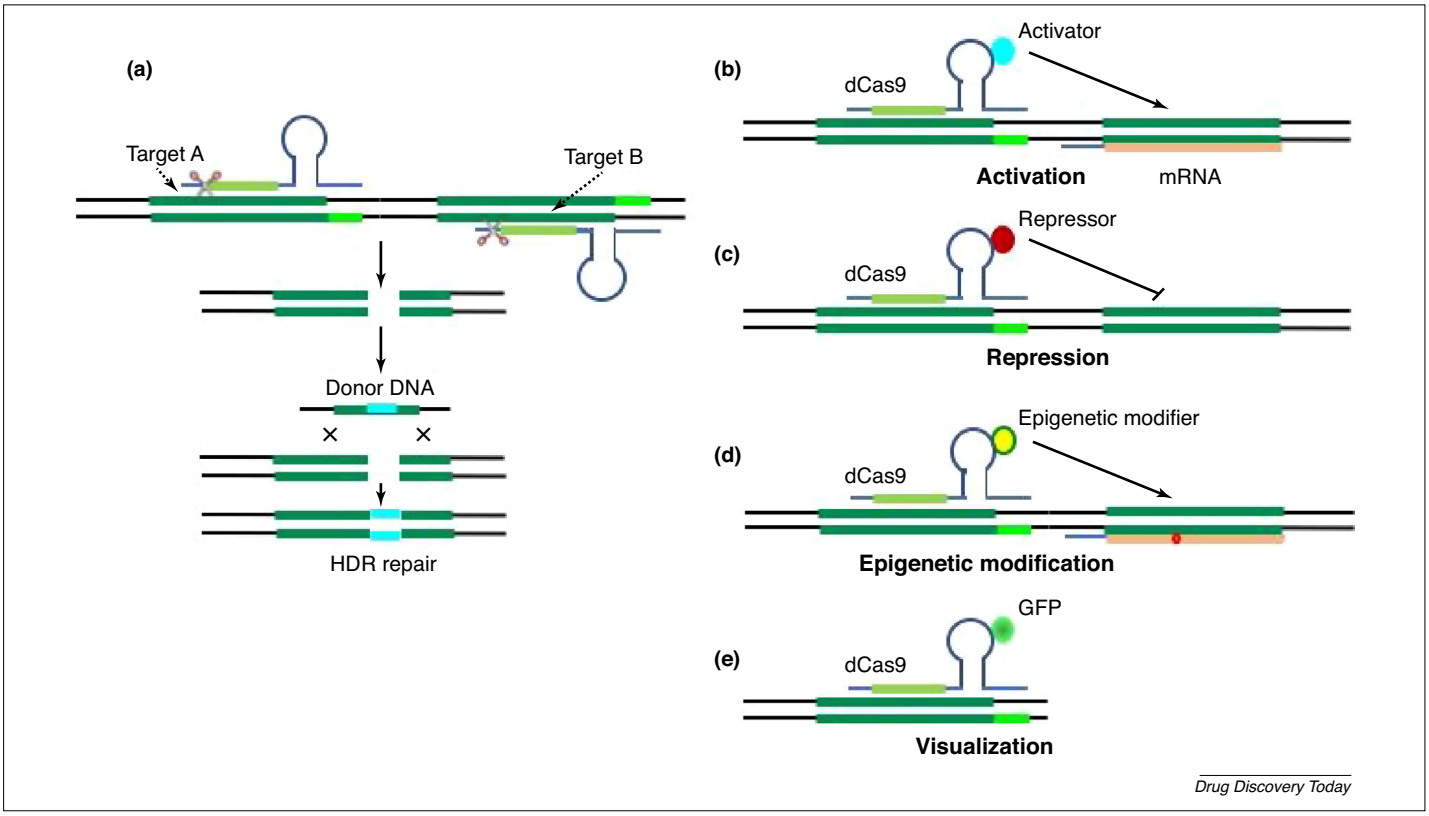

FIGURE 2

Mechanism of Genome editing through CRISPR/*Cas9*. (a) Genome editing and gene regulation by clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/*Cas9*). Following *Cas9*-induced DNA double strand breaks (DSBs), DNA is repaired through either nonhomologous end joining (NHEJ) or homology-directed repair (HDR), depending on the availability of the donor DNA template. NHEJ usually leads to small insertions or deletions (indels), whereas HDR results in the recombination of the donor DNA template into the DSB site. (b) Wild-type *Cas9* shows off-target effects that can be minimized through different strategies, such as the use of two *Cas9* nickase enzymes instead of one wild-type *Cas9*. **(c)** Transcription activation by recruiting transcription activators to the CRISPR complex in the presence of a nuclease-deactivated form of *Cas9* (d*Cas9*). (d) Transcription repression mediated by d*Cas9*. By binding to the coding sequence, d*Cas9* can block the progression of RNA polymerase, thereby inhibiting transcription. **(e)** Fusion of d*Cas9* to the effector domain enables the sequence-specific recruitment of epigenetic modifiers for epigenetic modification. (f) Fusion of d*Cas9* also allows recruitment of fluorescent proteins, such as GFP, for genome imaging.

nuclease activity: HNH and RuvC-like domain. During its mechanism of action, tracrRNA coordinates with RNaseIII to cut pre-crRNA into crRNAs. This crRNAs then interacts with tracrRNAs, helping *Cas9* to recognize the specific DNA sites. The *Cas9*–RNA complex searches for DNA sequences randomly and requires a PAM sequence (NGG or NAG motif) to interrogate the flanking DNA sequences for complementarity of guide (g)RNA. Once the target cleavage site has been recognized, the HNH nuclease domain of *Cas9* cleaves the strand that binds to crRNA, whereas the RuvC-like domain cuts the other DNA strand to generate DSBs. After the creation of site-specific DSBs, depending on the cell cycle stage of the cell or availability of a donor template, two different repair mechanisms are triggered: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). In the absence of donor templates, NHEJ conjoins the broken sequences by causing insertions or deletions (indels) in repaired sequence. These indels result in a frame shift in the reading frame and in the creation of premature stop codons. However, in the presence of a donor template, the HDR pathway generates specific mutations, insertions, or deletions.

CRISPR/Cas9-mediated genome editing

The CRISPR/*Cas9* system is a flexible and powerful tool not only for genome editing, but also for gene regulation, with research focusing on improving its applicability for each [7]. Different research groups have identified unique factors that can affect the productivity of CRISPR/*Cas9*.

