



# CRISPR/Cas9 system: a reliable and facile genome editing tool in modern biology

Darakhshan Javaid<sup>1</sup> · Shahid Yousuf Ganie<sup>1</sup> · Younis Ahmad Hajam<sup>2</sup> · Mohd Salim Reshi<sup>1</sup>

Received: 17 March 2022 / Accepted: 17 August 2022  
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## Abstract

Genome engineering has always been a versatile technique in biological research and medicine, with several applications. In the last several years, the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology has swept the scientific community and revolutionised the speed of modern biology, heralding a new era of disease detection and rapid biotechnology discoveries. It enables successful gene editing by producing targeted double-strand breaks in virtually any organism or cell type. So, this review presents a comprehensive knowledge about the mechanism and structure of Cas9-mediated RNA-guided DNA targeting and cleavage. In addition, genome editing via CRISPR-Cas9 technology in various animals which are being used as models in scientific research including Non-Human Primates Pigs, Dogs, Zebra, fish and *Drosophila* has been discussed in this review. This review also aims to understand the applications, serious concerns and future perspective of CRISPR/Cas9-mediated genome editing.

**Keywords** CRISPR/Cas9 · Genome editing · gRNA · Double strand break · Model animals

## Introduction

The gene therapy research began in the late 1980s and early 1990s. Genome editing, often known as gene therapy, is a method of repairing or substituting an undesired gene or a dysfunctional gene inside a cell. Although, gene therapy may be applied in both animals as well as plants but it is most commonly associated with the human gene therapy. There are a number of diseases found in humans which are caused by the mutations in one or the other genes or are caused by the unfavourable expression of genes. Against these genetic diseases, the gene therapy offers enormous potential to treat or completely cure these genetic diseases in a splendid way. As a result, gene therapy has gained increasing attention from the scientific and pharmaceutical sectors [1].

There are thousands of protein coding and noncoding genes which are encoded by more than a metre of DNA in the nuclei of the human cells. A key goal of human genome research has always been to decode the activities of individual genes and define the involvement of major regulatory elements. While data from large-scale genetic research began to uncover the links between genetic variations and disorders decades ago, identifying the mechanisms that cause specific diseases in phenotypes and rectifying mutations to treat them requires changing the genome. However, the accurate knowledge regarding the genetic modification in the large genome has always remained a major challenge [2]. Conventionally, Small interfering RNA (siRNA) and Short Hairpin RNA (shRNA) have been used to modify the genome [3]. Furthermore, the technology of genetic engineering (that is, the modification of the genome at certain, predetermined locations) have made significant technological advances in the last few years and are now being used as useful resources in preclinical research that may one day help patients suffering from incurable diseases. So, the conception of the newest powerful tool for genome editing, the Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated protein 9 (CRISPR/Cas9) system, which allows one to modify or modulate a genome in a robust and versatile manner, is absolutely important for gaining a better

✉ Mohd Salim Reshi  
reshisalim@gmail.com

<sup>1</sup> Toxicology and Pharmacology Laboratory, Department of Zoology, School of Biosciences and Biotechnology, Baba Ghulam Shah Badshah University, Rajouri, Jammu and Kashmir 185234, India

<sup>2</sup> Department of Life Sciences and Allied Health Sciences, Sant Baba Bhag Singh University, Jalandhar, Punjab 144030, India

understanding about the functions of different genes in the human genome and in developing more beneficial therapeutics [4].

Programmable protein-based genome editing technologies, such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator–Like Effector Nucleases (TALENs), have traditionally been designed and widely used in the field of genetic engineering, allowing for the precise targeting and cleavage of DNA at specific genomic locations, resulting in double-strand breaks (DSBs) and ensuring genome editing [5]. These protein-based genome engineering methods, on the other hand, recognise target DNA sequences based on the protein sequences. So, tedious protein engineering and optimization are therefore required for each target sequence of DNA and delivering many of these proteins into cells for immediate multiplexed genetic manipulation is quite challenging. For such challenges, their application to large-scale genome modification or genetic tests has been minimal [2].

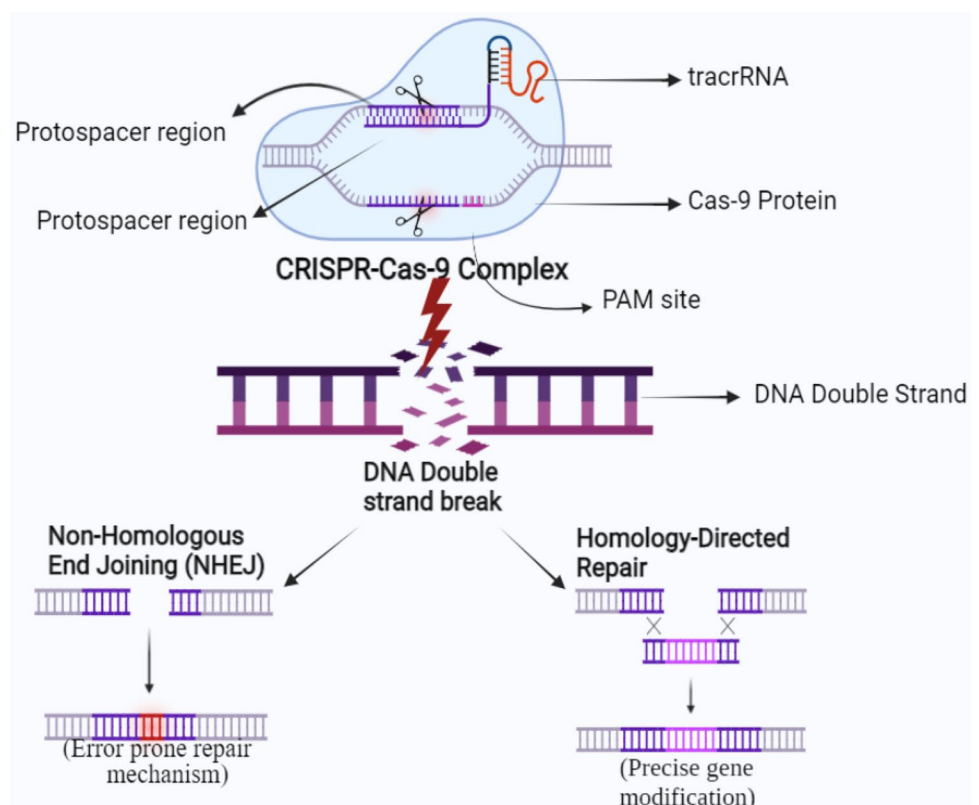
However, a newly discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated enzyme (Cas) technology and its rapid growth are changing the field of gene therapy and thereby making gene therapy really adaptable for the treatment of most of the human genetic abnormalities [1]. The CRISPR/Cas9 system, provides a straightforward RNA-guided approach for inducing specific mutations at a particular location. CRISPR is a form of genome editing technology that enables the

researchers to alter DNA. This alteration in DNA can induce changes in the phenotype of an organism, such as eye colour or any other disease vulnerability. CRISPR allows for relatively easy genome editing. CRISPR is much quicker and less expensive than other editing methods. CRISPR is democratising science by dropping the degree of complexity needed to perform practical scientific experiments, allowing everyone to participate [6].

