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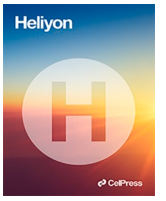

Review article

Emerging Gene-editing nano-therapeutics for Cancer

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ABSTRACT

Remarkable progress has been made in the field of genome engineering after the discovery of CRISPR/Cas9 in 2012 by Jennifer Doudna and Emmanuelle Charpentier. Compared to any other gene-editing tools, CRISPR/Cas9 attracted the attention of the scientific community because of its simplicity, specificity, and multiplex editing possibilities for which the inventors were awarded the Nobel prize for chemistry in 2020. CRISPR/Cas9 allows targeted alteration of the genomic sequence, gene regulation, and epigenetic modifications using an RNA-guided site-specific endonuclease. Though the impact of CRISPR/Cas9 was undisputed, some of its limitations led to key modifications including the use of miniature-Cas proteins, Cas9 Retron precise Parallel Editing via homologY (CRISPEY), Cas-Clover, or development of alternative methods including retron-recombineering, Obligate Mobile Element Guided Activity(OMEGA), Fanzor, and Argonaute proteins. As cancer is caused by genetic and epigenetic alterations, gene-editing was found to be highly useful for knocking out oncogenes, editing mutations to regain the normal functioning of tumor suppressor genes, knock-out immune checkpoint blockade in CAR-T cells, producing 'off-the-shelf' CAR-T cells, identify novel tumorigenic genes and functional analysis of multiple pathways in cancer, etc. Advancements in nanoparticle-based delivery of guide-RNA and Cas9 complex to the human body further enhanced the potential of CRISPR/Cas9 for clinical translation. Several studies are reported for developing novel delivery methods to enhance the tumor-specific application of CRISPR/Cas9 for anticancer therapy. In this review, we discuss new developments in novel gene editing techniques and recent progress in nanoparticle-based CRISPR/Cas9 delivery specific to cancer applications.

1. Introduction

Cancer, one of the most challenging life-threatening diseases, is characterized by the uncontrolled proliferation of genetically altered (mutated) cells in the human body [1,2]. Alterations include single or multiple genetic mutations leading to abnormal expression and translation of associated proteins [3,4]. These mutations may cause the formation of oncogenes (BCR-ABL, k-RAS, BRCA), upregulate the expression of growth factor-associated genes (EGFR, VEGF, etc.), and downregulate tumor suppressor genes (p53, Rb, PTEN, APC, etc.) [5–9], shut-down programmed cell death (apoptosis) [10], evade immune response [11], altogether result in the uncontrolled growth of cells. Understanding the genomic changes that initiate the malignant transformation, metabolism,

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drug-resistance mechanism, immune evasion, and overall progression, is of great importance to developing new innovative strategies to treat this disease. The recently invented CRISPR/Cas9 gene editing has made remarkable advances in gene therapy due to its specificity and simplicity [12]. While CRISPR-Cas9 is a powerful and versatile gene-editing tool, new alternative gene editing tools and approaches are investigated to address the current limitations associated with conventional CRISPR/Cas9. Some of those modified or alternate gene-editing mechanisms under evaluation for mammalian gene-editing applications include retron recombineering, CRISPEY, Cas-CLOVER, Omega, Fanzor, Argonaute, and compact Cas proteins [13,14].

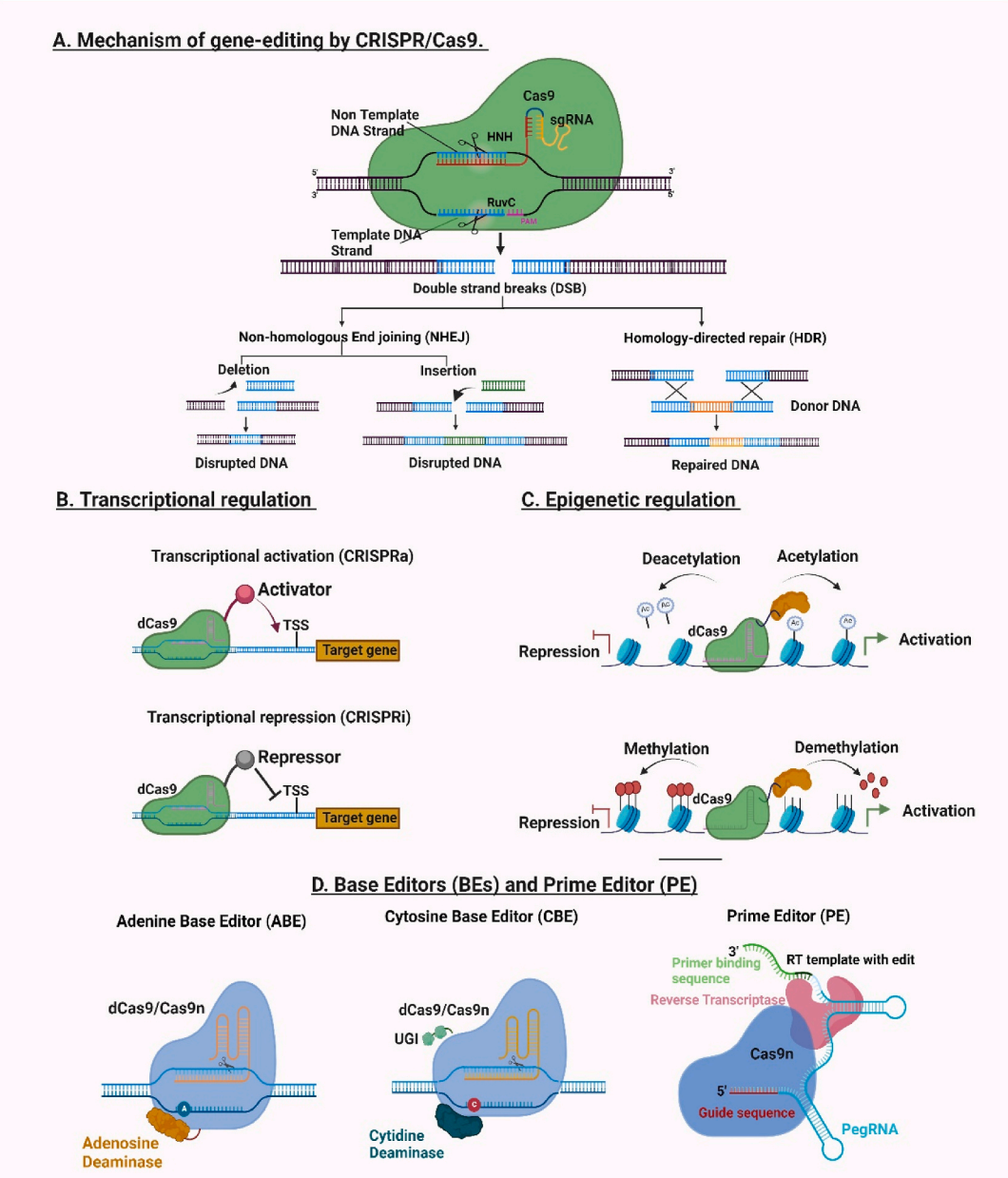

Fig. 1. Schematic of representation of A) mechanism of gene-editing using CRISPR/Cas9 via the double-strand breaks. The Cas9/sgRNA ribonucleoprotein (RNP) complex recognize and binds the complimentary sequence of guide RNA and cleaves the target DNA in the template and non-template strand using RuvC and HNH domains of Cas9 to produce double strand breaks (DSBs). Followed by the formation of DSBs, cell's DNA repair machinery repair the break via either the non-homologous end-joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. B&C) Mechanism of transcriptional and epigenetic regulation by dCas9. Dead Cas9 (dCas9) fused with active domains of other proteins perform different functions such as transcriptional and epigenetic regulation of gene expression. D) Base editors (BEs) and Prime editors (PEs). dCas9 or Cas9 nickase (Cas9n) fused with adenine and cytidine base editors function as sequence specific base editors. Cas9n fused with a reverse transcriptase and prime editing guide RNA (pegRNA) having guide sequence and reverse transcriptase template with specific gene modification are known as prime editors (PEs).

In just 11 years of its discovery, for the first time, in November 2023, The UK Medicines and Healthcare products Regulatory Agency (MHRA) approved a CRISPR/Cas9 gene-edited therapy, CASGEVY™(exagamglogene autotemcel) developed by Vertex pharmaceuticals and CRISPR Therapeutics for the treatment of sickle cell disease (SCD) and β-thalassemia [15]. This historic milestone in CRISPR/Cas9 based gene therapy gives great hope for its use in many other diseases. In the last decade, CRISPR/Cas9-based gene therapy has made rapid progress in cancer treatment, and ~25 clinical trials have been initiated in the last 5 years to treat different types of cancers. The majority of current clinical trials focus on ex vivo gene editing where the cells are collected from the patient or healthy donor, modified in the laboratory, and infused back into the patient [16]. However, to fully utilize CRISPR/Cas9 as a therapeutic tool, the availability of off-the-shelf gene-edited cells or direct, personalized editing inside the patient's body is necessary. One of the most critical factors in direct in vivo gene editing is the requirement of site-specific delivery devices for CRISPR/Cas9 [17]. The most widely used viral vectors have many limitations such as immunogenicity, chances of site-directed mutagenesis, limited packing capacity, cost of manufacturing, etc. [18,19]. In recent years, research community has shown increasing interest in the development of more biocompatible and biodegradable nanocarriers for CRISPR/Cas9 delivery [20]. Recent approval to initiate a phase 3 clinical trial of in vivo lipid nanoparticle-based CRISPR/Cas9 (NTLA-2001) (NCT06128629) for the treatment of Transthyretin (ATTR) Amyloidosis by the US FDA gives great promise in nanoparticle-based CRISPR/Cas9 delivery [21]. Targeted approach of tissue specific gene-editing using nanoparticles ensures long term benefit with reduced side effects [22].Recently Choudhry R et al. reviewed recent developments in the integration of nanoparticles for CRISPR/Cas9 delivery to improve functionalities such as intracellular delivery, tissue/cell targeting, stability, biocompatibility etc. [23].

In this review, we report the recent progress in novel alternate approaches to gene editing including the modified CRISPR/Cas9 and also discuss the recent development in the last two years in nanoparticle-based CRISPR/Cas9 delivery systems, specifically for the treatment of different cancers.