Target-site selection and sgRNA design

Here, we focus on target-site selection, gRNA design, and the different online tools available to get the most out of CRISPR/*Cas9*. Given that CRISPR/*Cas9* systems are highly programmable and are not confined to genome editing, the designed single guide (sg)RNAs should be as efficient and specific as possible. For this purpose, a pool of sgRNAs needs to be screened for their activity in each new study. The accelerating use of CRISPR/*Cas9* systems for different purposes has enabled scientists to identify a range of sequence features in and around the target sequences that anticipate the efficiency of the sgRNA. For example, for expression from a U6 promoter, a 5′ end of a sgRNA that is appended with a guanine (G) is required [1]. In addition, a G in the first or second position

closest to PAM facilitates *Cas9* loading [1], and adenine (A) is preferred in the middle of the sgRNA [1]. Thus, G-rich and A-depleted sgRNAs have been reported to be both efficient and stable. Usually, mismatches of one to five base pairs (bp) at the 5′ end of sgRNAs are more tolerated than those at the 3′ end, and single and double mismatches are tolerated to various extents depending on their position along the gRNA–DNA interface. These design principles are based on the theory that G-rich sequences fold into stable noncanonical structures (G-quadruplexes) *in vivo* and contribute to sgRNA stability. However, the base preference is predominantly dependent on the target site in most cases. Researchers have also identified features of the Sp*Cas9* PAM that enhance its reproducibility [8]. For example, cytosine (C) is favored and thymine (T) is disfavored as the variable nt of NGG and in mammalian cells. An extended PAM sequence of CGGH is more advantageous for the generation of DSBs using Sp*Cas9*. Given that the NGG PAM motif occurs once every eight bp within the genome, any gene of interest can be targeted with CRISPR/*Cas9* technology. sgRNAs for different applications have different preference; for example, the sequence preference for nuclease dead *Cas9* (d*Cas9*) fusion-mediated inhibition/activation (CRISPRi/a) is significantly different from that of genome editing. In CRISPRi/a experiments, 19 nt-long sgRNAs have the highest efficiency and perform better than both truncated sgRNAs, which are 17–18-nt spacers long or elongated sgRNAs, with 20 nt spacers. With the increase in proposed criteria for sgRNA design, a large number of computational tools are now available to facilitate the design of sgRNA. Most of these tools support either the Sp*Cas9* system or multiple orthogonal *Cas9* systems from other bacteria. A comprehensive list of online sgRNA design software that enables a comparison between these systems is provided in Table 2. Of these software, CRISPR design, E-CRISPR, and CROP-IT are the most commonly used.

Off-target effects of the CRISPR/Cas9-system

Despite the promise of the CRISPR/*Cas9* system, its use is hampered by mutations resulting from off-target effects. The main sources of these off-target effects are seed sequence, PAM, delivery of *Cas9*, cell type under study, epigenetic status of the DNA being edited, and double-strand repair pathways [9]. There are different methods to detect these off-target effects, including T7E1 assays, deep sequencing, *in silico* prediction, whole-genome sequencing (WGS), Chip-seq, Guide-seq, high-throughput, genome-wide, translocation sequencing (HTGTs), integrase-defective lentiviral vectors (IDLV), digested genome sequencing (digenome-seq), fluorescence *in situ* hybridization (FISH), and circularization for *in vitro* reporting of cleavage effects by sequencing (CIRCLE-seq) [9–11]. Even though our ability to detect these off-target effects is improving, these approaches can not precisely detect off-target effects *in vivo*. For example, a recent study showed that the same sgRNA that repaired a mutation leading to blindness in mice also introduced several unanticipated mutations into the genome, emphasizing the need for off-target assessment [12]. Off-target effects can be minimized by the following strategies: sgRNAs in genes, enhancers, and promoters should be chosen as far as is possible to improve the target cleavage efficiency; the amount of *Cas9* and sgRNA that will be delivered for genome editing needs to be titrated; the D10 mutant nickase version of *Cas9* should be used instead of wild-type and should be paired with two sgRNAs that will each cleave only one strand [9,13]. The specificity of target DNA editing can also be improved by fusing catalytically inactive *Cas9* with a FokI nuclease domain. Thus, further improvements in the specificity of the Crispr/*Cas9* technology will drive forward both basic and clinical research.

Applications of CRISPR/*Cas9*

The CRISPR/*Cas9* system has shown great promise in different genome-editing applications. Here, we briefly summarize these applications, with a focus on it use in generating *in vivo* models and drug discovery. Although this genome-editing tool has mostly been used in reverse genetics research to determine the role of various genes in different diseases, it has also been used to generate disease models for genetic disorders in many animal models. Its easy of use also makes large-scale genome screening possible to explore gene function. Along with its use in regular genome modifications, wild-type *Cas9* nucleases can also be turned into

TABLE 2

Online tools for designing CRISPR/*Cas9* gRNA[a]

Tool	Online source	Institution	Refs
ZiFiT	http://zifit.partners.org/ZiFiT	MGH/Harvard, USA	[85]
CRISPR design	http://crispr.mit.edu	Massachusetts Institute of Technology, USA	[86]
CRISPR direct	http://crispr.dbcls.jp	DBCLS, Japan	[87]
CRISPR RGEN tools	www.rgenome.net	Seoul National University, South Korea.	[88]
CHOPCHOP	http://chopchop.cbu.uib.no	University of Bergen, Norway	[89]
E-CRISPR	www.e-crisp.org/E-CRISP	DKFZ German Cancer Research Center, Germany	[90]
sgRNA Designer	http://broadinstitute.org/rnai/public/analysis-tools/sgrna-design	The Broad Institute of Harvard and MIT, USA	[91]
CRISPR MultiTargeter	www.multicrispr.net	Dalhousie University, Canada	[92]
CRISPR-ERA	http://crispr-era.stanford.edu/InitAction.action	Stanford University, USA	[93]
sgRNA Scorer	https://crispr.med.harvard.edu/sgRNAScorer	Harvard University, USA	[94]
CRISPRscan	http://crisprscan.org	Yale University, USA	[95]
CROP-IT	http://www.adlilab.org/CROP-IT/homepage.html	University of Virginia, USA	[96]
Benchling	http://benchling.com	Broad Institute of MIT and Harvard, USA	[97]
Deskgen	http://deskgen.com	DESKGEN™ CRISPR Libraries Cambridge, USA	[86]
DNA 2.0 CRISPR gRNA Design Tool	www.dna20.com/eCommerce/Cas9/input	ATUM California, USA	[98]
EuPaGDT	http://grna.ctegd.uga.edu	University of Georgia, USA	[99]
GenScript gRNA Design Tool	www.genscript.com/gRNA-design-tool.html	GenScript New Jersey, USA	[100]

[a] Modified from Ref. [84].

domain dead versions (d*Cas9*) by simply inactivating the catalytic domains (Fig. 2c–f).

CRISPR/Cas9-mediated gene knockout

CRISPR/*Cas9* can be used to generate knockout (KO) cell lines and *in vivo* animal models. For example, CRISPR/*Cas9* based on lentiviral vectors can be used to generate KO cell lines by depleting one or more genes simultaneously. These cell lines can then be confirmed by the use of a suitable loss-of-function assay. For example, KO of *Nanog* and *Nanogp8* in prostate cancer DU145 cells led to a significant loss of malignant potential, indicating that both these genes function as oncogenes in prostate cancer [14]. Similarly, KO of three tumor-suppressor genes (*Kras*, p53 and *Lkb1*) and introduction of a point mutation in Kras *G12D* at its genomic locus by using an adeno-associated virus (AAV)-9 vector significantly increased lung tumor growth in *Cas9* transgenic mice [15]. Given the expansion in the use of CRISPR/*Cas9*, different gRNA libraries are now available for genome-wide loss-of-function studies. For example, a lentiviral vector-based genome-scale library with 122 417 sgRNAs was built to target 19 052 human genes. Similarly, to address tumor growth and metastasis in lung cancer, 624 sgRNAs were screened to reveal the genes that expedite lung metastasis in a non-metastatic mouse cancer cell line [16]. More recently, a two-cell-type CRISPR screen with 123 000 sgRNAs was used to determine why tumors from some patients are resistant to immunotherapies, whereas others are nonresponsive [17]. Further validation of this study will be helpful in identifying the mechanism of immune escape and development of new immunotherapy drugs.