## Structure of CRISPR

In a very short period of time after the conception of CRISPR/Cas9 system, it has transformed the entire field of targeted gene manipulation. This approach consists of two key components: Cas9, an endonuclease that induces a double-strand break (DSB) in DNA at a specified position, and a short guide RNA (gRNA) that directs Cas9 enzyme to the intended target and assures the precision and specificity of genome editing (Fig. 1) [7]. Based on the sequences and structures of the Cas protein, the CRISPR/Cas system is classified into three categories- Type-I system, Type-II, and Type-III system [6]. Only one Cas protein, Cas9, containing an NH<sub>2</sub> nuclease domain and a RuvC-like nuclease domain, is required for the type II CRISPR/Cas system [8]. CRISPR/Cas9 has indeed been a rapid and reliable method of genome editing. CRISPR/Cas loci in bacteria and archaea

**Fig. 1** CRISPR–Cas9-mediated genome engineering method. The sgRNA or crRNA–tracrRNA structure leads a Cas9 endonuclease to nearly any DNA sequence in the genome, and further instructs Cas9 to create a double-strand break (DSB) in targeted genomic DNA. These DNA double strand breaks can then be repaired either by Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR)



encode RNA-guided adaptive immune systems that can eradicate the invading DNA [9]. In case of *Streptococcus pyogenes*, the type II CRISPR locus is comprised of the Cas9 nuclease and two non-coding RNAs, TracrRNA (trans-encoded crRNA) and a pre-crRNA array comprising nuclease directed sequences separated by identical direct repeats [10]. The in vitro reconstitution of the *S. pyogenes* CRISPR system demonstrated that crRNA coupled to a normally trans-encoded tracrRNA is sufficient to lead Cas9 protein to highly selective cleavage site of target DNA sequences complementing the crRNA [11]. This reorganisation as a single transcript (single-guide RNA (sgRNA) or guide RNA (gRNA)) contains all of the features required for Cas9 association and recognition of the target sites of DNA. Cas9 may be programmed to cleave double-stranded DNA at any genomic location determined by the guide RNA sequence and a Proto-Spacer Adjacent Motif (PAM) using sgRNA. The PAM is a crucial targeting element that also functions as a mechanism that distinguishes between self and non-self and thus preventing the CRISPR locus from being targeted [12]. It has been discovered that a single mutation in the PAM may limit Cas9 cleavage activity in vitro, allowing bacteriophages to bypass the host immune response [13]. Many alternative PAM requirements exist for type II CRISPR systems, which can affect their utility and targeting efficacy. For example, a 5'-NGG-3' Protospacer Adjacent Motif (PAM) is required for the most extensively engineered system i.e., *Streptococcus pyogenes* (SpCas9), where N can be any nucleotide. So, in bacteria, CRISPR/Cas9 may be utilised without modification, but in case of humans, it needs the development of a human-codon-optimized Cas9 protein with a specific nuclear localization signal. Furthermore, the crRNA and tracrRNA must be expressed separately or as a single chimaera through the use of an RNA polymerase III promoter, such as the human U6 promoter [8, 10, 11]

## Molecular mechanism of CRISPR/Cas9 system

CRISPR uses a general defence strategy of bacteria and archaea to cleave DNA at a specified position [14]. When bacteria are infected by a virus, they record a portion of the viral DNA in their own DNA and wrap it in Clustered Regularly Interspaced Palindromic Repeats (CRISPR). By storing a piece of the virus's genetic code, the bacteria may "remember" it. When the same virus infects a bacterial cell again, it cleaves the viral DNA and kills it using the CRISPR-associated protein number 9 (CAS9). In the laboratory, the same CRISPR/Cas9 technology is used to identify and cut a specific DNA sequence by creating an RNA sequence that conforms to the DNA that is to be changed. The Cas9 enzyme (a bacterial RNA-guided endonuclease)

then scans the genome for conserved three-nucleotide species-specific protoadjacent motifs (PAM) across the target genome [15]. When the Cas9-gRNA complex binds, it recognises DNA compatibility with the guide RNA and causes site-specific DSBs to create a blunt end, generally 3 bp before the PAM region. These double-strand breaks in DNA can subsequently be repaired via either Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) (Fig. 1) [16]. Non-homologous end-joining (NHEJ) is an error-prone mechanism in which severed ends are simply ligated together. This repair route can result in tiny insertions and deletions (indels) that impair the function at cleavage site. Homology-directed repair (HDR), on the other hand, uses homologous DNA sequences as templates for perfect repair [17].

## CRISPR-Cas9 delivery and genome editing in different animal models using this technology

There are several techniques (Fig. 2) being used to introduce CRISPR-Cas9 components into target cells like physical methods (through microinjection, electroporation etc.), viral delivery (through Adeno Associated Virus (AAV) and Lentivirus vehicles), non-viral delivery (by lipid nanoparticles or liposomes, polyplexes, Cell Penetrating Peptides (CPPs), gold nanoparticles etc.) are the most popular delivery techniques. These approaches for delivering CRISPR-Cas9 components into particular cells are briefly described below.

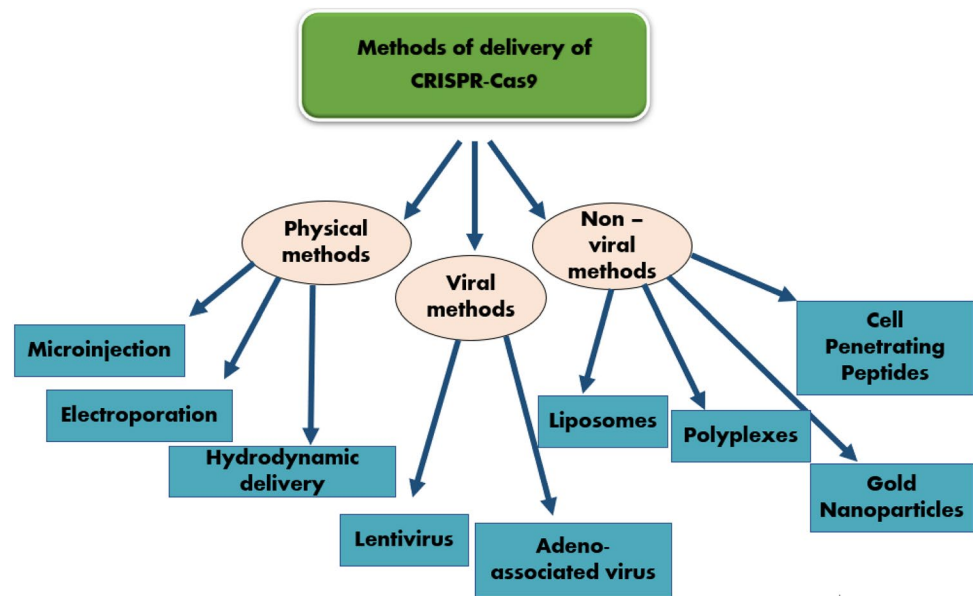
### Microinjection

In this technology, plasmid DNA encoding both the Cas9 protein and the sgRNA, mRNA expressing Cas9 and sgRNA, or Cas9 protein plus sgRNA may be directly injected into individual cells to deliver CRISPR components. A cell membrane is punctured with a 0.5–5.0  $\mu\text{m}$  diameter needle under the microscope and payloads are delivered directly to a desired spot within the cell. This method avoids the obstacles associated with administration via extracellular matrix, cell membranes, and cytoplasmic components. Because the use of a microscope to target individual cells (and precisely inject payloads to specific areas inside them) prevents the use of microinjection in a real in vivo scenario, it is best suited for in vitro and ex vivo studies only [18].

### Electroporation

Electroporation is a long-standing physical approach for delivering gene editing tools into a population of cells. This method employs pulsed high-voltage electrical currents to transiently create nanometre-sized holes inside the

**Fig. 2** Some common CRISPR-Cas9 delivery strategies



cellular membranes of cells suspended in buffer, allowing components with hydrodynamic diameters of tens of nanometres to enter the cell [19]. Electroporation can be used to efficiently distribute CRISPR/Cas9 reagents. Some recent investigations have revealed that electroporation may deliver CRISPR/Cas9 synthesised as ribonucleoprotein (RNP). The introduction of the reagents as RNP results in more improved efficacy, which has been demonstrated to enhance mutagenesis efficiencies and lower the degree of mosaicism detected inside the modified zygote. Electroporation is most typically utilised *in vitro*, although *ex vivo* uses are also possible, as with microinjection [20].