2. Mechanism of CRISPR/Cas9

CRISPR/Cas is an adaptive immune system found in most bacteria and archaea to provide immunity against invading pathogens [24]. The prokaryotic immune system was repurposed as an RNA programmable site-specific genome engineering tool in 2012 for modification of DNA sequence in mammalian systems [25]. A well-established and most widely used RNA-guided DNA endonuclease is the type-II Cas9 protein from Streptococcus pyogenes (SpCas9) [26]. Cas9 nuclease requires a 5′-NGG-3′ sequence named a protospacer adjacent motif (PAM) downstream of the target DNA that is necessary for the identification and cleavage of the target sequence [27]. CRISPR requires two RNAs: mature CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Jinek et al. have shown that CRISPR/Cas9 can also use single chimeric RNA made by the fusion of 20 nt crRNA and 80 nt tracr RNA, named single guide RNA (sgRNA) [25]. This prokaryote-derived site-specific Cas9 protein along with a programmable RNA called single guide RNA (sgRNA) generates double-strand breaks (DSBs) in the complementary DNA sequence as shown in Fig. 1A [26,28–30].

DSBs in eukaryotic cells are repaired either by non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway

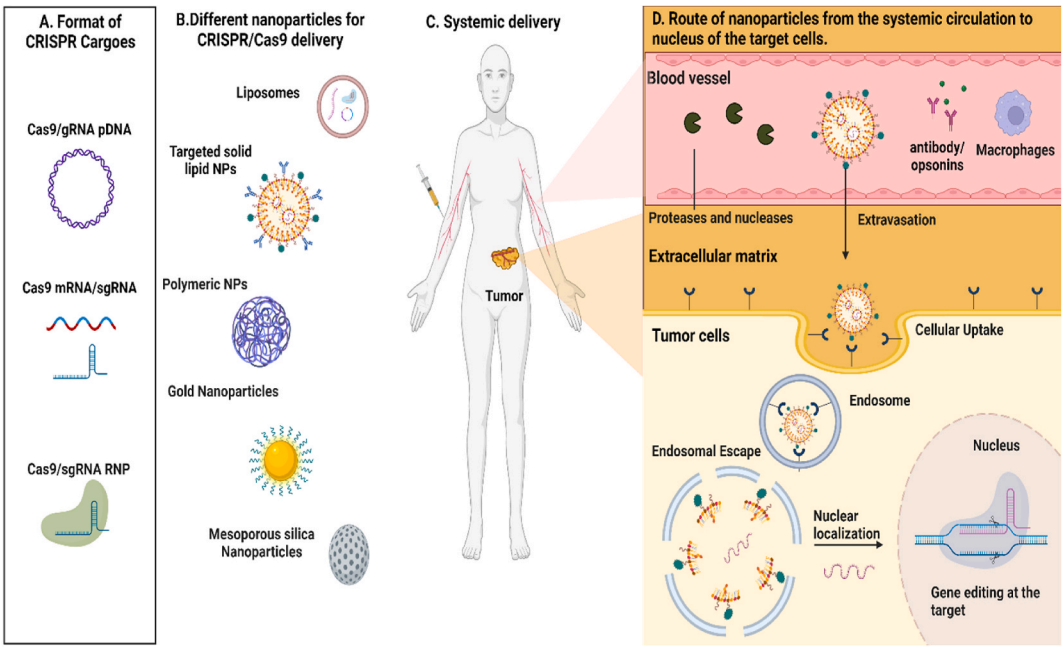

Fig. 2. Diagrammatic representation of systemic delivery of nanoparticle-based CRISPR/Cas9 gene therapy. A) Different formats of CRISPR cargoes include plasmid DNA, mRNA and Cas9/sgRNA ribonucleoprotein (RNP) complex. B) representative nanoparticles for CRISPR/Cas9 delivery include liposomes, targeted lipid nanoparticles, polymeric NPs, gold NPs and organic silica.

[31]. NHEJ pathway brings together the cleaved strands by a multiprotein complex and ligates both ends together resulting in the insertion or deletion (indels) of some of the bases of DNA. NHEJ is an error-prone DNA repair mechanism. The indels in the target site may result in the loss of function of a particular gene [31]. HDR pathway requires a template DNA with homologous sequence for repair [32,33]. HDR is utilized mostly for gene insertion or correction of mutated genes, Fig. 1A [34].

Other than the NHEJ/HDR-based gene editing applications, intensive research expanded the use of CRISPR/Cas9 by modifying the Cas9 enzyme. By introducing point mutations in both RuvC and HNH domains of the Cas9 protein, a dead Cas9 (dCas9) with no enzymatic activity was developed, that acts as an RNA-guided DNA binding protein [35]. Coupling dCas9 with functional domains of other active proteins enables many functions such as transcriptional and epigenetic regulations of gene transcription as shown in Fig. 1B and 2C [36,37]. In CRISPR interference (CRISPRi), dCas9 fused with active domains of transcriptional repressors such as histone demethylase LSD1 and human transcription repressor KRAB, repress gene transcription when directed to promoters or enhancers using specific guideRNAs [38,39]. Similarly, CRISPR activation (CRISPRa) is a mechanism of targeted activation of specific gene expression by binding to the promoter of genes using dCas9 fused with transcriptional activators like VP64, Rta, p65, and HSF1 [39,40]. Dead Cas9 (dCas9) is also used for epigenetic modification of the DNA or histones to control gene expression as shown in Fig. 1C dCas9 fused with active domains like p300 (dCas9-p300) of histone acetyltransferase (HAT) and CREB-binding protein (dCas9-CBPHAT) targeted to promoters or proximal or distant enhancers increase gene expression [41,42]. dCas9 can also be used for methylations of specific regions by fusing with DNA methyl transferase 3A (dCas9-DNMT3A) to repress gene transcription [43].

Base and prime editing are another advanced application of CRISPR/Cas9 that enables precise alteration of gene sequences without making DSBs (Fig. 1D) [44]. For example, dCas9 fused with cytidine base editors (CBEs) convert C to T and adenine base editors (ABEs) convert A to G when directed to specific locations in the gene [45]. To improve the base editing efficiency instead of dCas9, Cas9 nickase is also used, which nicks non-template DNA strands to initiate cell repair machinery [46]. Recently base base-edited CAR7 T cells have been investigated in patients with relapsed childhood T-cell leukemia in the clinics [47]. Prime editing is a 'search and replace' mode of gene editing using CRISPR, that can be used to make small insertions, deletions, and base swapping without making DSBs and without the need for a donor template [48]. For this, prime editing uses a fusion protein of Cas9-Nickase and an engineered reverse transcriptase (RT) called prime editor (PE) along with a modified prime editing guide RNA (pegRNA). pegRNA possesses a guide sequence specific to the target and a reverse template that encodes the desired edit as shown in Fig. 1D [49].

3. Delivery of CRISPR/Cas9 components

To achieve an effective therapeutic outcome, the CRISPR/Cas9 components are to be delivered to the target cells. CRISPR/Cas9 components can be delivered in different formats to the cells such as plasmid DNA encoding Cas9 and sgRNA, mRNA of Cas9 and sgRNA, or a Cas9/sgRNA ribonucleoproteian (RNP) complex [50] as shown in Fig. 2A. CRISPR/Cas9 can be delivered via physical methods, viral vectors, and non-viral vectors [51]. Electroporation and viral vectors are the most widely used delivery vehicles in clinical applications, especially for ex-vivo modes of gene editing therapy (Fig. 3) as they have high transfection efficiency [52]. In ex-vivo mode, these vectors do not compromise patient safety, especially in terms of off-target mutations as well as the immune response from CRISPR/Cas9 components and the delivery vectors. Though viral vectors are mostly used in current clinical investigations, they have major drawbacks like limited packaging size, immunogenicity towards the viral vectors, random integration of the viral genomes, high cost of production, etc. [53]. Delivery of CRISPR/Cas9 components for in vivo gene editing remains a critical challenge in effective therapeutic applications. Non-viral delivery vehicles based on nanoparticles (Fig. 2B) have received significant attention as they are ideal systems for nucleic acid and protein delivery with high biocompatibility, and low immunogenicity and more

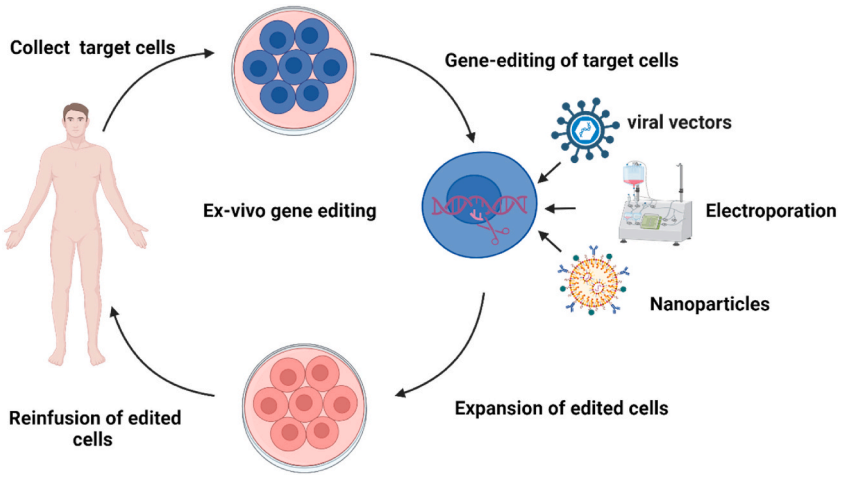

Fig. 3. *Ex-vivo* gene editing approach in clinical trials. Target cell population are isolated from blood collected from either a healthy donor or from patients. Cells are gene edited using different methods such as viral vectors, electroporation and using nanoparticles. The edited cells are selected and enriched in the laboratory and infused back to the patient.

interestingly they can be designed for precise targeting [54].

3.1. Viral vectors for CRISPR/Cas9 delivery

Viral vectors have played a significant role in gene therapy since the 1980s and have experienced setbacks during the initial stages [55]. After a long time, in 2017 the US FDA approved the first viral-vector-based gene therapy (Kymriah) for the treatment of acute lymphoblastic leukemia (ALL) [56]. The success of Kymriah has led to the exploration of different viral vectors for the treatment of several diseases including CRISPR/Cas9-based gene editing applications. Viral vectors are not an ideal choice for in vivo delivery of CRISPR/Cas9 components as they possess major limitations like immunogenicity, limited packaging capacity, host integration that leads to sustained Cas expression that can ultimately result in severe off-target effects, etc [57]. However, with their proven clinical track records for excellent delivery efficiency, viral vectors like adenoviruses (AdVs), adeno-associated viruses (AAVs), and lentiviruses (LVs) are being investigated in CRISPR/Cas9 genome engineering for the treatment of different disease conditions including cancer [58].