CRISPR/Cas9-mediated transcriptional regulation

CRISPR/*Cas9*-mediated transcriptional regulation, either as activation or repression, is an innovative approach to study loss-of-function and gain-of-function of a specific gene. Regulation is usually achieved by fusing nuclease-inactive d*Cas9* to different transcription regulation domains. For repression, d*Cas9* binds to DNA elements and keeps transcription in check. Different genome-wide transcriptional library screens of both loss-of-function and gain-of-function mutations are now available that work by targeting the promoter region guided by gRNAs. Using these screens, different drug-resistant genes were identified melanoma [18], whereas, in another study, Gilbert *et al*. used the d*Cas9*-KRAB system to repress gene expression and concluded that this strategy can be applied to genome-wide genetic screening [19]. Using a genome-wide transcriptional activation and repression library, other researchers identified various genes that are essential for cell differentiation and survival [31].

CRISPR/Cas9-mediated chromosome translocation

Recently, the CRISPR/*Cas9* system was also harnessed to address complicated diseases, such as cancer, which result from perturbation in multiple genes simultaneously and, in some cases, translocation of a chromosome region; for example, fusion of *EML4-ALK* in lung cancer [15,20]; fusion of *PAS3-FOXO1* in human alveolar rhabdomyosarcoma, [21], and fusion of *BCAM-AKT2* in ovarian serous carcinoma [23]. With advances in CRISPR/*Cas9* technology, these chromosome translocation events can be modeled in different *in vitro* and *in vivo* settings with a high degree of efficiency. As an example, by using pairs of sgRNAs that co-target genes, the paracentric inversion of *EML4–ALK* [22], pericentric inversion of *KIF5B–RET* [23], and translocation of *CD74–ROS1* [20] have been modeled in human lung adenocarcinoma cell lines. A similar approach was used in acute myeloid leukemia and in Ewing's sarcoma to model *RUNX1–ETO* and *EWSR1–FLI1* translocations, respectively [24].

CRISPR/Cas9-mediated epigenetic control

Progress has been made in the use of the CRISPR/*Cas9* system in epigenetic control. Complex genome functions are controlled by the highly dynamic landscape of different epigenetic scenarios. The demarcation of epigenetic modifications, such as histone acetylation and DNA methylation, inside a mammalian cell is maintained and established by enzymes. For example, epigenetic modifications that fine-tune histones are not only critical for the regulation of transcription, but also have a major role in various biological functions. Other genome-editing tools have been used on a small scale for targeting epigenetic-modifying enzymes [25]. However, using the CRISPR/*Cas9* system, *Cas9* epigenetic effectors (epi*Cas9*s) can now be used to install or remove specific epigenetic marks at specific loci.

Application of CRISPR/Cas9 in genomic structure studies

The CRISPR/*Cas9* system has also been used to envision endogenous genomic loci in living cells to track their dynamics *in vivo*. Chen *et al*. were able to label the DNA sequence, complementary to the sgRNA in mammalian cells, by fusing GFP to d*Cas9* [26]. A year later, another study group enhanced the fluorescent signal for CRISPR imaging using the d*Cas9*-SunTag system. [27]. Along with the dynamic tracking of endogenous genomes, this genome-editing technology can also inform about the proteins that are bound to DNA. This is done by fusing d*Cas9* with affinity protein tags and immunoprecipitation to pull-down the proteins. These bound proteins are then characterized by proteomic studies.

Therapeutic applications of CRISPR/Cas9

The pathogenesis of human diseases, such as hereditary movement disorders, neurodegenerative disorders, infectious disease, cancer, and many others, has been linked to the malfunctioning of different genes. For the development of gene therapy to treat such diseases, there is a need an easy-to-use and precise genome-editing tool. CRISPR/*Cas9* fulfills these criteria and has shown promising results in this regard. Examples include gene encoding the fumarylacetoacetate hydrolase (*Fah*) in hereditary tyrosinemia [28], *Crygc* in cataracts [29], *DMD* in Duchenne muscular dystrophy (DMD) [30], *HBB* in β-thalassemia [31], *CFTR* in cystic fibrosis [32], and *SERPINA1* in α1 antitrypsin deficiency [33] (Table 3). In addition, Huntington's disease (HD) [34], autosomal dominant cerebellar ataxia [35], spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 [36], Parkinson's disease (PD) [37], Alzheimer's disease (AD) [37], amyotrophic lateral sclerosis (ALS) [37], HIV [38], and cancer [39] are also potential therapeutic targets for CRISPR/*Cas9* technologies [40]. Furthermore, in June 2016, the US National Institutes of Health (NIH) approved, for the first time, the use CRISPR/*Cas9* to edit T cells in human clinical trials for cancer therapies [41].

TABLE 3

Potential of CRISPR/*Cas9* in gene therapy[a,b]

Disease	Targeted gene/DNA	CRISPR correction approach	Model organism	Refs
DMD	*Dystrophin*	Injection into zygote of gRNAs, *Cas9* mRNA, and ssODN as a template for HDR-mediated gene repair	Rat, Rhesus monkey, mouse	[30]
Cystic fibrosis	*CFTR*	Cotransfection of plasmid expressing *Cas9* and sgRNA together with a donor plasmid encoding wild-type *CFTR* sequences	Intestinal stem cells	[32]
Hereditary tyrosinemia	*FAH*	Lipid nanoparticle-mediated delivery of *Cas9* mRNA with AAVs encoding an sgRNA and a repair template, or hydrodynamic tail vein injection of plasmids expressing *Cas9*, sgRNA, and ssDNA donor	Mouse	[43]
Cataract	*Crygc*	Injection into zygotes of *Cas9* mRNA, sgRNA, and ssODN as a template for HDR-mediated gene repair	Mouse	[56]
Lung cancer	*KRAS*, *p53*, and *LKB1*	Intranasal and intratracheal delivery of AAV	Mouse	[39]
Selinexor resistance in cancer	*XPO1*	Transfection with neon transfection system	T-ALL Jurkat Clone E6-1 cells	[7]
Major mental illness	DISC1	Frame shift mutation in exon2 (homozygous); frame shift mutation in exon8 (homozygous/heterozygous)	iPSCs	[37]
Autism	*CHD8*	KO (heterozygous)	iPSCs	[35]
HD	*HTT*	Insertion of 97 CAG repeats into exon 1	iPSCs	[34]
Microdeletion and micro duplication syndromes	16p11.2 and 15q13.3 copy number variants	575-kb deletion, 740-kb deletion, 740-kb insertion	iPSCs	[48]
Epilepsy	*SCN1A*	Insertion of td tomato into GAD67 to fluorescently label GABAergic neurons	iPSCs	[49]
Fragile X syndrome	*FMR1*	Deletion of CGG repeats at the 5′-UTR of FMR1	iPSCs	[50]
β-Thalassemia	*bglobin*	Homologous recombination mediated by footprint-free piggyBac system	iPSCs	[51]
Urea cycle disorder	*OTC*	One AAV expressing *Cas9* and another expressing gRNA and donor DNA	Mouse	[79]
Walker–Warburg Syndrome	*Ispd*	Injection of into zygotes *Cas9*n RNA with sgRNAs together with a single DNA template encoding two loxP sites	Mouse	[102]
Hepatitis B virus (HBV)	HBV cccDNA	Plasmid transfection or lentiviral transduction for *in vitro* assays; hydrodynamic injection of plasmids encoding *Cas9* and sgRNAs for *in vivo* assays	Huh7, HepG2.2.15 cells and mouse	[103]
HIV-1	HIV-1 LTR	Transfection of plasmid encoding *Cas9* and sgRNA	Microglial, promonocytic, and T cells	[38]
Epstein–Barr virus (EBV)	Latent EBV	Nucleofection of plasmid encoding *Cas9* and sgRNA	Human Burkitt's lymphoma cells	[52]
Human papillomavirus (HPV)	HPV oncogenes E6 and E7	Transfection of plasmid encoding *Cas9* and sgRNA	Human cervical cancer cells, SiHa	[53]

[a] Modified from Ref. [101].