### Hydrodynamic delivery

Hydrodynamic delivery is an *in vivo* delivery approach that includes quickly injecting a large volume (nearly 8–10% body weight) of fluid carrying the gene editing cargo into an animal's circulation, mostly through the tail vein in mice. Since, blood is incompressible, the huge bolus of liquid causes a rise in hydrodynamic pressure, which momentarily increases permeability into endothelium and parenchymal cells, allowing the cargo (naked DNA, plasmid and proteins) to penetrate a cellular membrane and enter into the cells [21]. This form of cargo delivery is prevalent in the liver, but also includes cells from the kidneys, lungs, muscles, and heart. The appeal of hydrodynamic delivery is that it is technically straightforward and does not require any external delivery components to successfully introduce gene editing components into cells. Since the concept of delivery relies on transiently boosting pressure in a confined system and pushing cargo past normally impermeable barriers,

hydrodynamic delivery is generally employed solely for *in vivo* applications [22].

### Adeno-associated virus (AAV)

The employing of AAV in gene therapy provides a continuous source of the given DNA because of the fact that AAV delivered genomic material may reside in cells constantly and consistently as exogenous DNA or as directly integrated into the host DNA. HEK 293 T cells are commonly used to generate CRISPR/Cas9 AAV particles. After creating particles with precise tropism, they are utilised to infect the target cell line in the same manner as natural viral particles do. This is what eventually allows for the persistence of CRISPR/Cas9 components in the infected cell type, and it is what makes this method of delivery particularly suitable for circumstances when long-term expression is desired [23].

### Lentiviruses

Lentiviruses (LVs) are another viral vector utilised to deliver CRISPR/Cas9. Because LV vectors have a larger cloning capacity (8 kb) than AAV vectors, both Cas9 and sgRNA may be cloned into a single LV vector. LV production is also less difficult than AAV production. These benefits suggest that LV vectors are an excellent carrier for *in vitro* and *ex vivo* delivery. The difficulty related to LV systems is random integration into host cell genomes. The integration of LVs near oncogenes may result in their activation, leading in cancer. This rules out using LV-mediated CRISPR/Cas9 delivery for *in vivo* gene editing in clinical studies [24]. In terms of mechanics, this sort of CRISPR/Cas9 delivery is identical to AAV delivery. Transformed HEK 293 T cells



generate complete viral particles including the desired Cas9 and sgRNA. These viral particles are then subsequently used to infect the target cell type [18].

### Lipid nanoparticles/liposomes

Since, lipid nanoparticles have long been utilised as delivery vehicles for a broad range of substances to cells, and they have displayed the potential for nucleic acid delivery as well. As we know that outside the cells, nucleic acids are often unstable, and due to their highly anionic nature, they do not easily cross the cell membrane. Nucleic acids, on the other hand, may be transported to cells with reasonable ease by encapsulating them within often extremely cationic liposomes [24]. Because lipid nanoparticles do not contain any viral components, safety and immunogenicity problems are reduced. They can also be used *in vitro*, *ex vivo*, and *in vivo*, allowing for comprehensive testing on a wide range of cell populations. There are two techniques of using lipid nanoparticles to transport CRISPR/Cas9 components: delivering Cas9 and sgRNA genetic material (either plasmid DNA or mRNA) or providing Cas9: sgRNA RNP complexes. In terms of delivering Cas9 mRNA and sgRNA, this approach is functionally equivalent to micro-injection [25]. Several labs, however, have had great success using Cas9: sgRNA RNP complexes. Because Cas9 and the sgRNA as a ribonucleoprotein complex are extremely anionic, CRISPR/Cas9 appears to be particularly well-suited to this sort of delivery. This enables them to be packed using methods commonly used for delivering nucleic acids [26]. Lipofectamine, a commercially available lipid nanoparticle technology, is the most extensively used lipid nanoparticle system. Lipofectamine is a cationic liposome formulation that binds to negatively charged nucleic acids, allowing the complex to bind to negatively charged cell membranes and lead to endocytosis [24].

### Lipoplexes/polyplexes

Other nanocomplexes that rely on electrostatic interactions have been used to deliver CRISPR/Cas9 gene editing components. The commercially available FuGENE-6 reagent, a non-liposomal solution including lipids and other proprietary components, is a frequent technique. Kennedy et al. employed FuGENE-6 to deliver Cas9 and sgRNA expressing plasmid DNA to cervical cancer cells, inactivating the human papillomavirus E6 or E7 gene, leading in cell-cycle arrest and eventual cell death [27]. Miller et al. reported the production and development of zwitterionic amino lipids (ZALs). ZALs were complexed with Cas9 mRNA and sgRNA to produce nanoparticles that transfected mice effectively, concentrating largely in the liver, kidney, and lungs [28].

### Cell penetrating peptides (CPPs)

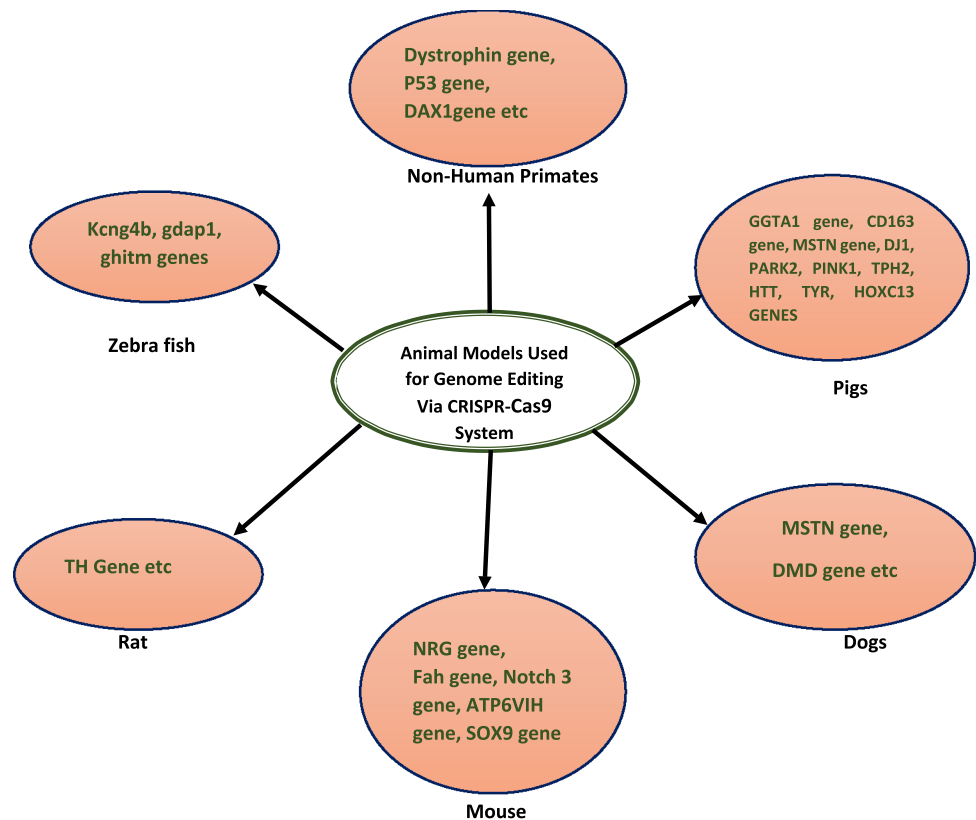
CPPs are often short peptides of polycationic, amphipathic, or non-polar amino acids. Each family of CPPs may help different types of proteins enter different cell types, and different combinations of CPPs and the target molecule for uptake will frequently result in varying uptake levels. CPPs are easily employed for *in vitro* and *ex vivo* studies, although substantial optimization for each cargo and cell type is often necessary. CPPs are not commonly used to administer components *in vivo* because of the degree of precision necessary for this optimization [18]. In the specific case of CRISPR/Cas9, the CPPs are usually covalently attached to the Cas9 protein directly, which is then complexed with the sgRNA and delivered to cells. Using confocal microscopy, Axford et al. (2017) revealed cellular and sub-cellular localisation of CPP administered CRISPR/Cas9 RNP [29].

### Gold nanoparticles (AuNPs)

Cas9 RNPs may be effectively delivered using gold nanoparticles (AuNPs) for gene editing. As AuNPs are chemically inert, they do not elicit an immunological response after administration [24]. Gold nanoparticles are widely employed in *in vitro*, *ex vivo*, and *in vivo* studies. It has been demonstrated that by tailoring Cas9: sgRNA RNP and AuNPs to interact, a complex is formed that can be effectively transported to cells and produce a targeted mutation at a rate of around 30% [30]. Lee et al. (2017) also reported the use of AuNPs to deliver Cas9: sgRNA RNP to DMD mice [31].