3.1.1. Adenoviral vectors (AdVs)

Adenoviruses (AdVs) are small, non-enveloped, linear double-stranded DNA viruses with a genome of ~36 kb, of which up to 30 kb can be replaced with therapeutic gene expression cassettes [59]. This characteristic offers substantial packaging capacity, along with other features such as high transduction efficiency, and reduced integration frequency [55]. However, they lack targeting capacity and exhibit higher immunogenicity primarily due to pre-existing immunity [55,60]. AdVs exhibit low-frequency integration, which has the advantage of reducing off-target effects, which is essential for an ideal CRISPR/Cas9 delivery vector [61]. Their high packing capacity enables the delivery of large sized commonly used SpCas9 protein and one or more sgRNAs together in a single vector. DJ Palmer et al. developed a helper-dependent (HDAdV) all-in-one adenoviral vector carrying Cas9 protein, sgRNA, and also the donor DNA template [62]. A high capacity AdV (HCAdV) was engineered to be devoid of all viral genes, was developed to carry CRISPR/Cas9 protein and gRNAs [63]. This HCAdV was evaluated to target different genes in different disease conditions such as the Human papillomavirus (HPV) oncogenes HPV18-E6 or HPV16-E6 in HPV-positive cells. In this study the vectors showed gene editing only in HPV-positive cells but not in HPV-negative cells. They also demonstrated their ability to target the dystrophin gene responsible for Duchenne muscular dystrophy (DMD) and the HIV co-receptor C-C chemokine receptor type 5 (*CCR5*) genes [63]. In a separate study, another AdV carrying Cas9 protein and eight multiple guide RNA were used to target the hepatitis B virus (HBV) gene integrated into the HepG2 cells for the treatment of hepatocellular carcinoma (HCC) and also for other HBV-positive disease conditions [64]. Recently, a study loaded AdVs carrying Cas9-sgRNA for complete disruption of PD-L1 gene in T-cells into silk gels, for local in vivo retention of viral vectors and also to mask them from recognition by the host immune system [65].

3.1.2. Adeno-associated viruses

Adeno-associated Viruses (AAVs) are non-enveloped single-stranded DNA viruses that exhibit the ideal characteristics of an effective viral vector including low immunogenicity, broad tropism, etc [66–68]. AAVs-based gene therapy has shown successful clinical outcomes for various genetic diseases. Luxturna (voretigene neparvovec) is the first AAV-based gene therapy drug approved by the FDA to treat inherited retinal dystrophy [69]. Recombinant AVVs (rAVVs) are devoid of proteins responsible for host genome integration, which makes them suitable delivery vector CRISPR/Cas9 components [70]. Several preclinical studies use AAV-based CRISPR/Cas9 genome engineering for the treatment of multiple diseases. The hepatic lactate dehydrogenase (LDH) gene was disrupted using the AAV-CRISPR/Cas9 tool for the treatment of primary hyperoxaluria type 1 (PH1) with no associated hepatotoxicity or off-target effects [71]. Simultaneous editing of two genes (PINK1 and DJ-1) in the brains of monkeys was successfully achieved using adeno-associated virus serotype 9 (AAV9) – CRISPR/Cas9 tool for producing Parkinson's disease animal models [72]. AAVs are used for targeted knock-in of desired genes by delivering donor template DNA for the production of animal models and model cell lines [73]. Other than the most commonly used Cas9 protein from Streptococcus pyogenes (SpCas9), engineered Cas proteins of CRISPR/Cpf1 system, archaeal Un1cas12f1, Neisseria meningitides Cas9 (NmeCas9) are also evaluated as alternatives of SpCas9 for efficient delivery using AAV vectors [74–76]. The limited packaging capacity of AAVs is the major drawback when it has to deliver large-size Cas9 proteins along with guideRNA or donor DNA templates into cells. Methods like co-delivery of Cas9 and guide RNA in two separate plasmids are practiced. A high-capacity adenoviral (HC-AdVs) that is devoid of viral genes can be used as a single vector for CRISPR/Cas9 delivery [77]. The currently available AAV serotypes were found to have limited efficiency in human T cells [78]. Nyberg WA et al. have developed an AAV variant termed Ark313 with high transduction efficiency for mouse T lymphocytes. This has for effective target-specific knock-in of transgenes including CAR targeting human CD19 [79]. Production cost and labor required for AAVs are significantly high compared to adenoviral vectors, rendering them an expensive option for gene therapy [80].

3.1.3. Lentiviral vectors

Lentiviruses (LVs) are single stranded RNA (ssRNA) viruses with high capacity for nuclear trafficking [81]. Their capacity for nuclear trafficking enables them to transduce genetic materials into non-dividing cells including neurons [82]. LVs have been largely used for preclinical and clinical applications [83]. FDA has already approved Lentiviral vector based genetically modified T cell therapies named Kymriah, Yescarta, and Tecartus for the treatment of $CD19^{+}$ B-cell malignancies [56,84,85]. Entry of LVs into the host cells is mediated by the interaction of envelope proteins with cell surface receptors [82]. Reverse transcription of ssRNA and integration of viral genetic material into the host genome are the two critical steps in the life cycle of LVs [86]. Other than the problems associated with insertional mutagenesis, integration of CRISPR/Cas9 genome engineering components into the host genome results in

a permanent expression of Cas protein, which lead to unintended off-target effects and non–specific double-strand breaks in the DNA. Hence it is important to use non-integrating/integrase-deficient LVs (IDLV) for CRISPR/Cas9 delivery. Uchida N *et al* have developed a non-integrating all-in-one LV for the delivery of Cas9 protein, gRNA and, donor DNA template for the treatment of sickle cell disease (SCD) [87]. Cell-specific targeting ability is another advantage of LVs. Systemic administration of E1/E2-pseudotyped LV vectors with Cas9 and guide RNA for kinesin spindle protein (KSP) selectively interact with Huh7 tumors and resulted in effective knock down of kinesin spindle protein, *KSP* gene with limited innate immune response [88]. Lentiviral vectors of non-integrating and cell-specific qualities can be a good platform for delivery of CRISPR/Cas9 components into different tissues including cells of the central nervous system [82].

Each of these viral vectors has specific advantages and disadvantages. Ongoing research is focused on addressing each of those challenges to improve the safety of viral vector-based genome engineering, leveraging their high transfection efficiency across various cell types and tissues. Advances in the engineering of viral vectors and alternative non-viral vectors for CRISPR/Cas9 delivery are continuously being explored to maximize the potential application of CRISPR/Cas9 in therapeutic settings.

3.2. Nanoparticles based delivery of CRISPR/Cas9

Today, nanotechnology-based nucleic acid and protein delivery are becoming increasingly attractive as they can overcome various difficulties associated with typical viral vectors [89]. Nanoparticles have already demonstrated their potential for gene therapy. FDA has already approved 3 lipid nanoparticle-based gene therapeutics for use in humans, which include ONPATTRO (siRNA), and IVT-mRNA-based COVID-19 vaccines by Pfizer-Biotech and Moderna, viz. Cominarity and Spukevax (mRNA-1273), respectively [90,91]. The success of the mRNA nano-vaccine led to the award of the Nobel Prize in medicine for Katalin Kariko and Drew Weismann in 2023 [92]. In addition, recently Intellia Therapeutics plans to initiate a phase 3 trial of nanoparticles (NTLA-2001) for in vivo CRISPR/Cas9 delivery for the treatment of transthyretin (ATTR) amyloidosis with

cardiomyopathy (CM)(NCT04601051) [21,93]. Another nanoparticle from the same company, named NTLA-2002 is under clinical evaluation for the treatment of hereditary angioedema (HAE)(NCT05120830) [94]. These studies will open up the path to more

Table 1
List of nanoparticle based CRISPR/Cas9 used in preclinical studies for anti-cancer therapy that are reviewed previously.

Nanoformulations	Cargo format	Target cells	Target Gene	Gene Editing (GE) efficiency/ Outcome	Reference
Cationic lipid coated gold nanoparticles (LACP) with photothermal therapy	pDNA	Melanoma cells (A375 cells)	*PLK-1*	65 % downregulation of *PLK-1in vitro*	[99]
Targeted ionizable lipid nanoparticles	Cas9 mRNA and sgRNA	Glioblastoma (GBM 005 cells)	*PLK-1*	70–80 % GE in vivo.	[100]
Lipid polymer hybrid nanoparticle (LPHNs) modified with cRGD peptide	pDNA	Glioblastoma	*MGMT*	35 % GE *in vitro*	[101]
Polyethylene glycol phospholipid modified cationic lipid (PLNP)	pDNA	Melanoma cells (A375 cells)	*PLK-1*	>67 % GE in vivo	[102]
Peptide modified lipid nanoparticle (R8-dGR-Lip)	pDNA	Pancreatic cancer (BxPC-3 cells)	*HIF-1α*	Downregulation of HIF-1α protein	[103]
Chain-shattering Pt(IV)backboned polymeric NP	pDNA	Colorectal cancer (PC-3 cells)	*EZH2*	32.2 % GE *in vitro* and 21.3 % GE in vivo	[104]
Zwitterionic polymer -inspired material (ZEBRA)	pDNA	HeLa cells	*PLK-1*	80 % cell apoptosis *in vitro*, tumor volume reduction in vivo.	[105]
Liposome template hydrogel NPs	Cas9 protein and minicircle gRNA	Glioblastoma (U87, Gs5 cells)	*PLK-1*	Cell growth inhibition: 79.3 % in U87 cells and 80.2 % in GS5 cells.	[106]
Dual locking nanoparticle (DLNP)	pDNA (Cas13a)	Melanoma (B16F10)	PDL-1	Specific GE% is not available	[107]
Hyperbranched poly(β-amino ester) polyplex NPs	pDNA	Cervical cancer	*HPV E7*	Inhibited tumor growth in vivo.	[108]
PEI-β-cyclodextrin cationic polymer NPs	pDNA	HeLa Cells	*HBB* and *RHBDF1*	19.1 % in *HBB*locus and 7 % in *RHBDF1in vitro*	[109]
Mesoporous organo-silica Nanoparticles	Co delivery of Cas9/ sgRNA RNP and Sorafenib	Hepatocellular carcinoma	*EGFR*	>60 % *in vitro*, 85 % tumor inhibition	[110]
pH sensitive cationic nano-liposome	pDNA	Cervical cancer (SiHa cells)	*HPV16 E6* and *E7*	72 % GE in E7 locus and 69 % GE in E6 locus *in vitro*	[111]
pH- responsive Gold Nanocluster	pDNA	HeLa Cells	*HPV E6*	34 % GE *in vitro*.	[112]
pH-responsive polymer nanoparticle	Co delivery of pDNA and Paclitaxel	Colorectal cancer (CT26), Melanoma (B16F10), Fibosarcoma (L929)	*Cdk-5*	Specific GE% is not available	[113]
Stimuli-responsive chitosan based nanocomplex	Co delivery of pDNA and Paclitaxel	Hepatocellular carcinoma (HepG2 cells)	*VEGFR2*	38.6 % *in vitro* and 33.4 % in vivo	[114]
Ultrasound control HMME@Lip-Cas9	Cas9/sgRNA RNP	Hepatocellular carcinoma	*NFE2L2*	58.77 % GE*in vitro*.	[115]

nanoparticle-based gene-editing therapy in the coming years.