[b] Abbreviations: cccDNA, covalently closed circular DNA; CFTR, cystic fibrosis transmembrane conductor regulator; Crygc, crystallin gamma C; Dmd, dystrophin; FAH, fumarylacetoacetate hydrolase; HBB, hemoglobin beta; LTR, long terminal repeat; OTC, ornithine transcarbamylase; sgRNA, single guide RNA; ssDNA, single-stranded DNA; ssODN, single-stranded oligodeoxynucleotide.

CRISPR/*Cas9* library screens for drug target discovery

The identification of unknown genes and determination of their function is usually done using high-throughput genetic screening. By using this approach, one can determine which genes are responsible for a particular phenotype and, hence, could used for drug target discovery.

RNAi screens for drug target discovery

Many basic biological mechanisms and signaling pathways have been discovered through DNA mutagenesis-based genetic screens. However, these screenings have some major issues, such as heterozygous mutants formed from these screens with unknown random mutations. The advent of RNAi helped to overcome these limitations by targeting specific mRNA molecules for degradation. RNAi-based high-throughput genetic screens have provided important information about gene function, although are still hampered by limitations such as inefficient knockdown and major off-target effects.

Cell-based CRISPR/Cas9 screens

CRISPR/*Cas9* systems have been used extensively in large-scale functional genome studies [42]. These systems have advantages, such as the complete loss-of-function mutations in genomic DNA instead of partial loss of protein expression and can target the

whole genome, including enhancers, promoters, introns, and intergenic regions [1]. CRISPR/*Cas9* can also be used to create viral sgRNA libraries by synthesizing target specific oligonucleotides that are then cloned as a pool to generate a viral library from which viral particles are produced to transduce cells [16,43]. CRISPR/*Cas9* KO, CRISPR-mediated repression (CRISPRi) and activation (CRISPRa) libraries have made the entire genome available for loss–unction and gain-of-function screens. Both positive and negative selection screens can be performed using CRISPR/*Cas9* systems. Positive selection screens are usually performed to identify genes that can render an organism resistant to treatments. For example, disruption of *HPRT1* through CRISPR/*Cas9* or point mutations in *ERCC3* rendered cells resistant to 6-thioguannie and triptolide [44]. Similarly, CRISPR/*Cas9*-mediated C528S mutation of *XPO1* provided resistance to selinexor, suggesting that XPO1 is a drug-resistant target [7]. By contrast, negative selection screens are usually used to address factors that render cells unfavorable for selection [43]. Different groups have carried out research to identify genes that are required for cell survival in different models. Along with CRISPR with wild-type *Cas9*, CRISPRi and CRISPRa have been explored as tools for functional genome screening to modulate gene expression. Through these mediations, instead of inactivating genes through indels after DSBs, CRISPRi specifically and efficiently inhibits the transcription of target genes. By tethering d*Cas9* to a transcriptional activation domain, it can activate the expression of target endogenous genes [19]. Both CRISPRi/a libraries were also applied on a large genome scale to identify mediators for cellular sensitivity to a cholera–diphtheria fusion toxin.

CRISPR/Cas9 screens in cancer

CRISPR was applied for upregulating long noncoding RNA transcripts and identifying genes that cause resistance to a BRAF inhibitor in melanoma [43]. The efficiency of such screens can be further improved by using cell lines that express stable *Cas9* [45]. Primary cells generated from *Cas9* transgenic mice can be used for this purpose. Current research is focusing on the generation of CRISPR sgRNA library-based KO mice for genetic screening [16,42]. One research group applied a pooled CRISPR screening approach to generate bone marrow-derived dendritic cells from *Cas9* mice and screened them for regulatory factors of innate immune circuits that are responsible for host responses to pathogens [46]. The pooled *Cas9*-sgRNA-integrated cell lines can also be used *in vivo* to assess different physiological outputs. Targeting specific sites also affects the efficiency of CRISPR/*Cas9*. For example, targeting of exons that encode functional protein domains generates more null mutations. Such CRISPR screens are usually applied to cancer studies [16]. Known oncogene addictions have also been successfully uncovered through CRISPR screens. For example, in the chronic myelogenous leukemia cell line KBM7, *BCR* and *ABL* are lethal hits that harbor a BCR–*ABL* translocation [1], while, in the colorectal cancer cell lines DLD-1 and HCT116, *KRAS* and *PIK3CA* are lethal hits [19]. CRISPRa screens in A375 cells showed that BRAF inhibition can be bypassed by overexpressing those genes that reactivate the mitogen-activated protein kinase (MAPK) pathway [45]. Thus, CRISPR/*Cas9* is a powerful tool for the creation of isogenic cancer cell lines that harbor defined, combinatorial genetic lesions.

Development of disease models using CRISPR/*Cas9*

iPSCs as models for gene manipulation

Genetically modified cells and animal models have proven critical for understanding the function of a gene and its role in the pathogenesis of human diseases. Compared with other molecular genetic approaches, the CRISPR/*Cas9* system is expediting the development of biological research tools. Cellular models are generated via CRISPR/*Cas9* systems by introducing plasmids carrying *Cas9* and sgRNA into target cells [45]. Among such models, induced pluripotent stem cells (iPSCs) are a valuable tool for the study of particular mutations. In 2015, scientists developed a cellular model of Friedreich's ataxia, which results from reduced levels of the gene encoding frataxin [47]. Given that iPSCs have the ability to differentiate in almost any kind of cell in the body, by carrying out genome editing in these cells, one can study genetic variants in different types of tissue in a cell culture dish [34,35,48–51]. The main use of iPSCs is in regenerative medicine, where these are useful in replacing diseased or unhealthy cells with healthy cells [34,35,48–51]. For this purpose, primary cells are obtained from the patient, genetically manipulated with CRISPR/*Cas9* technology, differentiated into identical cells and then put back into the same patient. Different groups have used CRISPR/*Cas9* systems to correct genetic mutations in patient-derived primary cells [7,32,38,52,53]. iPSCs with KOs for different genes have also been used in loss-of function studies [46]. CRISPR/*Cas9* systems have also been utilized to introduce specific mutations into iPSCs through HDR-mediated genome editing [33]. Taken together, the use of CRISPR/*Cas9* systems in iPSCs has augmented our knowledge of diseased primary cells (Tables 3 and 4).

Mice as models for gene manipulation

Along with *in vitro* models, different *in vivo* models are also available for genetic modification. Among them, mice model are the most-commonly used. Genetically modified mice are crucial for understanding not only the function of a gene, but also the cause of pathogenesis in human diseases. Traditional transgenic mouse models are generated through homologous recombination in mouse embryonic stem cells (ESCs) [54]. These cells are then microinjected into murine blastocysts for germline transmission. However, this process is time consuming. The CRISPR/*Cas9* system serves as a simple, fast, more precise, and efficient technology in this regard, through which more than one gene can be edited simultaneously in the germline or zygote stage to generate genetically modified mice [22,55]. This system has also been used to generate transgenic mouse models using other approaches, as discussed below.