The capacity to make highly exact changes to a living organism's DNA sequence, therefore changing its genetic composition, is known as gene editing. Because it is now possible to make mutants in practically any genetic background and in a range of species, the advent of CRISPR/Cas9 has opened up a whole new world of possibilities (Fig. 3) [32]. CRISPR-Cas9 was developed in 2012 by George Church, Jennifer Doudna, Emmanuelle Charpentier, and Feng Zhang as a method to alter some particular regions of genomes. CRISPR was selected Breakthrough of the Year in 2015 by science because of its potential to revolutionise Genome Editing. This genome editing method allows scientists to change the genomes of numerous animals (Table 1) more accurately than classical transgenesis, with minimal off-target implications [33]. These genome editing approaches have a wide range of applications. Some of the possible applications in animals include increase in the productivity of livestock and disease resistance [34], developing new animal models to study human diseases [35], protecting native species by eradicating invasive species, reducing or even eliminating vector-borne diseases like malaria, and possibly even resurrecting extinct species [36].

**Fig. 3** CRISPR-Cas9 based genome editing in a variety of animal models



### In non-human primates

In scientific and medical research, laboratory animals have long proven to be valuable. Regardless of the fact that mice have long been the most frequent experimental animal, Non-Human Primates (NHPs) have proved to offer significant advantages over other species as a crucial model system for the study of human illnesses. Many characteristics of NHPs are very comparable to those of humans, including genetics, developmental biology, societal behaviours, physiology and mental abilities. The similarity of DNA sequences between NHPs and humans can approach 98.77%, compared to 90% for the rodents [37]. As a result, NHPs are an excellent choice for studying common human health issues like heart disease, diabetes, liver diseases and other diseases related to nervous system (brain). So, NHPs are a unique model in order to study the human brain problems due to their resemblances in brain structure and function with humans [38].

Several studies used CRISPR/Cas9 to alter the genomes of NHPs. For example, in 2014, knock out cynomolgus monkeys were created by using CRISPR/Cas9 to target one-cell embryos, demonstrating the viability of this genome editing technique in NHPs for the very first time [39]. The CRISPR/Cas9 technology was utilised to produce a variety of monkey models, for example; the first double-mutant cynomolgus monkeys expressing RAG1 (Recombination Activating Gene 1) and PPAR (Peroxisome Proliferator-Activated Receptor

gamma), without off-targeting mutagenesis [40]. Another example is the Duchenne Muscular Dystrophy (DMD) monkey model, which was generated by deleting the dystrophin gene using CRISPR/Cas9 technology. Early muscle deterioration was found in the monkeys, which might be utilised to create early treatment strategies for this sickness [40]. Midic and colleagues also used the haemoglobin (HBB) gene as a test locus for CRISPR/Cas9 in rhesus monkeys in 2017 to investigate targeting efficiencies, anomalies, timing, and effects of genetic mosaicism and reported a high targeting efficiency (80–100%) in embryos [41]. Wan and colleagues used an optimised CRISPR/Cas9 method to inject monkey zygotes, which resulted in a p53 biallelic (homozygous) mutant monkey that could be used as a model for cancer research (tumorigenesis) [42]. Kang et al. (2015), demonstrated yet another effective gene deletion in cynomolgus monkeys. They successfully removed the DAX1 (Dosage sensitive sex reversal, Adrenal hypoplasia crucial region, on chromosomal X, Gene 1), and the DAX1-deficient animals showed aberrant testis architecture and unaltered Sertoli cell production [43]. In addition, Sasaki and his co-workers reported a precise gene knock-in marmoset models in 2019 [44, 45].

All of the Non-Human Primate models mentioned here were created by microinjection, which leads in a higher degree of mosaicism and restricts the ability to create such animals which are genetically homogeneous. Liu et al., in

**Table 1** CRISPR-cas9 mediated gene manipulation in different animal models

S.No.	Animal model	Disease	Gene manipulated/targeted	Technique	References
1	Non-Human Primates (NHPs)	Duchenne Muscular Dystrophy (DMD)	Dystrophin gene	CRISPR-Cas9 mediated deletion of Dystrophin gene	[40]
		Tumour	P53 gene	Microinjection of optimised CRISPR-Cas9 into monkey zygotes	[42]
2	Pig	Adrenal Hypoplasia Congenita (AHC)	DAX1 gene	Microinjection	[43]
		Fabry's Disease	GGTA1 gene	Direct embryo injection	[48]
		Chronic inflammatory and infectious diseases/Renal cell Carcinoma	CD163 gene	SCNT and CRISPR-Cas9 embryo injection technique	[50]
		Muscle Hypertrophy	MSTN gene	CRISPR-Cas9 Technology	[42]
		Neurodegenerative disease	DJ1, PARK2, PINK1 genes	CRISPR-Cas9 and TALENS	[53]
		Behavioural and neuropsychiatric diseases	TPH2 gene	Gene deletion via CRISPR-Cas9 technology	[54]
		Huntington's Disease	HTT gene	HTT gene knock-in via CRISPR-Cas9	[55]
		Skin disease	TYR gene	Biallelic alteration of TYR with CRISPR-Cas9	[57]
3	Dog	Ectodermal Dysplasia 9	HOXC13 gene	Deletion of HOXC13 with CRISPR-Cas9	[58]
		Muscle Hypertrophy	MSTN gene	Zygotic injection of Cas9 mRNA and sgRNA catalysed with autologous embryo transfer	[60]
4	Mouse	Duchenne Muscular Dystrophy	DMD gene	Systematic administration of CRISPR gene editing components	[61]
		Immunodeficiency disease	NRG gene	IVF and CRISPR-Cas9 technology	[64]
5	Rat	Tyrosinemia	Fah gene	IVF and CRISPR-Cas9 technology	[64]
		Lateral Meningocele Syndrome	Notch3 gene	Microinjection of Cas9 mRNA and gRNA	[65]
		Osteoporosis	ATP6VIH gene	CRISPR-Cas9 genome editing technology	[66]
		Acampomelic/campomelic Dysplasia	SOX9 gene	CRISPR embryo microinjection	[67]
		Parkinson's disease	TH gene	Intra cranial injection of AAV vectors expressing spCAS9 gRNA	[68]

the year 2018 created the first two cynomolgus monkeys using Somatic Cell Nuclear Transfer (SCNT) [46]. Using this delicate yet complex method, scientists may be able to avoid some of the variability issues and hasten the development of genetically similar monkey models for human disorders. As a consequence, the successful somatic cloning technology and the quickly expanding genome editing system that is the (CRISPR/Cas9 system) might be utilised to support the production of customised NHP models and boost the future viability of using NHP models in biomedical research [47].