In-vivo delivery of CRISPR/Cas9 components remains a great challenge in effective therapeutic applications. In-vivo delivery is carried out either systemically or locally. In systemic delivery (Fig. 2C), nanoparticles have to overcome many extracellular and intracellular barriers (Fig. 2D). For effective gene-editing of target cells, nanoparticles should protect CRISPR/Cas9 cargoes from proteolytic degradation in the physiological fluids, escape the immune recognition by mononuclear phagocyte system (MPS) and renal glomerular filtration. Once it reaches the target tissue, nanoparticles have to pass the vascular endothelium consisting of endothelial cells and penetrate the tumor microenvironment by passing through a dense extracellular matrix and heterogeneous cell population to reach target cells. Target cells internalize the particle mostly via endocytosis. The cargo has to be released from the endosome before if fuses with a lysosome to escape lysosomal degradation. Endosomal escape is a very critical step in non-viral vectors. CRISPR/Cas9 components have to enter the nucleus in order to carry out gene editing [95].

NPs. C)In-vivo systemic delivery of nanoparticles and D) route of nanoparticles from the systemic circulation to the nucleus of the target cell. From the blood vessels, NPs extravagate into the extra cellular matrix of the target tissue. Upon binding to the cell surface receptors, NPs get endocytosed into the cells. From endosomes, NPs are release into the cytosol by a process knowns as endosomal escape. The cargo is then entering the nucleus to perform the gene editing at the target DNA.

The most widely used nanoparticles for CRISPR/Cas9 delivery include lipid nanoparticles (LNPs), Polymer-based nanoparticles (PNPs), inorganic nanoparticles, and nanoparticles of other different structures and compositions [96]. The list of different nano-particles for CRISPR/Cas9-based gene editing for cancer therapy that are already discussed and reviewed are listed in Table 1, as these NPs were discussed extensively in recent reviews, [54,96–98]. In this review, we focus on the latest nano-systems reported from 2022 onwards.

4. CRISPR/Cas9 in cancer

Over the last two decades, cancer therapy has made significant progress beyond the traditional surgery, radiation, and chemotherapy regimen [116]. These advancements include molecularly targeted therapy using small molecule inhibitors, immunotherapy using immune check-point targeted monoclonal antibodies, CAR-T cell therapy, vaccines, and gene therapy [116,117]. Years of research have found the role of several gene mutations related to cancer initiation and progression [118]. CRISPR/Cas9 has great potential for cancer treatment as it can manipulate any gene expression by knocking out/knocking in, activating/deactivating, modifying the bases, or by epigenetic modifications [119]. The simplest and most widely used application of CRISPR/Cas9 in cancer treatment is the knockout of oncogenic genes [120]. Targeted insertion of specific sequences to replace the mutated sequence and thus regain the normal functions of tumor-suppressor genes is another application of CRISPR/Cas9 [121]. The point mutations in oncogenes can be corrected using the base editing technology of CRISPR/Cas9 [122,123]. CRISPR/Cas9 is also used for large-scale screening to identify novel oncogenic gene targets and understand the biological relevance of different genes in tumorigenesis, drug resistance, etc. [124–126]. In addition, CRISPR/Cas9 was explored to produce tumor cell and animal models for preclinical testing of the therapeutic effects of different therapies. Currently, in clinics, CRISPR/Cas9 is investigated extensively to improve the anti-tumor efficacy of immunotherapy [127].

4.1. Application of CRISPR/Cas9 gene-editing in cancer-immunotherapy

Tumor has developed mechanisms to evade immune surveillance using various methods. Down-regulating the tumor-associated antigen (TAAs) expression, expressing high levels of the immune checkpoint proteins, by recruiting suppressive cells like myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg), immune suppressive cytokines, and also by altering the metabolic condition of the tumor microenvironment (TME) [128,129]. Immunotherapy stimulates the body's immune cells to generate a tumor-specific response to identify and kill tumor cells using various methods such as immune checkpoint blocks (ICBs), adaptive cell transfer (ACT), chimeric antigen receptors (CAR-T cells, cancer vaccines, DC cell therapy, antibody-drug conjugate, etc. [130].

CAR-T cell therapy is cellular immunotherapy that engineer T cells to express chimeric antigen receptors that can recognize and bind neoantigens expressed in cancer cells to kill them [130]. Already six CAR-T cell therapies have been approved by the US FDA for antitumor therapy T cell therapy [131–135], T cells from a patient (autologous) or a healthy donor (allogenic) are isolated, engineered in the laboratory to express the CAR receptors, and infused back into the patients [136]. Engineered T cell activity in the human body is seriously affected by the overexpressed immune checkpoint protein which leads to T cell exhaustion and dysfunction of T cells at the tumor site. Immune checkpoints include programmed cell death protein – 1 (PD-1), cytotoxic T lymphocyte 4 (CTLA), T cell immunoreceptor with Ig and ITIM domains (TIGIT), etc. [137]. Programmed cell death-1 (PD-1) expressed in T cells interacts with the receptor PD-L1 expressed in cancer cells [138]. This interaction induces T cell apoptosis and inhibits T cell proliferation, acting as a major obstacle in CAR-T-based cell therapy leading to impaired clinical outcomes [139]. CRISPR/Cas9 was utilized in several current CAR-T cell therapies to improve the clinical outcome by eliminating immune checkpoint blockades by knocking out PD-1 in T cells [140]. A representative method of CRISPR/Cas9-based ex-vivo gene editing is shown in Fig. 3. The first clinical trial of CRISPR/Cas9 for cancer treatment was conducted in patients with non-small cell lung cancer in 2016 (NCT02793856) using PD-1 KO T cells [136]. Though the study was inconclusive in determining the efficacy of PD-1 KO T cell therapy, it provided insight to address other critical challenges associated with PD-1 KO CAR-T cells such as T cell anergy or exhaustion due to chronic exposure to tumor antigens or previous anti-cancer therapy, lack of proliferation and short life span of edited T cells inside the body, etc. [136].Later, many other genes were targeted in CAR-T cells using CRISPR/Cas9 and are under evaluation in currently ongoing clinical trials, details are given in Table 2.

In the case of autologous T cell therapy, the lack of availability of enough T cells from patients who have received extensive chemotherapy or in patients who is immune supressed due to infections with HIV or aging limits the potency of T cell therapy [131]. Autologous cell therapies have several other limitations, including manufacturing failure rates, wait times, and supply constraints. So the central focus of CRISPR research is the production of allogenic CAR-T cells. In such cases, allogenic CAR-T cells from healthy donors provide a better choice of treatment, as these cells would be "off-the-shelf" and ready to be used for patients with urgent requirements. The challenges in the use of allogenic T cells is the presence of major histocompatibility complex (MHC) class I and T-cell receptor (TCR) in T cells leading to alloreactivity and graft-versus-host disease (GVHD) [131,132]. Multiplex CRISPR/Cas9 is used to overcome the challenges of GvHD and improving the efficacy of CAR-T cell therapy in the following ways [133,134].

- Delivery of CAR construct using viral vectors results in random integration of the CAR into the DNA. CRISPR/Cas9 can precisely insert CAR construct into the TCR alpha constant (TRAC) locus for consistent expression of CAR proteins.
- T cell receptors (TCR) on T cells recognize and kill the antigen-presenting cells to provide immunity. TCR in donor T cells may recognize patient cells as foreign and result in GVHD. CRISPR/Cas9 is used to knock down the TCR on T – T-cells to reduce the risk of GvHD.
- MHC I can be recognized by TCR of the patient's own T cells and result in the rejection of donor CAR-T cells. In order to avoid that CRISPR/Cas9 is used to eliminate MHCI expression by disrupting the β2M gene.
- HLA Class-I negative cells are recognized and lysed by natural killer (NK) cells through the 'missing self' response. NK-cell-dependent lysis can be inhibited by expressing minimal polymorphic HLA-E protein. CRISPR/Cas9 can insert transgene to express the fusion of B2M and HLA-E protein (HLA-E-B2M).

Although CRISPR/Cas9 gene editing achieved promising outcomes in clinical studies [134], it still needs to be evaluated in a long-term follow-up to have a deeper understanding of its long-term effectiveness and safety. Many obstacles remain to be addressed in the current CRISPR/Cas9 technology. Off-target effects and highly efficient non-viral delivery systems have two major immediate requirements [135].

4.2. Recent progress in nanoparticle based gene editing for cancer-immunotherapy

Immune checkpoint blockade (ICB) therapy by monoclonal antibodies is a clinically successful well-known strategy for cancer immunotherapy [164]. However, ICB with mAbs results in transient blockage of immune checkpoints, resulting in insufficient cytotoxic $CD8^+$ T lymphocytes (CTLs) infiltration and T cell exhaustion [165]. The promising advantages of CRISPR/Cas9 in immunotherapy include the opportunity to permanently disrupt inhibitory genes to provide durable therapeutic immune responses, knockout of immune checkpoint proteins, activate proinflammatory genes, etc. Different nanoparticle-based CRISPR/Cas9 is used to modulate gene expression for effective anti-tumor immunotherapy.

PD-L1 and T cell immunoreceptor with immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (TIGIT) and their ligand poliovirus receptor (PVR) are promising immune checkpoint targets in immunotherapy [166,167]. Folate receptor (FRα) specific nanocarriers made of lipidoligo-amino amides (Lip-OAA) in combination with folic acid (FolA)-PEG nanoparticle deliver Cas9/sgRNA RNP to target two immune checkpoint genes PD-L1 and PVR in cancer cells [168]. FolA-mediated targeting along with dual knockout of PD-L1 and PVR resulted in enhanced $CD8^+$ T cell recruitment to the TME and tumor growth inhibition compared to the individual knockouts or the non-targeted delivery groups. A reactive oxygen species (ROS) and pH-dual responsive, core-shell tecto-dendrimers (CSTD) loaded with gold nanoparticles (Au -CSTDs) that deliver CRISPR pDNA knock-out PD-L1 in cancer [169]. Au-CSTD is made of gold NPs entrapped in lactobionic acid (LA) – modified poly-(amidoamine) dendrimers as the core. The core was surface coated with phenylboronic acid (PBA) by forming an ester bond with PBA and LA, to achieve specific accumulation in tumor tissue overexpressing sialic acid. Tumor-targeted disruption of PD-L1 enhanced ICB-based cancer immunotherapy. Au NPs are used for better X-ray contrast in computed tomography imaging of tumor accumulation studies [169]. However, from the perspective of human translation, the use of non-biodegradable Au NPs is a disadvantage, especially because of its known property of accumulation in the liver and spleen as part of mono-nuclear phagocytic clearance [170]. Lu Y et al. used triple-function magnetic nanoparticles made of mesoporous polydopamine (PDA) encapsulated with Fe3O4 to deliver Cas9 RNP to knockout PD-L1 [171]. The system accumulates at the tumor site using the magnetic targeting potential of Fe3O4 NPs. PDA generates mild photothermal therapy (PTT) effect upon laser irradiation, induces immunogenic cell death(ICD) and activates T cell-mediated immune response.