Germline CRISPR mouse models

Manipulation of a single gene or many genes simultaneously can be accomplished through CRISPR/*Cas9* in the germline and also at the zygote stage (Fig. 3a) [22,54,56]. This can be performed in different ways; for example, by injecting sgRNAs and *Cas9*-encoding mRNA into the fertilized eggs of mice that efficiently produce mice carrying biallelic mutations either in one or more genes [57]. The same research group also showed that CRISPR/*Cas9* can be applied for precise HDR-mediated genome editing [57]. The authors expanded this approach to generate mice carrying a conditional allele, a reporter gene, and a tag in endogenous genes in a one-cell zygote [39]. In addition to generating germline mice,

TABLE 4

CRISPR/*Cas9* animal models for complex human disease phenotypes

Disease	Animal/age/tissue	Gene	Delivery	Purpose/mutation	Refs
Acute myeloid leukemia	Mice C57Bl/6/adult/HSPCs or fetal-liver HSCs	*TET2*, *DNMT3A*, *RUNX1*, *NF1*, and *EZH2/Mll3*	Intravenous injection of *Cas9*-edited human HSPCs or HSCs/*ex vivo*	Generation of mouse models of myeloid malignancies	[104]
Adrenal hypoplasia congenital and hypogonadotropic hypogonadism absent sexual maturation, DMD	Cynomolgus monkey/embryo or 5–8-years old	*DAX1 Nr0b1*, *Pparg*, *Rag1*, *DMD*	Microinjection of *Cas9* mRNA and sgRNA into one-cell monkey embryo	Generation of gene-modified cynomolgus monkey/germline	[77]
Albinism indel	*Xenopus* G0 embryos	*pdip*	Injection	Targeted gene disruption in *Xenopus*/ germline	[69]
Alveolar rhabdomyosarcoma	Mouse/limb myoblasts	*PAX3–FOXO1*	Electroporation	Modeling alveolar rhabdomyosarcoma in mouse myoblasts/*Pax3–Foxo1* chromosome translocation	[21]
Burkitt lymphoma	Adult mouse/HSPCs from EM-Myc/ Arf/-EMMyc	*Mcl1* and *p53*	Intravenous injection of *Cas9*-edited HSPCs	Dox-inducible Burkitt lymphoma model/*ex vivo*	[105]
Cardiomyopathy	Mouse *Cas9* transgenic/adult/heart	*Myh6*	Intraperitoneal injection with AAV-9	Mouse model for adult cardiac-specific gene deletion/somatic	[63]
Cataracts	Mice BALB/c/Crygc/mutant/zygotes	*Cryg*	Co-injection	HDR-induced correction of mutant *Crygc* gene/germline	[56]
Cholesterol regulatory gene modification/metabolic liver disease	Mice C57/BL6/5–6 weeks/liver; mouse spfash/postnatal day 2, 8–10 weeks/liver	*Pcsk9*, *ApoB/OTC*	AAV9/AAV8 tail vein injection	Sa*Cas9* can mediate genome editing *in vivo* with high specificity/correction of lethal human metabolic disease following *in vivo* genome editing/somatic	[79]
Cystic fibrosis	CFTR mutant intestinal stem cells	*CFTR*	Lipofectamine transfection	Functional repair of CFTR	[32]
DMD/rat modeling	Mouse C57BL/10ScSn-*Dmdmdx*/J/ zygotes/muscular skeletal tissue/ adult or postnatal	*DMD*	AAV-9/AAV-8 intraperitoneal, intramuscular, retro-orbital injection/electroporation	Correction of genetic defect in *Dmd*/ somatic or germline	[65]
	HEK293T/myoblasts	*DMD*	Lipofectamine 2000 transfection	Correction of dystrophin mutations/Indel, 336-kb deletion	[30]
	Rat Wistar-Imamichi strain/zygote, Sprague–Dawley, F344/Stm, Jcl: Wistar	*DMD/ApoE*, *B2m*, *Prf1*, *Prkdc/Rosa26 Sirpa*, *Dnmt1,3a,3b/Il2rg/*	Microinjection	Generation of rat muscular dystrophy model/disruption of four genes/germline	[68]
Friedreich's ataxia	T-REx293-cFXN cells	*FXN inducible*	Lipofectamine 2000 transfection	Cellular model to follow Friedreich's ataxia development	[47]
Gene-modified pigs/indel PD/1-nt substitution Warrdenburg disease, B cell-deficient pigs	Chinese Bama miniature pigs/ zygotes or 35-days old	*Npc1l1*, *Parkin*, *DJ1*, *PINK1,/Sox10*, IgM heavy chain gene	Oocyte (pig)/injection, somatic cell nuclear transfer (SCNT) technology	Generation of gene-modified pigs via injection of zygote/germline	[75]
Genetic deafness	Mice (Atoh1-GFP)/postnatal day 1/ inner ear cochlea	*Atoh1-GFP*	Cationic liposomes injection	Genetic deafness mouse models/somatic	[81]
Hematopoietic malignancies	$CD34^+$ HSCs and $CD4^+$ T cells transplanted into NSG	*B2 M* and *CCR5*	Electroporation	Applicability for hematopoietic cell-based therapy	[46]
Hemophilia A deficiency	iPSC cells/mouse	*FVIII/F9*	Lipofectamine 2000 transfection/ Fah mutant mouse/tail vein injection	Functional correction of large factor VIII gene in hemophilia A/chromosomal inversions/11-nt substitution	[78]
HCC and ICC	Mouse Alb-Cre, KrasLSL-G12D/. or CCl4/20 weeks	*Trp53*, *Smad4*, *Pten*, *Cdkn2a*, and *Apc*, *Brca1/2*	Hydrodynamic tail vain injection	Multiplex-mutagenesis for high-throughput functional cancer genomics in mice/somatic	[42]
Intestinal hyperplastic polyps/colon cancer	Mouse Doxycycline-inducible *Cas9* C57B6/129/ES cell blastocyst	*p53*, *Apc*, *Pten/R26-rtTA* and *col1A1*	Plasmid DNA blastocyst injection, hydrodynamic plasmid delivery	Develop conditional, genetic 'deletion' models/inducible, *Cas9* knock-in	[107]
Liver steatosis, nonalcoholic steatohepatitis, Chronic hepatitis B	Mice/NRG, FVB/NJ female C57BL/6 female/8–10 weeks/liver	*Pten/p53* and *CTNNB1*/Pcsk9/Cebpa/ *HBV*	Tail vein injection of AAV/ Hydrodynamic	Modeling human liver diseases in mouse/ indel somatic	[62]

TABLE 4 (*Continued*)