### In pigs

Pigs have indeed been extensively utilised for scientific research in recent decades because of the advantages of their breeding habits and handling benefits as well as the fact that they have fewer ethical problems than NHPs. Pigs are an excellent preclinical research animal model

because they reach sexual maturity at a young age (i.e., 5–8 months), have a short gestational period of around 114 days, and deliver numerous off springs (around 10–12 piglets each litter) [38]. Since, the genome editing technologies have quickly captured the interest of animal scientists and have thus been applied in pigs as well. For example, in the year 2014, Sato et al., were successful in knocking out the GGTA1 gene from the pigs using the CRISPR/Cas9 technique [48]. The CRISPR/Cas9 technology along with the direct embryo injection were successfully employed in pigs in order to create the genetically modified animals [49]. Again in 2014, Whitworth et al. used Somatic Cell Nuclear Transfer (SCNT) and the CRISPR/Cas9 embryo injection technique to generate CD163 or CD1D knock-out pigs [50]. Feng et al. (2015), edited the genome with the CRISPR/Cas9 technology and created Induced Pluripotent Stem (IPS) cells [51]. Using CRISPR/Cas9, a targeted integration system was created at the H11 genomic safe harbour gene in pigs. This method can be used to efficiently and

effectively inoculate any gene into the H11 site, resulting in high-level gene expression [52]. In 2015, Wang et al., used the CRISPR/Cas9 technology to conduct a specific repair of the porcine MSTN (Myostatin) gene. In addition to this, a large number of models of pigs have been developed in order to study neurological disorders. CRISPR/Cas9 and TALENs, for example, were used to create three pig lines that represented Parkinson's disease (PD): The DJ1 knockout, the PARK2/PINK1 double knockout, and the Parkin/DJ-1/PINK1 triple knockout. These pigs were employed as study models for the pathophysiology as well as the development of therapeutic intervention of Parkinson's disease [53]. Behavioural and neuropsychiatric disease models have also been created using mutant pigs. A population of pigs having deleted Tryptophan Hydroxylase-2 (TPH2) with CRISPR/Cas9 had significantly lower levels of serotonin (5-HT), as well as worse growth and survival rates prior to weaning [54]. A Huntingtin (HTT) knock-in pig model of Huntington's Disease was also generated by utilising the CRISPR/Cas9 technology, and the pigs that not only showed the abnormalities in their movement and behaviour, but also displayed the selective degeneration of striatal medium spiny neurons at a very early stage, perfectly mimicking the selective neurodegeneration that can be seen in people with Huntington Disease [55]. Besides this, Pigs have also proved to be the excellent models for studying the skin disorders. Pig skin has a comparable structure to that of the human skin in terms of thickness of the skin, interface of the dermis and epidermis, content of hair follicles, pigmentation, composition of collagen, lipid and blood of the dermis [56]. Because of these commonalities, nuclease-mediated genome editing was used to alter many genes linked to pigmentation or skin condition. For example, once Tyrosinase (TYR) was biallelically mutated with CRISPR/Cas9 technique, the mutant pigs had typical albinism, including loss of pigments in the skin, eyes, and hair [57]. Pigs were also used to model Ectodermal Dysplasia-9 (ED-9) after Hoxc13 was deleted using CRISPR/Cas9 technology. Loss of hair loss, fewer number of hair follicles, and abnormal structure of hair follicle was observed in the Hoxc13-knocked-out pigs, but normal structure of skin, skeleton and growth was observed, which is quite comparable with the phenotypes of people with ED-9 [58]. So, the creation of pig mutants to mimic human diseases has considerably extended due to the advent of new genome editing technologies and it will surely aid in the knowledge of pathophysiology and in the development of treatment strategies for human diseases in future.

### In dogs

Canines also have a lot in common with that of the humans in terms of metabolism, physiological function and anatomical structure. More than 450 genetic illnesses of dogs

provide naturally occurring disease models, with over half of these exhibiting remarkable clinical parallels to the diseases found in humans, which may be explored to learn more about the aetiology of the diseases and the development of preclinical therapeutic strategies [59]. Zou et al. (2015) developed MSTN (Myostatin) biallelic knockout dogs for the first-time using zygote injection of Cas9 mRNA and sgRNA combined with autologous embryo transfer. At 4 months, it was found that one of the puppies exhibited a double-muscle phenotype in the thighs, suggesting the feasibility of developing dog models for biomedical research [60]. The hereditary disorders in the natural phenotypes of dogs have also contributed in the discovery of genes and genetic pathways associated with diseases such as Duchenne Muscular Dystrophy (DMD) [61]. It has been reported that systemic delivery of CRISPR gene editing components significantly restored dystrophin gene expression in a DMD dog model and it was found that the histology of the muscles in the dog improved, indicating that gene editing techniques may be used to treat people with DMD [62].

### In rodents

It is quite obvious that the rodents, which include mice and rats, are the most commonly used animal models for illnesses created by genome editing [63]. The CRISPR-Cas9 technology allows scientists to create mutant mice models that were previously impossible to modify genetically. Li et al. (2014), created an immunodeficient mouse model with NRG (NOD-Rag1-/-IL2RgammaC-null) knock-out and a Tyrosinemia animal model with Fah gene knock-in using in vitro fertilisation (IVF) and CRISPR-Cas9 technologies [64]. Through microinjection of Cas9 mRNA & gRNA, a human Lateral Meningocele Syndrome (LMS)-related mutant mouse model of the Notch3 gene was generated. The LMS-related mutant mouse model showed a significant reduction in the volume of the cancellous bone and a significant drop in the trabecular number, mimicking the skeletal manifestations of Lateral Meningocele Syndrome [65]. Furthermore, a number of studies have described mouse models related to osteoporosis which are created by obliterating the ATP6V1H gene, a V-ATPase subunit that plays a key role in the biochemical and physiological activities of osteoclasts, via CRISPR/Cas 9 technology [66]. Mochizuki et al. (2018) used the CRISPR embryo microinjection approach to develop an ACD (Acanthotic Campomelic Dysplasia) and CD (Campomelic Dysplasia) mouse models by deleting the cartilage-specific SOX9 enhancer. This model has been found to be comparable to the clinical features of genuine ACD/CD patients and was expected to be useful in ACD/CD therapeutic studies in future [67]. Furthermore, Back et al. (2019) created a rat model of Parkinson's disease by obliterating the Tyrosine Hydroxylase (TH) gene from the



dopaminergic neurons of the brain through intracranial injection of AAV vectors expressing SpCas9 and gRNA. This Parkinson's disease rat model was intended to be utilized in a variety of studies envisioned at finding treatments for such condition [68].

### In zebra fish

The zebrafish has been widely used for the fundamental developmental biology research as well as the models for human illnesses due to its transparency, fertility, and availability of well-developed genetic and cell biological tools. The use of CRISPR/Cas9 to create missense point mutations of residues conserved between humans and zebrafish can be very valuable for disease modelling, as research in zebrafish can be much more cost-effective and scalable than studies in other vertebrate model animals, such as that of mice [69]. In zebrafish, the CRISPR-Cas9 system has been used effectively for high-throughput mutagenesis screening, knockout, conditional knockout, knock-in and multiplex knockout [70]. CRISPR/Cas9 mutagenesis in zebrafish has been demonstrated to be highly successful, with up to 86.0% efficiency and heritability. The efficacy of the CRISPR/Cas9 system has enabled for the targeted knock-in of a protein tag provided by a donor oligonucleotide, with high knock-in efficiencies. Mutation rates at possible off-target positions are found to be only 1.1–2.5%, confirming the specificity of the CRISPR/Cas9 system of genome editing [71]. An improved CRISPR-Cas9 genome editing approach has been reported in order to construct the precise deletion mutant alleles of some of the genes of zebrafish like *kcng4b*, *gdap1*, and *ghitm*. This method entails injecting two complementary gRNAs accurately delimiting the target area, together with the purified Cas9 protein, into one cell embryos of *D. rerio*. The addition of two complementary gRNAs has been found to improve the specificity of CRISPR-Cas9 and allows for the generation of predictable and precise changes in the *D. rerio* genome [72].

### In *Drosophila*

Multiple genes and the specific sites in the genome in *Drosophila* have been disrupted, deleted, replaced, tagged, and edited by utilising the CRISPR-Cas9 system of genome editing. The Cas9 expression vectors in *Drosophila* cell lines have also been described. The Actin5c and U6 promoters have been reported to be used to produce the Cas9 and sgRNA components, respectively. Because of the indels caused by poor NHEJ, a very efficient mutagenesis in over 80% of cells in *Drosophila* has been found. It has also been shown that short oligonucleotide donors can be used to insert small sequences, but longer homology arms can be

utilised to insert a 1.8 kb cassette with up to 4% efficiency [73].