In T cell-mediated immune response, the CTLs recognize neo-antigens presented in major histocompatibility complex (MHC-1) expressed on the cancer cells, resulting in direct killing of the cancer cells [172]. It is reported that the level of MHC-1 expression in cancer cells decreases as the tumor grows, which impairs the recognition by CTLs [172,173]. A combination of CRISPR activation (CRISPRa) to upregulate MHC-1 expression and traditional CRISPRKO to disrupt the PD-L1 expression in cancer cells enhance $CD8^+$ cytotoxic T cell infiltration to tumor tissue, enhanced dendritic cell maturation and antigen presentation, and prevented tumor recurrence and metastasis [174]. This was achieved using a pH and photo dual-activatable binary CRISPR nanomedicine (DBCN) made of a thioketal linked polyplex core and an acid-detachable polymer shell which effectively delivers different gene-editing components specifically to tumor tissue in response to the acidic TME and releases the components upon laser irradiation [174]. The need for laser irradiation for the photo-trigger is a challenging proposition for human translation.

Another critical challenge of immunotherapy, as well as CRISPR-based gene-editing in solid tumors, is the lack of accessibility to the interior of the solid tumor mass due to the physically dense, stroma-rich tumor microenvironment, which inhibits the accessibility

Table 2
List of CRISPR/Cas9 based clinical trials for anti-cancer therapy as of Sep 2023, arranged according to their last date of update.

CRISPR Modifications	Cell type	Target disease	Delivery method	Phase/study status	Sample No	NCT Number	Sponsors	Last updated	Ref
TRAC KO CAR insertion at TRAC locus β2M KO β2M-HLA-E insertion at β2M locus	Autologous anti-BCMA CAR-T cell. (CB-011)	Relapsed/refractory multiple myeloma	NA	Phase I, Recruiting	50	05722418	Caribou Biosciences, Inc.	August 2023	[141]
TRAC KO CAR insertion at TRAC locus PD-1 KO	Allogenic Anti-CD19 CAR-T cell	Relapsed/Refractory B Cell Non-Hodgkin lymphoma	NA	Recruiting	72	04637763	Caribou Biosciences, Inc.	August, 2023	[142]
TRAC KO CAR at TRAC locus β2M KO	Allogenic CD19 CAR-T cells (CTX110)	Relapsed/Refractory B-Cell malignancies	Electroporation	Recruiting	227	04035434	CRISPR Therapeutics	August 2023	[143]
TRAC KO CAR at TRAC locus β2M KO	Allogenic CD19 CAR-T cells (CTX112)	Relapsed/Refractory B – cell malignancies	Electroporation	Phase I/II Recruiting	120	05643742	CRISPR Therapeutics	August 2023	[144]
CD52 KO TRAC KO CAR insertion at TRAC locus	Allogenic CD19 T cells (TT52CAR19)	B – cell Acute Lymphoblastic Leukemia (B-ALL)	Lentiviral vector	Phase I, Active, not recruiting	10	04557436	Great Ormond Street Hospital For Children NHS Foundation trust	May 2023	[145]
TRAC KO CAR at TRAC Locus β2M KO	Allogenic CD70 – CAR- T cells (CTX130)	Advanced Relapsed or refractory Renal cell carcinoma	Electroporation	Active, not recruiting	107	04438083	CRISPR therapeutics	May, 2023	[146]
TRAC KO CAR at TRAC locus β2M KO	Allogenic CD70^{-} CAR- T cells (CTX131)	Relapsed/Refractory Solid Tumors	Electroporation	Phase I/II, Recruiting	250	05795595	CRISPR Therapeutics AG	April, 2023	[147]
PD-1 KO	MUC1-CAR T cell	Advanced breast cancer	NA	Phase I/II Completed	15	05812326	Sun Yat-Sen university	April, 2023	[148]
CISH KO	TILs	Metastatic Gastrointestinal cancer	NA	Phase I/II Recruiting	20	05037669	Intima Biosciences	March, 2023	[149]
CISH KO	TILs	NSCLC	NA	Phase I/II, Not yet recruiting	70	05566223	Intima Biosciences, Inc.	December, 2023	[146]
PD-1 KO	Primary T cell	NSCLC	Electroporation	Phase I Completed	22	02793856	Sichuan university	January, 2021	[150]
PD-1 KO TCR KO	Mesothelin CAR- T cells	Mesothelin Positive multiple solid tumors	NA	Phase I, Unknown	10	03545815	Chinese PLA General Hospital.	August, 2020	[151]
HPK1 KO	Autologous CD-19 CAR T cells	CD19^{+} Leukemia/Lymphoma	Lentiviral (CAR) Electroporation (HPK1)	Phase I, Recruiting	40	04037566	Xijing Hospital	July, 2019	[152]
PD-1 KO	Primary T cell	Esophageal cancer	NA	Completed	21	03081715	Hangzhou Cancer Hospital	June, 2019.	[153]

(*continued on next page*)

Table 2 (*continued*)

CRISPR Modifications	Cell type	Target disease	Delivery method	Phase/study status	Sample No	NCT Number	Sponsors	Last updated	Ref
PD-1 KO	Primary T cell	Metastatic renal cell carcinoma	NA	Phase I/withdrawn (no funding)	0	02867332	Peking university	March, 2019	[154]
PD-1 KO	Primary T cell	Prostate cancer	NA	Phase I /Withdrawn (no funding)	0	02867345	Peking university	March, 2019	[155]
PD-1 KO	Mesothelin- CAR-T cells	Mesothelin positive multiple solid tumors	NA	Phase I/Unknown status	10	03747965	Chinese PLA general Hospital	November, 2018	[156]
PD-1 KO	MUC1- CAR T cell	Advanced Esophageal Cancer	Lentiviral (CAR insertion)	Phase I/II Unknown status	8	03706326	Hospital of Guangdong pharmaceutical University.	October, 2018	[154]
TRAC KO CAR at TRAC locus β2M KO	Allogenic anti-BCMA – CAR-T cells	Relapsed/Refractory Multiple Myeloma	Electroporation	Phase I, Active, not recruiting.	26	04244656	CRISPR Therapeutics.	July 2018	[157]
PD-1 KO	MUC1-CAR-T cells	NSCLC	Lentiviral-(CAR Insertion)	Phase I/II Unknown	60	03525782	The First Affiliated Hospital of Guangdong pharmaceutical University.	May 2018	[158]
TRAC and CD52 KO	$CD19^+$ CD20/CD22 CAR-T cells	Relapsed/Refractory haematological malignancies.	NA	Phase I/II Unknown status	80	03398967	Chinese PLA general hospital	January 2018	[155]
TCR KO β2M KO	Allogenic CD19 – CAR -T cells (UCART19)	Relapsed/Refractory $CD19^+$ Leukemia/Lymphoma	Lentiviral (CAR) Electroporation (CRISPR)	Phase I/II Unknown	80	03166878	Chinese PLA general hospital	June 2017	[159]
HPV E6/E7 KO	–	HPV – related cervical intraepithelial NeoplasiaI	NA	Not yet recruiting	60	03057912	Sun Yat-Sen university	June 2017	[160]
PD-1 KO	EBV-CTL cells	EBV positive advanced stage malignancies	NA	Phase I/II, Unknown	20	03044743	Hospital of Nanjing University Medical School	May 2017	[161]
CCR5 KO	Allogenic $CD34^+$ HSPCs	HIV – infected subjects with haematological malignancies	NA	Unknown	5	03164135	Affiliated hospital to Academy of Military Medical Sciences.	May 2017	[162]
TRAC KO CAR at TRAC locus β2M KO	Allogenic CD70 CAR-T cells (CTX130)	Relapsed/refracted T or B Cell malignancies	Electroporation	Phase I, Recruiting	45	04502446	CRISPR Therapeutics	April 2017	[163]

of nanoparticles and infiltration of immune cells to the interior of the tumor mass [175]. Tumor penetration of nanoparticles and T cell infiltration was enhanced by modulating the stiffness of the extracellular matrix (ECM) of tumor tissue by inhibiting focal adhesion kinase (FAK) using siRNA. Multiplexed dendrimer lipid nanoparticles (LNP) co-packed with anti-FAK siRNA, Cas9 mRNA, and sgRNA, reduced PD-L1 expression and ECM stiffness and hence significantly increased immune cell infiltration for effective immunotherapy in four mouse models of cancer [176].

Immunogenic cell death (ICD) stimulates long-lasting antitumor immune response by activating immature dendritic cells (DCs) upon exposure to damage-associated molecular patterns (DAMPs) [177]. Luo W et al. used a novel method to deliver CRISPR/Cas9 components into deep tumor tissue to induce ICD using facultative anaerobe Escherichia coli Nissle 1917 (EcN) bacteria along with photothermal therapy (PTT) [178]. Photothermal agent, polydopamine (PDA) coated EcN was further coated with cationic liposomes that are encapsulated with pCRISPR targeting Hsp90α using ROS responsive linkers (shortly EPP). Hypoxia-specific EcN releases liposomes upon reaching the deep tumor in response to acidic TME. mPTT–induced immunologic cell death (ICD), kills both tumor cells and bacterial cells and triggers an anti-tumor immune response by releasing TAAs that can be recognized by DCs. These processes of enhanced tumor penetration, gene editing, and immune activation in the hypoxic core of tumors reshaped the cold tumors into hot tumors [178]. Xing Y et al. developed a Cas9/sgRNA RNP nanocapsule (Cas9NC) which synergistically activates antitumor immune response by time-dependent DC47 disruption and immunogenic cell death [179]. The nano-capsule is made of a thin polymer shell of multiple functional monomers (cationic and anionic monomers, ICD monomer, blood-circulated monomer, tumor-targeted monomer and glutathione-degradable cross-linkers) to encapsulate Cas9 RNP through non-covalent interactions. Time-programmed laser irradiation coupled with stimuli-responsive nanocapsule first releases Cas9 RNP to knockout CD47. Extended laser irradiation results in the activation of ICD, leading to robust and durable anti-tumor immune responses in vivo [179].