Disease	Animal/age/tissue	Gene	Delivery	Purpose/mutation	Refs
Lung metastases/DLD1	Mice$^{Nu/Nu}$/adult/right side flank/ murine cells	*KrasG12D/+; p53$^{-/-}$; Dicer1$^{+/-}$/PKCb A509T*	Subcutaneous injection	Genome-wide *in vivo* screen in mouse model of lung metastasis/correction of PKCβ A509T/xenograft	[16]
Medulloblastoma/glioblastoma/ neocortical neurogenesis	Mouse WT, Crt-CD1 (ICR)/P53-/-/WT, C57BL/6N/P0, E13.5/fourth ventricle or cerebral ventricular zone	*Patch1/Pten*, *Trp53*, *Nf1/Eomes/Tbr2*	PEI transfection/electroporation	Brain tumor model through somatic gene transfer/somatic	[60]
Mouse models	Mice B6DBAF1 and B6-EGFP, CD1, C57BL/6NJ CB6F1/embryos	*Tet1/2/3*, *B2m*, *Il2rg*, *Prf1*, *Prkdc*, *Rag1*, *Smg9*, *Tenm1*, *F9/Y371D*, *bglobin Pcdh cluster*, *Notch1*, *Mecp2*	Oocyte (mouse)/injection	Generation of different immunodeficient mice/germline	[54]
Mouse therapeutic models	Mice Fah^{mut}/^{mut}/8–10 weeks	*Fah*	Tail vein nanoparticle injection	Therapeutic genome editing by combined viral and nonviral delivery *in vivo*/somatic	[43]
Model generation	HEK293T, murine erythroleukemia (MEL) cells	*EMX1*, *PVALB*, and *Chr.14*	Electroporation/transfection	Precise cleavage at endogenous genomic loci in human and mouse cells/inversion, deletion	[45]
Non-small cell lung cancer	HEK 293T cells	*CD74–ROS1* and *EML4–ALK*	Basic epithelial cell/transfection	Targeted genomic rearrangements/ translocation, Inversion	[20]
	Mouse Cre-dependent *Cas9* knock-in or KrasLSL-G12D, p53^{fl}/^{fl} or KrasLSL-G12S/C57BL/6J × 129SvJ/ adult/Lung	*p53* and *Lkb1*, *Kras/Nkx2.1*, *Pten*, *Apc*	AAV intratracheal delivery	*In vivo* rapid functional investigation of candidate genes/somatic	[39]
	Mouse WT, p53$^{+/-}$ or p53$^{-/-}$; CD1 and C57BL/6J (B6)/8 weeks/lung	*Eml4–Alk*	Adenovirus, lentivirus intratracheal delivery	*In vivo* generation of chromosomal rearrangements/translocation in lung	[15]
Pancreatic cancer	Mouse C57Bl/6/KT, H11LSL-G12D/, KrasLSL-G12D$^{-/+}$; R26LSL-Tom/ zygote	*Lkb1*, *Apc*, *Arid1a*,*1b*,*5b*, *Atm*, *Brca1*,*2*, *Cdkn2a-1*β,*2a2*,*2b*, *Trp53*, *Pten*, *Smad4*	Micronuclear injection	Pancreatic cancer modeling/somatic, *Cas9* knock-in	[22]
Pancreatic ductal adenocarcinoma	Mouse/Kras^{+}/LSL–G12D; Trp53loxP/ loxP/adult/pancreas	*p53*, *Kras G12D*, and *p57*	Lentiviral injection	Potential epigenetics-based therapy for pancreatic ductal adenocarcinoma/somatic	[23]
Rett syndrome/learning/memory	Mouse C57BL/6N/12–26 weeks/ brain dorsal dentate gyrus	*Mecp2*, *Dnmt1*, *Dnmt3a* and *Dnmt3b*	Stereotactic delivery/AAV injected	Enable reverse genetic studies of gene function in the brain/somatic	[61]
Tyrosinase disorders	Rabbit Dutch belted/embryos	*TYR*	Microinjection	Generation of rabbits carrying a targeted allele/germline	[73]
Tyrosinemia type i hereditary tyrosinemia	Mouse Fah^{mut}/^{mut}/adult/liver	*Fah*	Hydrodynamic tail vein injection	Correction of Fah mutation in mouse hepatocytes/somatic	[28]
β-Thalassemia	iPSCs/CD1 mice	*HBB*, *b41/42*	Electroporation/transfection	Reprogram fibroblasts of patient with β-thalassemia into transgene-free naive iPSCs/4-nt insertion	[106]