## Applications of CRISPR/Cas 9 genome editing technology

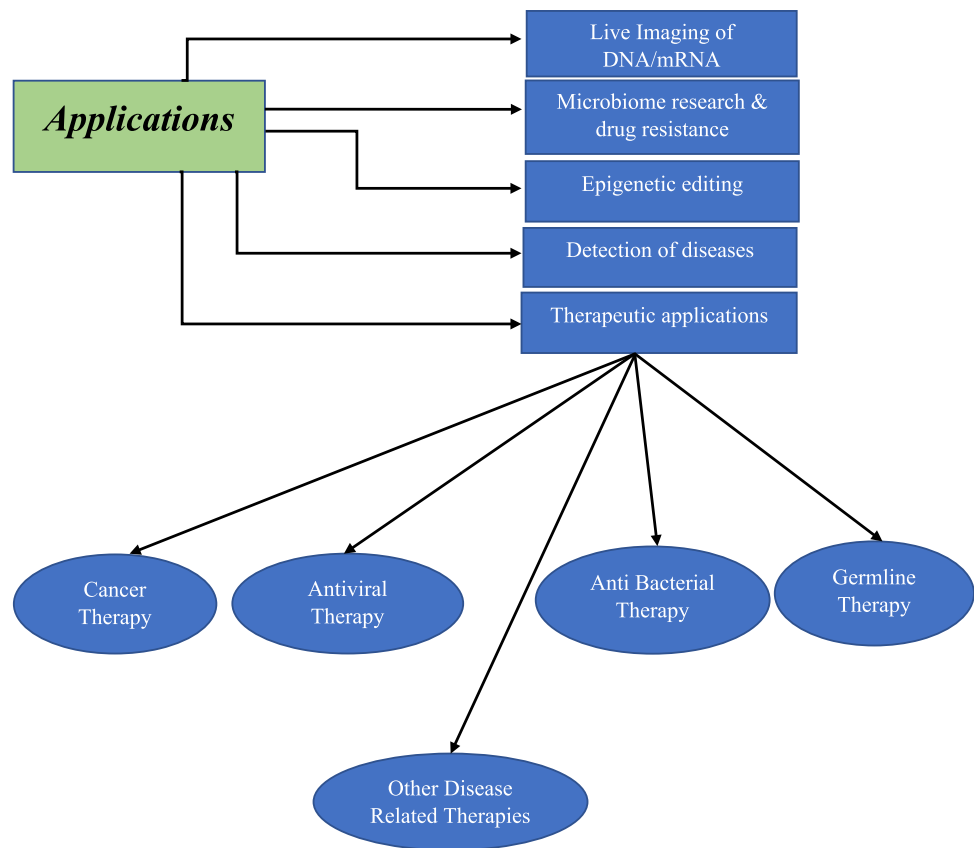
CRISPR-Cas9 technology offers a wide range of applications in the field of genomic research (Fig. 4). A few of these applications are briefly discussed below:

### Transcriptional regulation (activation and repression)

CRISPR/Cas9-based gene expression regulation provides scientists and research scholars with a revolutionary high-throughput tool for deciphering the functions of genes in molecular processes and activities of a cell. Single-guide RNAs have the ability to coordinate the recruitment of a nuclease-dead Cas9 protein as well as transcriptional Cas9-effector fusion proteins to the particular genomic locations, hence influencing gene expression [74]. Cas9 nuclease may be turned into deactivated Cas9 (dCas9), an RNA-programmable DNA-binding protein, by changing two key residues inside its nuclease domains. Furthermore, Cas9 nuclease may also be converted into deactivated Cas9 (dCas9), an RNA-programmable DNA-binding protein, by changing two key residues inside its nuclease domains [75].

In the most basic example, by targeting the gene of interest with an appropriately chosen sgRNA, dCas9 may inhibit transcription by sterically interfering with transcription initiation or elongation [76]. The location of the target promoter, as well as the kind of promoter itself, have a substantial impact on the degree of transcriptional suppression [77]. In prokaryotes, directing dCas9 to the DNA strand within a promoter or the non-template DNA strand downstream resulted in up to 1000-fold suppression [53]. In eukaryotic cells, however, steric repression is mild, with only up to 2- and 20-fold repression observed with natural promoters in mammalian and yeast cells, respectively [78]. In mammalian cells, synthetic promoters particularly tailored for direct repression by dCas9 can be suppressed up to 100-fold using CRISPR/Cas9 genome editing technology [76]. It has been found that, in eukaryotic organisms, combining dCas9 with transcriptional repressor domains may result in stronger downregulation of native promoters [79]. A fusion with the transcriptional repressor Mxi1 resulted in up to 50-fold suppression in yeast, while a dCas9 fusion with the histone demethylase LSD1 may be used to reduce transcription by distant enhancers in human cells [78, 80]. However, the most often used dCas9-KRAB fusion is both powerful as well as selective in yeast and mammalian cells [81].

**Fig. 4** Some important applications of genome editing via CRISPR-Cas9 system



The CRISPR-Cas9 system has been modified for transcriptional activation (CRISPRa), and many second-generation systems of CRISPRa including VPR, SunTag, and SAM have been created to recruit a number of transcriptional activators to a deactivated Cas9, which is guided to a transcriptional start site via base complementarity with a target guide RNA [82]. Engineering dCas9, sgRNA or both in order to attract the effectors of transcription to the DNA can also result in transcriptional activation. An activator was generated in *E. coli* by linking the native RNA polymerase subunit (omega) to dCas9 in a host lacking the subunit [83]. Furthermore, the most basic eukaryotic activators may be created by combining dCas9 with VP64 [84]. Konermann et al. (2015) created the Synergistic Activation Mediator by inserting two bacteriophage MS2 RNA hairpins into non-essential portions of sgRNA (SAM). Each MS2 hairpin binds two molecules of the MS2 Coat Protein (MCP), which is related to two transcriptional activators: p65 and Human Heat Shock Factor HSF1. The dCas9-VP64 fusion protein was found to be activated within a 2- to > 104-fold range when directed by this chimeric sgRNA, which is generally better than the activation of the same promoter with dCas9-VP64 guided by 8 tiled conventional sgRNAs [85]. Furthermore, it has been found that combining dCas9-based activation and repression screening can offer additional regulatory network information; this strategy enabled the discovery of complementary

pathways in glycosphingolipid biosynthesis linked to cholera and diphtheria toxin resistance. These findings suggest that dCas9 has the potential to be used in next-generation functional genomic screening [86].

### Live imaging of DNA/mRNA with CRISPR/Cas9

Genome editing technologies can result in chromosomal rearrangements such as translocations [87]. Because of fluorescent protein-fused, nuclease-deactivated dCas9 or single guide RNA (sgRNA) recruiting fluorescent protein-fused RNA-binding proteins, CRISPR-mediated live imaging of genomic loci is now conceivable [88]. It has been found that fluorophores can be used to scan both repetitive DNA elements and protein-encoding genes using inactivated dCas9, allowing us to understand how chromatin arranges itself during the cell cycle. In addition to live DNA imaging, CRISPR/Cas9 technology may be used for live RNA imaging. Changes to the gRNA sequence allow for the identification and tracking of mRNA. Using CRISPR-mediated RNA imaging tools, researchers were able to visualize the accumulation of ACTB (Beta-actin), CCNA2 (Cyclin A2), and TFRC (Transferrin Receptor) mRNAs in RNA granules. These new applications improve on existing approaches for live imaging within cells, allowing researchers to investigate dynamic biological processes involving DNA and RNA [89].

## Epigenetic editing with CRISPR/Cas9

Epigenome editing refers to the selective rewriting of the epigenetic markers [90]. In order to construct the core approach for CRISPR/Cas9-mediated epigenome editing, the Cas9 protein is fused with a transcription repressor or activator domain which was previously known as an epigenetic effector or simply epieffector [91]. For instance, using the dCas9-KRAB complex to target and induce locus-specific deposition of H3K9me3 in the HS2 enhancer region, researchers successfully silenced multiple globin genes in K562 cells when the fused epigenetic effector domain was Kruppel-associated box (KRAB). Similarly it was also found that when the fused domain was LSD1, researchers discovered downregulation of Tbx3 (a gene associated in pluripotency maintenance), deletion of H3K27Ac at the enhancer, and diminished pluripotency in embryonic stem cells [80]. Researchers have also used the dCas9-p300 core complex in order to activate the Myod gene at a regulatory region (located distal to the promoter), upregulate the Oct4 gene from a regulatory region (located anterior to the promoter), and cause transcription in three-quarters of downstream haemoglobin genes by targeting several DNase hypersensitive sites within the beta globulin locus control region [84]. In short, the dCas9-epieffector combo could methylate and demethylate DNA, rewrite histone marks by inducing methylation or acetylation at the nucleosome level, and can be altered to improve editing efficiency [92].