Metabolic activity of tumor microenvironment (TME) plays a critical role in $CD8^+$ T cell dysfunction and potential antitumor activity [180]. Recently, Bian et al. reported the link between amino acid metabolism and T-cell dysfunction [181,182]. Cancer cells that overexpress SLC43A2 compete with $CD8^+$ T cells for methionine, deficiency of which leads to dysfunctioning and death of $CD8^+$ T cells [169]. Mn2+/Zn2+ bimetallic metal-organic framework (MOF) nanoparticles modified with hyaluronic acid target SLC43A2 to remove the methionine competition pressure from $CD8^+$ T cells [183]. Hyaluronic acid modification of nanoparticles enhances tumor accumulation. The release of large amounts of nutrient metal ions (Mn2+ and Zn2+) stimulated the cGAS/STING pathway to increase T cell infiltration and also produced a large amount of ROS to induce tumor cell death [170]. Another important target for metabolic engineering is lactate dehydrogenase (LDHA). A cationic lipid nanoparticle-based CRISPR/Cas9 knockout of LDHA in combination with PD-L1 antibody in a melanoma tumor model achieved synergistic anti-tumor response and improved the survival of the animals [184].Yang et al. reported a novel two-step strategy for tumor-specific delivery of CRISPR/Cas9 components and activation of innate and adaptive immunity [185]. A TME-biodegradable nano-gel made of hollow manganese dioxide (Ac4ManNAz/H-MnO2@Gel) selectively labels tumor cells with azido groups via metabolic engineering and releases manganese ions (Mn2+) to activate the cGAS-STING pathway to improve antitumor immunity. Simultaneously dibenzocyclooctyne (DBCO) modified liposome carrying pCRISPR (DBCO Lipo/p) is delivered to target protein tyrosine phosphatase N2 (PTPN2) gene to enhance adaptive immune response [185].

Repolarization of anti-inflammatory M2 macrophages into proinflammatory M1 macrophage phenotypes has been an attractive strategy for cancer immunotherapy [186]. However, repolarized M1 macrophages can revert to the M2 phenotype as they constantly receive macrophage colony-stimulating factors (M-CSF) released by tumor cells. The colony-stimulating factor 1 receptor (CSF1-R) expressed in macrophages binds with M-CSF, resulting in the activation of signalling pathways responsible for tumor-suppressive M2 phenotype [186,187]. Also, overexpression of CD47 on cancer cells interacts with the signal regulatory protein α (SRPα) on macrophages and inhibits phagocytosis of tumor cells [188]. X-CC9 is an X-ray-guided and triggered system that controls the expression of CRISPR/Cas9 gene-editing components in the tumor to permanently repolarize TAMs to M1 macrophages without the risk of reconversion [189]. X-CC9 uses a cationic polymer poly-etherimide-g-poly-propylene sulfide (PEI-PPS) to deliver CRISPR plasmids to target both CSF1-R and SIRPα genes. Upon X–ray irradiation this nanosystem gets activated and releases the gene editing components at the tumor site [189].

4.3. Nanoparticle based gene editing using CRISPR/Cas9 in other cancer models

4.3.1. Glioblastoma multiforme (GBM)

GBM is an aggressive tumor with a very low survival rate of 14 months or less [190]. Patients with GBM exhibit rapid recurrence after surgical removal, radiation, and chemotherapy [191]. Currently, CRISPR/Cas9 is used to study the significance of several genes involved in the tumor growth, stemness, recurrence, and resistance of GBM [192–194]. Sammarraie NA and Ray SK have listed all the possible gene targets involved in the tumorigenesis of GBM in their review [195]. Low permeability across the blood-brain barrier (BBB), poor in vivo stability of gene editing components, and lack of tumor targeting are limiting factors in the systemic delivery of CRISPR/Cas9 components to the brain. Different nanoparticles are used for the targeted delivery of anti-glioma therapeutics to the brain [196]. Our team has shown promising deep brain penetration and drug release up to 15 days using injectable nano-gels, which can be re-engineered for delivery of gene editing components locally in the brain [197,198].

Zou Y et al. developed a non-invasive and effective gene therapy approach for brain delivery using a nanocapsule with the potential to cross the blood-brain barrier [199]. Small-sized nano-capsules (~30 nm) are made of a disulfide-cross-linked polymer shell and functionalized with angiopep-2. Angiopep-2 binds with low-density lipoprotein receptor-related protein-1 (LRP-1), which is highly expressed in BBB endothelial cells and glioblastoma cells. Treatment of nano-capsules encapsulated with Cas9/sgRNA RNP targeting PLK-1 resulted in inhibited tumor growth and extended survival time in animal models. Nano-capsules without the disulfide

cross-linking or angiopep-2 failed to achieve BBB penetration and gene editing [199]. To improve the in vivo stability and endosomal release of the Cas9 RNP in glioma tissue, Ruan et al. developed a brain-targeted CRISPR/Cas nanomedicine using polymeric NPs (Ang-NP@RNP) functionalized with angiopep-2, guanidinium, and fluorine [200]. Guanidinium and fluorine groups improve the in vivo stability and enhance endosomal release of the encapsulated Cas9 RNP targeting PLK-1. Treatment with Ang-NP@RNP resulted in suppression of tumor growth and improved survival in orthotropic glioblastoma models [200].

CRISPR/Cas9-based loss–of function screening identified the role of glutathione synthetase (GSS) as a potential regulator of radioresistance [201]. High levels of GSS are associated with suppression of radiotherapy-induced ferroptosis in glioma cells. Extracellular vehicles (EVs) modified with angiopep-2 (Ang) and trans-activator of transcription (TAT) peptides encapsulating Cas9/sgRNA RNP exhibit high capacity for both BBB and tumor penetration [201].

The hypoxic microenvironment is another characteristic feature of solid tumors like glioblastoma, where the most aggressive and highly resistant cancer cells reside [202]. Hypoxia-response elements (HREs) sequence–driven gene expression by hypoxia-inducible Factor-I (HIF-I) are well studied in multiple systems including hypoxia-specific CAR-T cells and other therapies [203,204]. By utilizing this mechanism, Davis et al. achieved hypoxic specific expressions of therapeutic genes in GBM delivered using lipid nanoparticles (LNPs) [205]. The efficacy of the system was demonstrated using two therapeutic genes: the herpes simplex virus thymidine kinase (HSV-tk) suicide gene and the CRISPR/Cas9. The LNP-mediated delivery and HRE-regulated expression of Cas9 and sgRNA targeting PLK-1 genes exhibited significant reductions in cell viability in multiple cancer cell lines, including glioblastoma-patient-derived neurospheres in a hypoxia-specific manner [205].

4.3.2. Lung cancer

Lung cancer is one of the most common cancers, among which non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer [206]. Several genes and their aberrant expression result in acquired and multi-drug resistance. Researchers are constantly looking for novel gene targets involved in drug resistance and other tumorigenesis activities in NSCLC. One of the significant contributions of CRISPR/Cas9 was the genetic screening of potential gene targets in NSCLC [207,208].

For the first time, Wang Y et al. investigated the effects of Human MutT homolog 1 (MTH1) disruption in NSCLC using nanoparticle-based CRISPR/Cas9 [209]. MTH1 expression is crucial for cancer survival but not required for normal cell proliferation [210]. A multifunctional hyaluronic acid (HA) modified lipid nanoparticle loaded with pDNA condensed using protamine sulphate (PS@HA-Lip/pMTH1) was used to target MTH1 knockout. Treatment with PS@HA-Lip/pMTH1 showed targeted accumulation at the tumor site, a significant reduction in tumor growth, reduced liver metastasis, and prolonged survival compared to other treatment groups [209]. Dual targeting of MTH1 and another gene Apurinic/apyrimidinic endonuclease 1 (APE1) involved in base excision repair pathways using nanoparticles-based CRISPR/Cas9 in combination with photodynamic therapy (PDT) showed significant anti-tumor effects when both MTH1 and APE1 were targeted simultaneously compared to the individual knockout [211]. The nano platform called Ce6-Mn-Cas9, composed of His-tagged Cas9 bound to Chlorin-e6 (Ce6) - a photosensitizer, Manganese ($Mn2+$) encapsulated Pluronic (Poloxamer) F127 micelles. Side effects associated with PDT are a concern in the clinical application of this combinatorial therapy [211].

Survivin is an inhibitor of apoptosis that is over-expressed in most cancers with the highest expression in lung and breast cancers [212]. Wang et al. used a mannose/ethanolamine functionalized poly glycidyl methacrylate (PGEA-co-ManMA) star polycation termed GM for targeted CRISPR/Cas9 delivery in lung cancer cells that express high levels of mannose receptors (MR) [213]. In vivo, studies using GM2/pCas9-survivin treatment showed tumor growth inhibition without causing toxicity to other organs [213].

FOXM1 protein is an oncogenic transcription factor overexpressed in many cancers including NSCLC, correlated with cell proliferation, tumorigenesis, and metastasis (201). Khademi et al. designed a multifunctional delivery vector composed of cell targeting polymer (hyaluronic acid), a cell, and a nuclear targeting group (AS1411 aptamer) on the surface of a chitosan-based core encapsulated with pDNA (Apt-HA-CS-CRISRP/Cas9) to improve tumor accumulation and cellular uptake [214]. In vitro and in vivo gene knockout of FOXM1 using Apt-HA-CS-CRISRP/Cas9 reduced cell density and noticeable necrosis in the tumor tissue [214]. These studies have shown effective in vivo tumor-specific targeting of lung cancer using different nanoparticles and also demonstrated novel gene targets such as MutH1, Survivin, FOXM1 for gene editing-based therapy in lung cancer.

4.3.3. Breast cancer

Breast cancer exhibits the highest genetic diversity due to hereditary and somatic mutations, which result in increased cell proliferation and tumorigenesis [215]. Several preclinical studies used CRISPR/Cas9 gene editing tool to downregulate multiple oncogenes (cMYC, CXCR7, CXCR4, FASN, HER2, FOXA1, CDK7, UBR5), to upregulate tumor suppressor genes (PTEN, FOXP3, p53, RB1, NF1), and also to demethylate promoters of genes (BRCA1) involved in tumor progression in breast cancer [216].