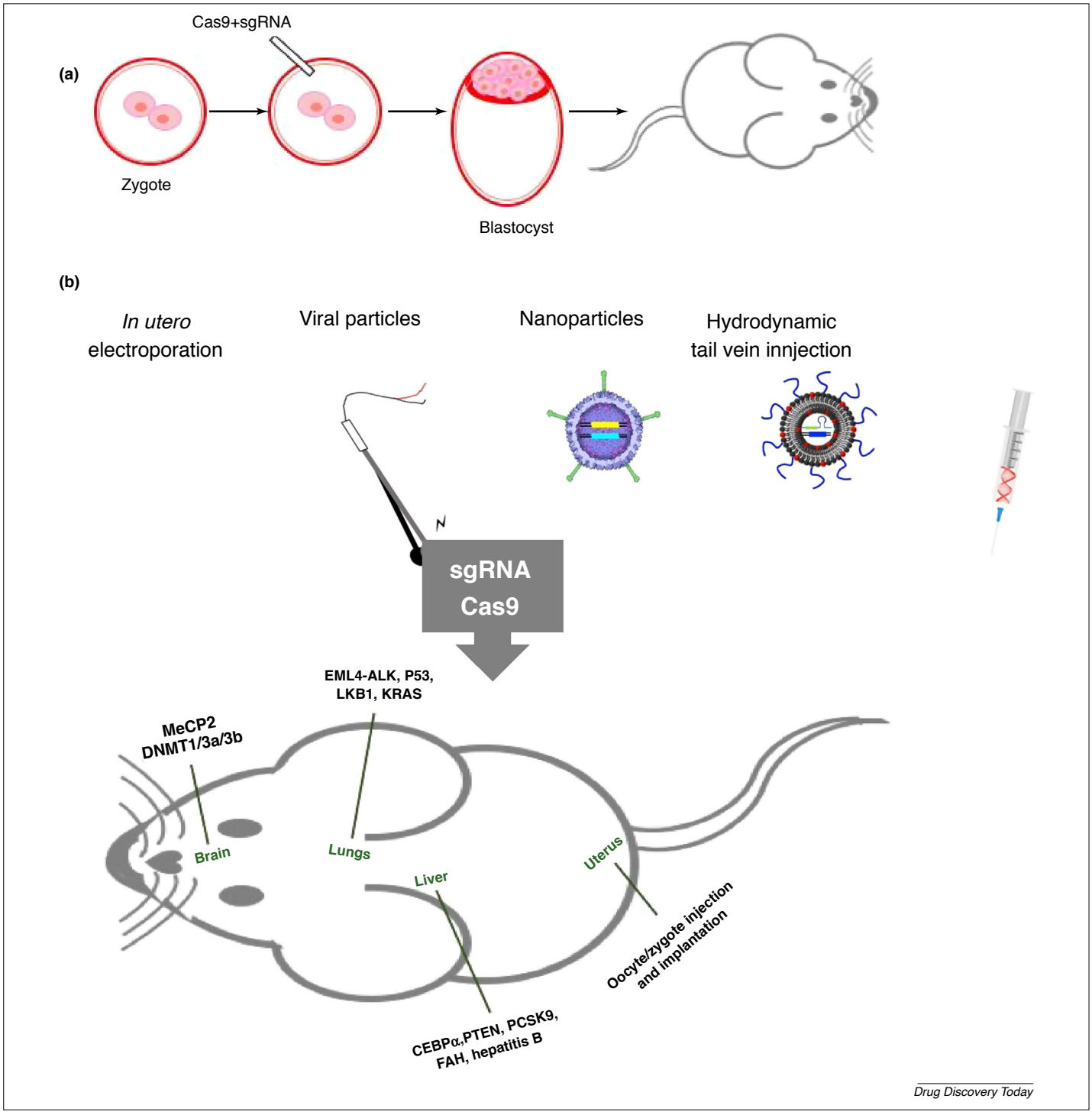

FIGURE 3

Generation of different mouse models through CRISPR/*Cas9*. (a) Germline clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/*Cas9*) mouse model: single guide (sg)RNA and *Cas9* are microinjected into mouse zygotes to generate germline mouse models. Mice generated through this approach can either have homology-directed repair (HDR)-mediated repair or carry indels that can cause mosaicism. (b) A somatic CRISPR mouse model: schematic of different approaches for the delivery of *Cas9* and gRNA to different mouse tissues. For example, hydrodynamic injection to the liver, *in utero* electroporation to brain, and viral or nanoparticle delivery to various mouse tissues. Different genes indicate the use of CRISPR/*Cas9* in gene therapy in such models.

this CRISPR/*Cas9*-mediated mouse-generating approach has also been used in animal models of genetic diseases for precisely correcting disease-associated alleles. For example, correction of cataracts was accomplished by co-injecting donor template RNA along with sgRNAs and *Cas9*-encoding mRNA [56]. Similarly, a muscular dystrophy phenotype was also rescued by injecting single-stranded (ss)DNA oligonucleotides along with sgRNAs and *Cas9*-encoding mRNA into zygotes of mdx mice, which harbor a mutation in the gene encoding dystrophin (*Dmd*) [58]. Given its simplicity, CRISPR/*Cas9* has become a method of choice for ge-

nome editing by 'in-house' transgenic core facilities and even by some less-experienced laboratories. Although it is a simple and quick approach to generating mouse models, the germline method does have drawbacks; for example, it cannot be used to generate nonsynonymous mutations or to study homozygous disruption of genes because the compound mutant mice will show allelic segregation in the F1 generation and genetic mosaicism because of the suppression of transcription and translation activity in the zygotes. Thus, *Cas9* mRNA translation into the active enzyme is delayed until after the first cell division [22,54,56,57].

Transplantation-based CRISPR mouse models

iPSCs or other cells, such as stem and progenitor cells, can be genetically manipulated *ex vivo* and then transplanted into a syngeneic recipient animal. Different research groups have shown success in this regard. For example, Malina *et al*. deleted *p53* by using sgRNAs targeting *p53* and compared the efficiency of this approach with one using short hairpin RNA (shRNAs); the authors found that the CRISPR-induced *p53* deletion was able to cause Em-Myc lymphomas *in vivo,* which was comparable to cells from *p53*-null animals [3]. By using pooled CRISPR lentivirus, other group mutated eight genes simultaneously in myeloid cancer lines [46]. This CRISPR-mediated transplantation-based *in vivo* approach is also applicable to high-throughput genome-wide screening for identifying genes that enhance metastasis and promote tumor growth [42,46].

Exogenous delivery-based CRISPR mouse models

Direct *in vivo* delivery of CRISPR/*Cas9* components into tissues of postnatal mice can be used to precisely manipulate multiple genes (Fig. 3b) [43,59]. This approach is more successful than transplant methods at depicting the sporadic nature of tumor initiation [60]. For example, deletion of a single *Ptch1* gene through direct delivery to a mouse brain led to the development of medulloblastoma, whereas deletion of three genes (*Trp53*, *Pten* and *Nf1*) simultaneously from a mouse brain caused glioblastoma [60]. In another brain study, scientists showed the tissue-specific editing of multiple genes through microinjection of CRISPR AAV into mouse hippocampus [61]. The same methodology has also been applied to other mouse tissues to generate model animals. For example, a liver tumor model was generated by hydrodynamic injection of a plasmid encoding *Cas9* and sgRNAs that target P*t*en and *p53* in the liver [62] and functional gene correction *in vivo* was achieved by co-injecting a donor template for oncogenic point mutations in *CTNNB1* [28]. Other mouse liver models have also been established through viral delivery [59]. Similarly, hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) models have also been generated for large-scale screening [42]. Cardiomyopathy disease models have also been established by generating cardiac-specific *Cas9* transgenic mice and delivering an sgRNA to target myosin heavy chain 6 (Myh6) [63]. Lung cancer models have also been established through exogenous delivery of *Cas9* sgRNA containing lentivirus to lung epithelium [64]. Along with establishing valuable mouse models, this exogenous delivery approach has also been used for therapeutic applications of CRISPR. For example, in DMD disease therapy, AAV-mediated delivery of either *SaCas9* or *SpCas9* and sgRNA to skeletal and cardiac muscle cells enabled deletion of the mutated exon from *Dmd* [65]. Similarly, a mouse model of hereditary tyrosinemia type I (HT1) was corrected *in vivo* using *Cas9* along with a wild-type donor template of *Fah* [28].

Inducible CRISPR mouse models

Naked DNA or viral elements cannot manipulate genes in certain cells and tissues, whereas CRISPR/*Cas9* can be used to this end. CRISPR-mediated conditional transgenic adult mouse models have been established that are capable of inducing tissue-specific *Cas9*-dependent mutagenesis [39,54]. A Cre-dependent CAGs-LSL-*Cas9* knock-in transgene mouse model is a prominent example of this methodology [39]. This approach has also been extended to generate inducible germline animals. CRISPR/*Cas9* has also revolutionized traditional Cre-driven mouse models by the inclusion of CRISPR-mediated targeting. Simple CRISPR-mediated modification in these Cre-dependent models enables them to express *Cas9* downstream of strong promoters, such as CAGs [54]. Such inducible models are able to target either single or multiple genes in both individual and multiples tissues and show the same phenotypes as those reported from conventional gene KO models [3]. These inducible models are not restrained by the mode of sgRNA delivery used to abolish expression of *Cas9* after manipulation of the gene of interest. Similar to exogenous delivery approaches, if *Cas9* is integrated into the genome stably, it can aid the packaging of *Cas9* cDNA into viral cassettes because not all viral entities can accommodate this package [61]. However, inducible models have some flaws, such as mosaicism induction from the *TRE3G* promoter and unexpected cellular outcomes in the *LSL-Cas9* mouse because of the constitutive and strong expression of *Cas9* [54]. These drawbacks are expected to be resolved soon as a result of the discovery of smaller *Cas9* derivatives that would be activated by small molecules in both inducible and transient mouse models.

Translocation-based CRISPR mouse models

Not all genetic disorders are caused by the simple mutation or deletion of individual or multiple genes; some diseases, such as cancer, are sometimes caused by complex genome anomaly, such as inversions, deletions, and translocations of large chromosome fragments [15]. Modeling such complex modalities is not easy with older genome-editing tools [2]. CRISPR/*Cas9* has recently been used to reproduce such complex disease models. For example, the *EML4–ALK* intrachromosomal inversion in lung cancer *was* modeled through CRISPR-mediated genome targeting [16,39]. To explore this mechanism of disease further, other research groups used CRISPR/*Cas9* tools for the intratracheal delivery of lentiviral or adenoviral constructs to target both *Eml4* and *Alk* introns and generate the fusion of *Eml4–Alk* that results in the development of lung adenocarcinoma [15,16,39]. In a nutshell, CRISPR/*Cas9* will be instrumental to further explorations of the causal relationships between human diseases and genomic structural variations through the generation of relevant mouse models.