## Therapeutic applications

CRISPR-Cas9 gene editing activity is extremely efficient for therapeutic applications in systems ranging from the stem cells of the humans to the animals used as models in the research field [93]. The application of this technology for medicinal reasons, such as gene therapy, has grown in popularity [94]. Some of the therapeutic applications of CRISPR-Cas9 genome editing system are briefly described ahead.

### Cancer therapy

CRISPR-Cas9 technology for cancer treatment has been applied in three aspects viz, cancer genome, cancer immunotherapy and epigenome modification and abolition or inactivation of carcinogenic infections caused by a certain group of viruses [94]. The use of the CRISPR/Cas9 technology to alter the genome for the creation of the Chimeric Antigen Receptor (CAR) is extensively regarded as one of the most significant advances in personalized cancer treatment. During CAR T cell therapy, autologous T cells are harvested, genetically altered to attack cancer antigens in vitro, and then returned to the patient. By altering the genes that code for T-cell inhibitory receptors or inhibitory substances,

CRISPR-Cas9 can be utilised to increase CAR T cell function [95]. The first clinical trial, which was conducted in lung cancer patients, looked at the safety and effectiveness of the CRISPR-Cas9-engineered PD1-knockout T cells [96]. Thus, the combination of CAR modification, immune checkpoint suppression, and CRISPR-Cas9 technology offers a therapeutic strategy with significant promise for improvement in the treatment of solid tumors [97]. Furthermore, CRISPR-mediated knock out of miRNAs can be used in cancer treatment since miRNAs play an important role in the regulation of cellular processes in both normal and pathological manifestations [98].

### Antiviral therapy

The prevention of viral infection or replication is included in antiviral genome editing. One of the most promising and emerging gene therapy techniques is programmable nuclease-mediated antiviral treatment. In order to inhibit viral infection, Cas9 nucleases can target viral genes or host genes that encode critical receptors, such as HIV-1, Epstein–Barr Virus, Hepatitis B Virus, Herpes Simplex Virus, Human Papillomavirus, etc. [99]. The deletion of CC chemokine receptor-5 (CCR5) gene in human primary T cells and CD34 Hematopoietic Stem and Progenitor Cells (HSPCs) is the most sophisticated CRISPR-Cas9-mediated antiviral treatment till date. CCR5 was, in fact, one of the first genes in eukaryotic cells to be targeted by Cas9 [100]. Knocking down the CXCR4 gene, a co-receptor for HIV entrance, through electroporation of Cas9 ribonucleoproteins (RNPs) in primary human CD4T cells was another method used to prevent viral infection [101]. C–X–C chemokine receptor type 4 (CXCR4) expression was lost up to 40% of CD4T cells when Cas9 RNPs were used. By employing the CRISPR-Cas9 system to change patient cells and then transplanting gene-edited cells back to the patient, these techniques offer a lot of promise for CRISPR-Cas9 in ex vivo cell therapy. These HIV-resistant T cells would not be infected, allowing the patient's HIV-resistant T cell population to grow. Several additional antiviral methods with significant promise in infectious disease therapy used CRISPR-Cas9 to directly target viral genomes, demonstrating the capability of suppressing viral replication without compromising the human genome [102].

### Antibacterial therapy

CRISPR-Cas9 nucleases can be employed as antibacterial reagents because they target bacterial genes and hence limit bacterial growth [103]. For instance, skin of the mice which were infected by a *Staphylococcus aureus* bacterial strain, were treated with a CRISPR-Cas9-encoding phage, this resulted in the bacteria being eradicated from the skin

[83]. This research clearly demonstrates the efficacy of Cas9-based antibacterial therapy in the treatment of human bacterial infections [93].

### Germ-line therapy

Germ-line therapy is an experimental treatment or prevention strategy that leverages genes. The first proof of germ-line genome editing via CRISPR-Cas9 was the repair of Duchenne Muscular Dystrophy (DMD)-causing mutations in a mouse zygote. To test the efficacy of CRISPR-Cas9 in germ-line gene therapy, Cas9 protein, sgRNA, and an ODN (oligodeoxynucleotide) template were co-delivered into dystrophic mdx mouse embryos. Microinjecting these components into the cytoplasm or pronuclei of zygotes resulted in successful HDR-mediated gene repair that corrected the disease-associated nonsense mutation in exon 23 of the dystrophin gene, restoring the synthesis of dystrophin protein [104]. Germ-line gene modification, as proven in cataract mouse research, increases the likelihood of altered genes being handed down to the progeny of transformed creatures. Co-delivery of Cas9 mRNA, sgRNA, and an ODN template to mutant mouse zygotes resulted in animals with normal eyes, suggesting repair of disease-associated mutant *Crygc* alleles [105]. Furthermore, the repaired *Crygc* gene was successfully handed down to these mice's offspring, demonstrating the feasibility of transmitting alleles altered by Cas9-mediated genome editing [93].

### Other disease-related gene therapies

The in vitro and in vivo models of a variety of genetic disorders have been used to evaluate the CRISPR-Cas9 system. Apart from treating cataracts and DMD. The CRISPR-Cas9 system has been used to treat a variety of diseases including Alfa-1-Antitrypsin deficiency, Beta-thalassemia, Cystic Fibrosis (CF), Hemophilia, Hyperammonemia, Limb-girdle Muscular Dystrophy, Myeloproliferative disorders, Hearing Loss, Severe Combined Immunodeficiency (SCID) and Tyrosinemia. These findings point to the fact that CRISPR-Cas9 genome editing tool can be used to treat a wide variety of animal diseases and possesses a tremendous therapeutic potential [93].

### Applications of CRISPR-Cas9 in microbiome research and drug resistance

CRISPR technology has the potential to tackle drug resistance while also expanding the scope of microbiome study. The makeup of the gut microbiome is increasingly implicated in both disease and wellness. CRISPR/Cas9 editing technology can be used to learn more about the molecular processes behind drug resistance and to target specific

oncogenes as a solo therapy or in combination with already available therapies [106]. The CRISPR/Cas9 system is used to resensitize tumour cells to treatment by correcting a resistant form of a targetable gene related to cancer. Furthermore, CRISPR/Cas9 can be utilised to disrupt resistance-related oncogenes like KRAS or to repair tumour suppressor genes like TP53, resulting in tumour cell resensitization [106]. According to a genome-wide CRISPR/Cas9 drug resistance screening research, Loss of *KEAP1* gives various drug resistance to distinct lung cancer cells, such as *H1299*, *CALU1*, and *HCC364*, through increased *NRF2* activity [107]. Furthermore, several research teams have used CRISPR-based techniques to better understand possible resistance pathways and improve effectiveness of sorafenib (a multikinase inhibitor, commonly used for the treatment of hepatic cancer) in Hepato-Cellular Cancer (HCC) [108]. It has also been demonstrated that HCC cancer cells are sensitized to sorafenib when the fibroblast growth factor receptor (FGFR4) is knocked off using CRISPR technology [109]. In addition, several research has used the CRISPR/Cas9 system to explore the function of different proteins including *ATRX*, *NOTCH1*, *PCMI* and *GLII* in response to various forms of treatments like chemo and radiation therapy in gliomas. For example; in primary glioblastoma cells, knocking down *GLII*, also known as glioma associated oncogene, in conjunction with Penfluridol (an antipsychotic medication), enhanced apoptosis of primary glioblastoma cells [110]. CRISPR/Cas9 technology has also been extensively utilized to study resistance pathways in breast cancer, either as a single gene modification tool or as a genome-wide screening approach. For example; Genetic ablation of APE1, a base excision repair enzyme, in TNBC (Triple Negative Breast Cancer) cells (HCC1937) led to resistance to Olaparib [111]. In summary, CRISPR-based studies have been found to be linked with drug response and resistance in the most common solid tumors, including breast, lung, liver and brain malignancies.