Over-expression of N-cadherin (N-cad, Cdh2) is correlated with poor patient prognosis in triple-negative breast cancer patients as they promote the epithelial to mesenchymal transition (EMT) [217]. Gao R et al. knocked out the Cdh2 gene using CRISPR/Cas9 using a biosynthetic nanobubble (Gas vesicles, GVs) combined with ultrasound [218,218]. Nanobubbles are made from Halobacterium NRC-1 or Anabaena flos-aquae, and modified with polyethyleneimine (PEI) to deliver pDNA encodes sgRNA targeting Cdh2 gene to Cas9 expressing 4T1 breast cancer cells upon stimulation with ultrasound. A significant reduction in the Cdh2 expression in the edited cells resulted in reduced tumor cell migration in the Cdh2 KO cells compared to the normal cells [218]. Similarly, Li Y et al. used a combination of pH-responsive lipid-polymer hybrid nanoparticles (PLNPs) and ultrasound-mediated microbubble destruction (UMMD) to enhance the efficacy of pH-responsive lipid-polymer hybrid nanoparticles (PLPNs) to deliver CRISPRi plasmids to silence microRNA-10b (miR-10b) in metastatic breast cancer models [219]. The PLNPs/miR-10b in combination with UMMD exhibited improved anti-metastatic efficacy compared to other treatment groups [219]. These strategies of combining gene editing using

ultrasound can be useful only in laboratory conditions for basic research applications and for ex-vivo-based gene editing.

Studies have shown that the combination of survivin shRNA and doxorubicin (DOX) has improved therapeutic benefits for various cancers [220]. Li Q et al. used CRISPR/Cas9 to knockout the survivin gene to resensitize the tumor to doxorubicin (DOX) treatment [221]. Co-delivery of sgSurvivin pDNA and DOX was achieved using a nano-vector composed of trimethyl chitosan named FTD NPs. Chitosan NPs modified with folic acid (FA) and 2- (Diisopropylamine) ethyl methacrylate (DPA) enhance their uptake by cancer cells. FTD NPs with pH sensitivity release DOX under acidic conditions. The co-delivery of sgSurvivin pDNA and DOX from a single delivery system exhibits an improved antitumor effect compared to single delivery of either DOX or sgSurvivin pDNA. The study also explored the antitumor efficacy between CRISPR/Cas9 and RNAi, and found no significant difference in the antitumor efficacy when survivin was targeted with CRISPR/Cas9 and siRNA [221]. The study also highlights the potential of nanoplatforms for the co-delivery of chemotherapeutic and gene editing components for cancer therapy, which is a promising strategy to be investigated in other cancer models.

4.3.4. Cervical cancer

Cervical cancer is the fourth most common type of cancer in females caused mostly by the infection with high-risk human papillomavirus (HPV) [222]. HPV E6 and H7 oncoproteins degrade the host tumor suppressor genes, p53 and pRb [222]. Viral vectors based CRISPR/Cas9 could completely remove the HPV E6 and E7 in cervical cancer and achieve a significant reduction in tumor growth and enhanced tumor cell apoptosis by upregulating the expression of p53, p21, pRb [223–225].

CRISPR/Cas9-based knock-out of HPV using liposomes promoted $CD8^+$ T cell infiltration in the tumor site, enhanced the expression of proinflammatory cytokines, and reduced regulatory T cells and myeloid suppressor cells [226]. Combinatorial treatment of CRISPRKO of HPV using liposomes with immune checkpoint inhibitors and anti-PD-1 antibodies showed significant antitumor effects compared to the anti-PD-1 antibody alone or HPV KO alone groups. This study highlights the significant therapeutic effect of the combination of HPV-targeted CRISPR-based gene editing and immune checkpoint blockade (ICB) in cervical cancer [226]. Ling K et al. used a pH-responsive nanoparticle composed of acetylated cyclic oligosaccharide (ACD) in combination with low molecular weight polyethyleneimine (PEI) to deliver Cas9mRNA and sgRNAs to knock out the HPV18 E6/E7 oncogenes [227]. Treatment with these nanoparticles in xenograft mice models resulted in reshaping the immunosuppressive microenvironment, leading to improved $CD8^+$ T cell survival and synergistic antitumor effects when combined with adoptive T-cell transfer therapy.

In another study, Khairkhah et al. used a cell penetrating peptide, LL-37 for systemic delivery of the CRISPR/Cas9 vector for targeting specific genes associated with HPV16, including E5, E6, E7, and p97 promoter genes [228]. The simultaneous knockouts of the E6 and E7 genes using CRISPR/Cas9 showed a significant reduction in tumor volume compared to individual knockouts. They also compared the efficiency of CRISPR/Cas9-mediated gene editing with the chemotherapy agent cisplatin. The tumor size was significantly smaller in the CRISPR/cas9-treated groups compared to the cisplatin-treated groups [228]. Liang Y et al. used a biomimetic mineralized CRISPR/Cas9 delivery system for multiplex gene editing to simultaneously target PLK-1, survivin, and HPV genes [229]. Mineralized NPs (Mg2PPi) were synthesized by a natural mineralization process to encapsulate Cas9 mRNA and multiple guide RNAs within the single nanoparticle with a high RNA loading capacity. Mg2PPi achieved efficient multisite gene editing in vivo, providing an ideal platform for multiplexed gene editing applications in cancer models [229]. Together these study provides promising insight for the development of novel gene therapeutic strategies for HPV-related cancers using the CRISPR/Cas9 system in cervical cancers.

4.3.5. Prostate cancer

Prostate cancer is the sixth leading cause of cancer death in men [230]. Erythropoietin-producing hepatocellular receptor A2 (EphA2) regulates the growth and survival of cancer cells through different signalling pathways in prostate cancer [231]. Nanoparticles made of calcium phosphate core and a cell-penetrating peptide, - TAT termed as "RNP@CaP-TAT" targeting EphA2 resulted in significant inhibition of cell migration and invasion but no significant apoptosis was observed [232]. ~50 % of cancers possess a mutation in the tumor suppressor gene, TP53. PEI-modified graphene quantum dots (PEI-GCD) are used to correct the mutated TP53 in prostate cancer model cells (PC-3) using CRISPR/Cas9 RNP complex and ssODN donor template and resulting in cancer cell apoptosis [233].

4.3.6. Liver cancer

Hepatocellular carcinoma (HCC) mostly arises as a result of many environmental and genetic factors such as liver cirrhosis, infections from hepatitis B virus (HBV), Hepatitis C virus (HCV), alcohol consumption, non-alcoholic fatty liver, etc. [234].

The liver is the primary organ of LNP accumulation following systemic delivery, hence delivery of nucleic acids into hepatocytes using LNP has a therapeutic advantage [235]. Li C et al. developed a lipid nanoparticle using ionizable lipid (iLP18) for efficient delivery of CRISPR/Cas9 for the treatment of hepatocellular carcinoma. The iLP181 LNPs effectively encapsulated plasmid encoding both Cas9 enzyme and guide RNA targeting PLK1 and achieved gene editing efficiency of 33 % in-vitro in liver cancer cells. A single dose of intravenous injection of iLp181/psgPLK-1 accumulated in the tumor for more than 5 days, resulting in significant inhibition of tumor growth in vivo with an ideal safety profile. These LNPs can be used for the treatment of other genetic diseases in liver tissue [235].

4.3.7. Gastrointestinal cancers

Gastrointestinal (GI) cancer affects the GI tract including different types such as gastric cancer, pancreatic cancer, colorectal cancer, etc. [236]. GI cancers showcase sequential accumulation of mutations in oncogenes during the cancer progression [236]. CRISPR/Cas9 is intensively used in the functional analysis of the combination of different gene mutations involved in colorectal cancer [237].

Table 3
List of recently reported nanoparticles based CRISPR/Cas9 gene editing in different cancers.

Nature of the Nanoparticle	Composition of NPs	Cargo used	Cell surface target	Gene target	Target cells	Gene editing (GE) %	Route of administration	Ref
Immunotherapy								
Lipid-oligoamino amides (Lipo-OAA) -FolA-PEG-DBCO NPs	Oligo(ethyenamino) amides (OAAs), FolA-PEG-DBCO	RNP	Folate Receptor – α (FR- α)	*PD-L1* and *PVR*	CT26	*PD-L1* (68.2 %) and *PVR* (61.7 %) *in vitro*.	Intravenous	[168]
ROS and pH responsive core-shell tecto dendrimers loaded with AuNPs (Au-CSTDs)	Au-entrapped Poly(amidoamine) G5 (PAMAM) Dendrimers and Phenylboronic acid (PBA) modified G3 PAMAM	pDNA	Sialic acid (SA)	*PD-L1*	B16F10	*PD-L1* 59.8 % *in vitro*.	Intravenous	[169]
Dual-activatable binary CRISPR nanomedicine (DBCN)	Thioketal –PEI-PBA -Pheophorbide (Pha) (TK-PPP) Glucose modified Poly (ethylene glycol) -*b*-polylysine ($mPEG_{113}$-*b*-$PLys_{25}$/Glu)	pDNA	Sialic acid (SA)	*MHC 1* activation and *PD-L1* KO	B16F10	*PD-L1* 34.2 % *in vitro* and 28.7 % in vivo.	Intravenous	[174]
Mesoporous polydopamine (mPDA) -Fe_3O_4 nanoparticles	Mesoporous PDA with Fe_3O_4, modified with mPEG and nickel	RNP	Magnetic targeting	*PD-L1*	B16F10	*PD-L1* 42.1 % *in vitro* and 25.1 % in vivo.	Intravenous	[171]
Multiplexed dendrimer Lipid Nanoparticles (LNPs)	5A2-SC8 ilipid, DOPE, Cholesterol, PEG-lipid	FAK siRNA, Cas9 mRNA and PD-L1 sgRNA	NIL	*FAK* inactivation and *PD-L1* KO	Ovarian and liver cancer models	Not available	Intravenous	[176]
Polydopamine-coated *Escherichia coli* Nussle 1917 (EcN) coated with pCRISPR encapsulated liposome (Lipo-P)	Polydopamine-coated *Escherichia coli* Nussle 1917 (EcN) coated with pDNA-Liposome (DOTAP, DOPE, DSPE-PEG, $DSPE\text{-}PEG_{2000}$-COOH)	pDNA	NIL	*HSP90α*	4T1	*HSP90α* 33.65 % *in vitro* and 25.08 % in vivo.	Intravenous	[178]
Cas9RNP Nano-capsule (Cas9NC) of multiple functional monomers	N-(3-aminopropyl) Methacrylamide (APM), Methacrylate (MA), Acrylated pheophorbide a (APPa), Acrylated PEG (APEG), folic acid -APEG, N.N′-bis (acryloyl)cystamine (BIS).	RNP	Folic acid receptor	*CD47*	B16F10, 4T1	*CD47* 36.4 % *in vitro* and 38.4 % in vivo.	Intravenous	[179]
Mn^{2+}/Zn^{2+} bimetallic Metal organic framework (MOF) nanoparticles	Zn^{2+}, Mn^{2+}, 2-methylimidazole (MM), Poly (allylamine hydrochloride), Hyaluronic acid (HA)	pDNA	CD44	*SLC43A2*	4T1	Not available.	Intravenous	[183]
Cationic lipid NPs	DOTAP, DC-Chol, DPhPE	pDNA	NIL	*LDHA*	B16F10 HeLa-GFP	31.3 % in GFP *in vitro*.	Intratumoral	[184]
TME-biodegradable Hollow manganese dioxide (H-MnO_2) nanogel + DBCO Lipo/p	Gelatin coated hollow MnO_2labelled with N-azidoacetylannosamine-tetraacylated (Ac_4ManNAz). Dibenzocyclooctyne (DBCO)coated liposome (DOTAP, DOPE, DBCO-PEG2000-DSPE)	pDNA	NIL	*PTPN2*	B16F10	*PTPN2* 28.2 % *in vitro*, 24.9 % in vivo.	Intravenous	[185]
X-ray guided and triggered cationic polymer (X-CC9)	Polyetherimide-g-poly (propylene sulphide) (PEI-PPS), 2,2″-diselenobis acetic acid,PEG with dual aldehyde	pDNA	NIL	*SIRPα*	RAW264.7	10 % in GFP gene, *in vitro*.	Intravenous	[189]
Angiopep-2 peptide coated polymeric NPs	Acrylate guanidine, *N, N′*-bis (acryloyl) cystamine and angiopep-2-PEG	RNP	LRP-1	*PLK-1*	U87MG	*PLK1:36.6%in vitro*, 33.8%in vivo.	Intravenous	[199]