Other animals as models for gene manipulation

Application of CRISPR/*Cas9* is not confined to cells and mice for generating human disease models; it has also been used to generate transgenic models of other organisms, such as zebrafish [66], rat [67,68], *Xenopus* [69], sheep [70], goat [71], rabbit [72,73], pig [74,75], and monkey [76,77]. These animal models can also be used to explore the mechanism of pathogenesis of different diseases. Large animal models that are physiologically and genetically closer to humans will serve as better models for not only modeling human diseases, but also developing therapeutic strategies against such diseases. For example, following CRISPR-mediated genome

editing, the porcine kidney epithelial cell line PK15 showed a 1000-fold increase in the transmission of a retrovirus to human cells, emphasizing the clinical application of organ xenotransplantations from pigs to human [74,75]. CRISPR-mediated monkey models are also available to study different diseases, such as hypogonadotropic hypogonadism and X-linked adrenal hypoplasia congenita [76,77]. Transgenic pig models have also been suggested for studying neurodegenerative disorders, such as HD and PD [75].

Delivery of CRISPR/*Cas9* constructs

To get the maximum out of CRISPR/*Cas9* technologies, along with refining their precision and efficiency, the delivery methods used need to be improved. Delivery methods differ depending on the cell type and application approach. For example, for small-scale regular application in cells, plasmids encoding sgRNA and *Cas9* are delivered through transient transfection modes, such as lipofectamine, nucleofection, and electroporation [20,21,45,46,78], because continuous expression is not needed once the desired genome manipulation is performed. However, transient transfection might not be suitable for repression assays because CRISPRi only affects transcriptional efficacy rather than both mRNA and protein stability [61–63,79]. Therefore, viral applications are better suited for CRISPRi. For large-scale high-throughput screening, delivery is carried out with a stable *Cas9* effector cell line using lentiviral and retroviral vectors before the introduction of a pool of sgRNA into these cells [1]. Given that a single sgRNA per cell is needed to minimize the signal:noise ratio, different viral vectors with low frequency are used for large-scale sgRNA-expressing arrays, including integrase-defective lentiviral vectors (IDLVs) [15], adenoviral vectors (AdVs) [15], and AAVs [61]. Viral deliveries are also used in most gene therapy studies. Among viral deliveries, AAV-based vectors are preferred because of their lack of pathogenicity, mild immune response, which depends on the recognition of unmethylated CpG dinucleotides in the bacterial DNA, and their ability to target nondividing cells [61]. However, the large size of regular *Cas9* limits the use of AAV-based delivery methodologies, for which shorter 1-kb coding *dCas9* was recently developed [80]. Other approaches have also been explored to decrease cell toxicity and enhance genome modification. For gene therapy, nonviral delivery methods, such as hydrodynamic injections, have shown promising results [28,42,62]. Thus far, the recommended cellular delivery option is the use of purified *Cas9*–sgRNA ribonucleoproteins (RNPs), which allow the fast action of the RNP complex in the nucleus during its short stay inside the cell [81]. RNP delivery of *Cas9* and sgRNAs can be achieved using different ways, including electroporation [21,45,46], microinjection [68,73], lipid-mediated transfection [32,81], nucleotide transfection reagents [32], cationic lipid transfection reagents [32,43], and cationic lipid-mediated delivery [32]. Compared with vector-mediated nucleotide delivery methods, RNP delivery methods [81] are less stressful to the cell and more precise. Different nanoparticles, such as lipids, liposomes, polymeric and inorganic nanoparticles and nanoemulsions have shown good results as delivery vehicles for delivering both genes and drug proteins, especially in solid tumors because of their enhanced permeability and retention effect [32]. Nanoparticles are advantageous compared with other delivery tools because they enhance the cellular uptake of nucleic acids that are otherwise unable to get inside the cell, as well as extending the circulation of cargo after intravenous administration and preventing the nucleic acid load from being degraded. Moreover, the efficient endosome escape, protein release, and nucleus delivery achieved using nanoparticles can be harnessed for CRISPR/*Cas9*-mediated genome editing. This has already been achieved to some extent through commercially available reagents, such Lipofectamine 2000 [21,45,46]; because this reagent is positively charged, it formed a complex with the negatively charged *Cas9*/sgRNA in human U2OS cells and in transgenic Atoh1-GFP mouse cochlea [82]. Cationic lipid nanoparticles have also proven helpful for protein and gene delivery [43,81]. Further improving their intracellular release and ability to avoid degradation will enhance their delivery efficiency. Intratumoral injection of *Cas9*/sgRNA-encapsulated DNA nanoclew also showed promising results in U2OS cells, suggesting the improved precision and efficacy of this approach for genome-edited protein delivery [83].

Concluding remarks

Genome-editing tools, such as ZFN and TALENs, have been important in the development of novel therapeutics over the past few decades. However, the fast development and implementation of CRISPR-*Cas9* has brought new promise to drug discovery. This technology has become the procedure of choice for genome editing in the laboratory. CRISPR-*Cas9* has enhanced our ability to perform systematic analyses of gene function, to reproduce animal models for complex human diseases phenotypes, and as a tool for gene therapy and screening of drug target candidate genes. We believe that CRISPR will have a positive impact on real-world drug discovery and will be key to the development of the next generation of transformational therapies and treatment paradigms. Despite such promise, further improvements of this approach are required to minimize off-target effects, enhance its efficacy in primary cells, and formulate safety guide lines for both guide RNA and *Cas9* delivery in human studies. Regulatory agencies need to speed up the regulatory process to establish guidelines for the implementation of this rapidly evolving technique. Ethical issues are a main concern and also need to be addressed urgently. The establishment of guidelines for the implementation and designing of CRISPR-*Cas9*-based preclinical and clinical studies is vital to translate efforts from the laboratory to the clinic. Initially, existing gene therapy frameworks could be amended according to the needs of CRISPR-*Cas9* and then be amended to keep up with scientific advances. Once regulatory aspects are available, these will help the pharmaceutical community to speed up the process of precise drug development. The delivery of components of the CRISPR-*Cas9* machinery needs to be shifted towards nonviral modes to avoid the undesirable effects of viral vehicles. Nanoparticles have shown great potential as such delivery vehicles because of their higher efficiency and reduced adverse effects. Over the past 5 years, CRISPR-*Cas9* has demonstrated tremendous potential in both cell- and animal-based disease models and has set the stage for drug discovery and development for future clinical applications. CRISPR-*Cas9*, along with facilitating drug development, is also likely to be able to address the need for organs for transplant by exploiting different animal models. Pig models have shown promising results in this regard. In the future, CRISPR sgRNA libraries might discompose noncoding genetic

elements to dissect the function of large genomic regions. This could lead to the discovery of general promoter architectures, distant enhancers, and any additional regulatory elements that can affect protein levels. CRISPR might also be able to dissect large, uncharacterized genomic regions for sequencing studies or genome-wide association studies. In addition, d*Cas9* could be fused to epigenetic modifiers to study the effects of methylation or certain chromatin states on cellular differentiation or disease pathologies. Given its potential, it is necessary for strict control measures to be put in place for the use of CRISPR-*Cas9* to avoid its misuse and to ensure that it is only used for positive therapeutic outcomes.

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