### Detection of diseases

By using sequence-specific target binding and cleavage as well as target-specific trans-cleavage, the CRISPR-Cas system has emerged as an intriguing and effective diagnostic tool for the detection of diseases. The trans-cleavage activity is appealing because it occurs numerous times for one target binding by the CRISPR-Cas complex thereby increasing detection sensitivity. Since, nucleic acids are the primary target of the CRISPR-Cas system, numerous kinds of nucleic acids related to pathologies, such as genomic DNA, mRNA, miRNA, SNP, and methylated DNA, have been effectively identified and quantified by combining the CRISPR-Cas system with target amplification [112]. Currently, "Severe Acute Respiratory



Syndrome Coronavirus 2” or “Coronavirus Disease 2019” (COVID-19) has become an international concern. Researchers have presented a coronavirus fast detection approach based on the CRISPR/Cas system. Cas12a and Cas13a have shown to be more effective in disease diagnosis. Cas12a is DNA-specific but Cas13a works with RNA which makes it convenient in detection of SARS-CoV-2 [113]. Recently in 2020, Zhang et al. published a Specialised High-sensitivity Enzymatic Reporter unlocking (SHERLOCK) technology, a CRISPR/Cas13-based nucleic acid detection approach for fast detection of SARS-CoV-2. Given the significant need for speedy diagnosis, the use of CRISPR/Cas13-based diagnostic tool or SHERLOCK for SARS-CoV-2 detection has been proven to be substantially faster than detection by qRT-PCR with high sensitivity in current global pandemic state of COVID-19 [114].

### Some concerns/challenges associated with CRISPR

Genome editing technology have been used to modify, control, determine and construct genomes in different animals, which might lead to new biomedical and agricultural uses [47]. Despite CRISPR/Cas9’s enormous potential in genome editing, certain critical difficulties must be addressed, such as off-target mutations, PAM (Proto-Spacer Adjacent Motif) dependency, gRNA synthesis and CRISPR/Cas9 delivery systems [115]. Besides, there are some ethical concerns that are associated with CRISPR-Cas9 genome editing. Some of the challenges are briefly explained below:

#### Off-target mutations

Since its beginning, the high rate of off-target effects caused by the CRISPR-Cas9 system has been a matter of severe concern [116]. Off-target effects have been found to be cell type specific and highly dependent on the proper operation of the cell’s DSB repair machinery [117]. Off-target mutations have been demonstrated to be uncommon in human pluripotent stem cells with functional DSB repair machinery, but widespread in human cell lines with dysfunctional DSB repair machinery [118]. Off-target mutations might result in cell death or transformation. To reduce CRISPR/cellular Cas9 toxicity, increasing emphasis is being placed on removing CRISPR/off-target Cas9 mutations [119]. Shortening sgRNA, adding extra nucleotides at the 50 end of the guide sequence, and even changing sgRNA structure have all been demonstrated to boost target specificity and minimise undesired mutagenesis at non-target regions [120].

### Methods of delivery

Delivering the programmable nuclease is one of the most difficult components of genome engineering. The most significant barrier to CRISPR/Cas9 usage is gene cargo delivery methods and an all-purpose delivery technique has yet to develop. Instead, several ways for the delivery of CRISPR into the cells have been discovered. Every technique has benefits and drawbacks, and some are more suited to particular types of delivery than others. Another stumbling challenge for distribution systems is ensuring that the technique utilised is both safe and specific. The vehicle of the CRISPR system is dictated by the experiment’s aims and might range from viral to non-viral approaches. Adenoviruses, baculoviruses, and integrative and non-integrative lentiviruses are all viable options for delivering enzymes and nucleases. AAVs, which are non-pathogenic human viruses that enable for long-term transgenic expression without genomic integration, are presently the most appealing gene delivery vectors [121]. However, their greatest drawback is that the cargo they may carry is limited in size, limiting their capacity to convey Cas9 or the vector’s guide sequences. Smaller Cas9 orthologs, such as those derived from *Staphylococcus aureus*, have been developed to circumvent this issue and avoid the usage of two separate vectors [122]. These orthologs are called the “editor’s option” for in vivo genome editing since they are easier to pack [123].

### gRNA production

The gRNA production is another critical element of CRISPR/Cas9-mediated genome editing. Due to considerable post-transcriptional processing and modification of RNA polymerase II-produced mRNA, it is currently difficult to employ RNA polymerase II for gRNA synthesis. RNA polymerase III, U3 and U6 snRNA promoters are now used to create gRNA in vivo. The U3 and U6 snRNA genes, on the other hand, are ubiquitously expressed housekeeping genes that cannot be used to generate tissue- or cell-specific gRNA. As a result, the unavailability of commercially available RNA polymerase III limits U3 and U6-based gRNA production [124].

### Ethical concerns

The potential application of CRISPR technology in human embryos is one of the most problematic parts of the technology. This disagreement is mostly caused by a misunderstanding of the stage of the human embryo, rather than by CRISPR itself. Despite the fact that some experts feel that testing on human embryos after 14 days is immoral [125]. It is difficult for any one party—government, laboratory, funding agency, panel of experts, court, religious organisation,



or other group—to determine the status of a human embryo and whether or not it possesses "personhood" [126].

## Perspective and future directions

However, CRISPR's therapeutic potential will continue to grow as technology advances and it gets more mechanistically accurate and can be administered with greater accuracy. The CRISPR field is advancing at a breakneck speed and therefore the technology's future prospects are bright and we anticipate that any limitations like off-target mutations, delivery methods etc. will be addressed and overcome as a result of the vast number of academics from many areas presently concentrating on it. CRISPR is even making its way into popular culture today, with casual allusions in a variety of media genres. CRISPR is, without a doubt, the modern genetic engineering's new face.

## Concluding remarks

CRISPR-Cas9 has revolutionised biomedical science by permitting single-nucleotide genetic changes in practically every cell type or creature. CRISPR's quick progress reflects its utility, simplicity, and effectiveness. As a result of the extensive use of subsequent applications based on CRISPR, it has evolved into a multipurpose platform. Its use has grown to include multiplexed edition, sequence-specific gene expression regulation, and genome-wide screening of various platforms. These new methodological advances have substantially broadened the technological choices for studying gene function and modelling in a wide range of animals and diseases. Furthermore, the combination of CRISPR-based genome editing and genome-wide association analysis may be critical in the creation of personalised treatments. Despite the rapid growth of CRISPR technology, many mechanistic concerns remain unresolved, as do a number of challenges. Current delivery techniques must be improved, and novel mechanisms for CRISPR element distribution to the target cell must be devised, in order to achieve acceptable levels of efficiency. Another major difficulty is the ethical concern around the use of CRISPR-Cas9 technology in humans, as well as the essential ethical and regulatory standards that must be developed to evaluate the permissible use of these technologies. Furthermore, efficiency must be linked with specificity, necessitating the development of innovative approaches to controlling targeted edition while minimising off-target effects. Finally, further research is needed before CRISPR-Cas9 can be extensively employed in basic and medical research and therapy.

**Acknowledgements** Authors are thankful to Central Library and Departmental Library (Department of Zoology) of Baba Ghulam Shah Badshah University, Rajouri, Jammu and Kashmir, India for providing access to different journals and books to write this manuscript. Authors are also thankful to Indian Council of Medical Research (GOI), Govt. of India (F.No. 59/7/2020/TRM/BMS, dated 25.03.2021) and University Grants Commission (UGC), Govt. of India (F.30- 441/2018 (BSR), dated 07-06-2018) for financial Assistance.

**Author contributions** MSR contributed to the article conceptualization, supervision and final draft preparation. Writing, editing, figures and tables, data collection and analysis were performed by DJ, SYG and YAH. The first draft of the manuscript was written by DJ and all authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

**Funding** No financial support was available for this study.

## Declarations

**Competing interests** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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