(*continued on next page*)

Table 3 (*continued*)

Nature of the Nanoparticle	Composition of NPs	Cargo used	Cell surface target	Gene target	Target cells	Gene editing (GE) %	Route of administration	Ref
Immunotherapy								
Angiopep-2, guanidinium and fluorine functionalized polymeric nanoparticle. (Ang-NP@RNP)	Angiopep-2 –poly (ethylene glycol) -*block*-poly (*N*-(3-methacrylamideo-Propyl) guanidinium) or Poly (ethylene glycol)-block-poly[(*N*-(3-methacrylamidopropyl) guanidinium-*co*2,2,3,3-tetrafluoropropyl methacrylate}	RNP	Lipoprotein Receptor Protein −1 (LRP-1)	*PLK-1*	U87MG	*PLK-1* 32 % *in vitro*.	Intravenous	[200]
Angiopep-2 and trans-activator of transcription (TAT) dual peptide modified extracellular vehicle (EV).	Angiopep-2, (TAT) trans-activator of transcription (TAT) - dual peptide modified EVs.	RNP	LRP-1	*GSS*	LN229	58.6 % *in vitro*, 61.8 % in vivo	Intravenous	[201]
Lipid nanoparticles	DLin-KC2-DMA, SOPC, Cholesterol, DMG-PEG	pDNA	NIL	*PLK-1*	U251, 293FT, HT-1080, H1299.	~30 % *in vitro*	–	[205]
Hyaluronic acid (HA) modified lipid nanoparticle (PS@HA-Lip/p*MTH1*)	DOTAP, DOPE, Cholesterol, DSPE-PEG-HA, Protamine sulphate (PS)	pDNA	CD44	*MTH1*	A549	33.1 % *in vitro*, 29.28 % in vivo.	Intravenous	[209]
Ce6 encapsulated pluronic (Poloxamer) F127 micelles (Ce6-Mn-Cas9)	Pluronic F-127, Chlorin e6, Manganese (Mn)	RNP	NIL	*MTH1* and *APE1*	NIH3 T3 and HEK-293	No GE% available	Intra-tumoral	[211]
Mannose/ethanolamine functionalized PGMA cationic polymers (GM).	Mannose/ethanolamine Functionalized Polyglycidyl methacrylate (PGMA) polymer	pDNA	Mannose receptors	*Survivin*	A549 and HEK293	No GE% available	Intravenous	[213]
Chitosan NP modified with HA and Aptamer (Apt-HA-CS-CRISPR/Cas9)	AS1411, Hyaluronic acid (HA), Chitosan (CS).	pDNA	Nucleolin, CD44	*FOXM1*	MCF-7, HeLa, HEK293, SK-MES-1	No GE% available	Intravenous	[214]
Gas Vesicle (GV) with ultrasound	Gas vesicles from *Halobacterium NRC-1* or *Anabaena flos-aquae,* Polyethylenimine (PEI)	pDNA	NIL	*Cdh2*	Cas9 and GFP expressing 4T1 cells	12.27 % *in vitro*.	Intravenous	[218]
pH-responsive lipid-polymer hybrid nanoparticles (PLNPs) with ultrasound-mediated microbubble destruction (UMMD).	PEI-PBA, Lecithin, Cholesterol, DSPE-PEOz.	pDNA of dCas9-KRAB and sgRNA	Sialic Acid.	microRNA −10b (miR-10b)	4T1 and MDA-MB231	No GE% available	Intravenous	[219]
pH-responsive trimethyl chitosan NPs (FTD NPs)	Folic acid (FA), 2-(Diisoproplamino) ethyl methacrylate (DPA) grafted trimethyl chitosan (TMC).	pDNA and Doxorubicin	Folic acid receptors (FA).	*Survivin*	4T1 and A549	No GE% available	Intravenous	[246]
Liposomes	DOTAP, DOPE, Cholesterol, DSPE-PEG.	pDNA	NIL	*HPV 16 E6/E7*	SiHa	Reduction of E6 and E7 expression	Intravenous	[226]
pH responsive acetylated cyclic oligosaccharide (ACD) with PEI.	Acetylated cyclic Oligosaccharide (ACD), PEI.	Cas9mRNA and sgRNA	NIL	*HPV 18 E6/E7*	HeLa cells	25.5 % at E6 and 17 % at E7 locus *in vitro*.	Intra-tumoral	[227]

(*continued on next page*)

Table 3 (*continued*)

Nature of the Nanoparticle	Composition of NPs	Cargo used	Cell surface target	Gene target	Target cells	Gene editing (GE) %	Route of administration	Ref
Immunotherapy								
Cell penetrating peptide (LL-37) NPs	LL-37 peptide	pDNA	NIL	*HPV 16 E5/E6/E7*	HeLa cells	80 % GE *in vitro*.	Intravenous	[228]
Biomimetic mineralized NPs	Magnesium Pyrophosphate (MgPPi), PEI, HA.	Cas9 mRNA and sgRNA	CD44	*Survivin, PLK-1, HPV*	HeLa cells	Survivin: 31.9 %, PLK1: 24.41 % HPV: 23.2 %	Intravenous	[229]
Ionizable lipid Nanoparticles(iLNPs)	Ionizable lipid-iLP181, Cholesterol, DSPC, DMG-PEG.	pDNA	NIL	*PLK-1*	HEK293A and HepG2-Luc	33 % in HepG2 cells *in vitro*	Intravenous	[235]
Chitosan/Hyaluronic Acid/ Protamine sulphate (CS/ HA/PS) polyplexes	Chitosan (CS), Hyaluronic acid (HA), Protamine sulphate (PS).	pDNA	CD44	*ERCC1*	HT-29	46.6 % delivery efficiency Reduction in ERCC expression		[242]
Polydopamine NPs	Polydopamine (PDA)	RNP	NIL	*HMGA2*	MKN-45 and MGC-803	95 % delivery efficiency and 82 % GE *in vitro*.	intravenous	[245]
TAT modified – calcium phosphate NPs	Transactivator of Transcription (TAT), Calcium chloride ($CaCl_2$)	RNP	NIL	*EPHA2*	PC-3	GE% not available	–	[232]
PEI - Graphene Quantum Dots (GQD).	Glucosamine HCL, PEI.	RNP and ssDNA.	NIL	*TP53* Gene correction	PC-3, HEK-293T,	GE% not available	–	[233]

CRISPR/Cas9 screening of novel genes in colorectal cancer leads to the identification of new pathways such as nicotinamide adenine dinucleotide kinase (NADK), ketohexokinase (KHK) that are activated in KRAS-mutated CRC [238] and genes involved in TGF-β resistance [239].

Oxaliplatin is the primary drug used in patients with metastatic colorectal cancer (CRC) [240]. Resistance to treatment with oxaliplatin alone or combinatorial treatment of oxaliplatin with irinotecan or 5-fluorouracil/leucovorin are significant barriers in the metastatic CRC treatment [241]. Bidabadi et al. used a system biology approach that included protein-protein interaction (PPI) networks and pathway enrichment analysis to identify the crucial genes and pathways involved in drug resistance [242]. They found a critical role of the excision cross complementation group 1 (ERCC1), a member of the nucleotide excision repair (NER) pathway in drug resistance and poor prognosis in CRC patients. They also developed a chitosan/hyaluronic acid/protamine (CS/HA/PS) polyplexes for CRISPR/Cas9-based disruption of ERCC1 to restore drug resistance in oxaliplatin-resistant cells. This study sheds light on the molecular mechanism of oxaliplatin resistance in CRC patients and demonstrates the potential of nanoparticle-based genome editing using CRIPSR-Cas9 to overcome chemo resistance in colorectal cancer [242].

Gastric cancer is the third leading cause of cancer death worldwide, which remains a major unsolved clinical condition [243]. High mobility group protein A2 (HMGA2) is a transcription factor overexpressed in most types of gastric cancer [244]. HMGA2 was knocked out using a polydopamine (PDA) based nanoparticle carrying Cas9/sgRNA RNP complex in gastric cancer with high efficiency [245]. In vivo, treatment with CRISPR/Cas9-3NLS/sgHMGA2@ PDA resulted in a drastic decrease in HMGA2 expression and a reduction in tumor volume in the treated animals. They demonstrated the use of a highly biocompatible nanoparticle system with higher gene editing efficiency that can be used for gene editing therapy for cancer as well as other diseases [245].

Details of each nanoparticle, including composition, format of cargo used, cell surface targets, gene, cell line, % of gene editing and route of administration of in vivo studies that are reviewed here are listed in Table 3.

5. Novel alternatives to CRISPR/Cas9 genome engineering tool

The Discovery of CRISPR/Cas9 revolutionized the field of genetic engineering, which had an undeniable impact on biomedical research but it possesses several limitations such as the requirement of PAM sequence, off–target effects occurring due to random DSBs, delivery challenges due to the large size of Cas9 enzyme, difficulty in simultaneous delivery of Cas9/sgRNA complex along with exogenous DNA for homology-directed repair, etc. This demands alternative or modified CRISPR tools to address these limitations and hence improve the potential application of CRISPR/Cas9 technology. A CRISPR/Cas9 modified technique named Cas-CLOVER was utilized in a clinical trial (NCT05239143) as an alternative to Cas9 protein to improve the challenges associated with off-target effects [247]. This section highlights some of the promising alternatives of gene editing methods including both CRISPR/Cas9-less and CRISPR/Cas9-modified futuristic gene editing tools.

5.1. CRISPR/Cas9-less genome engineering tools

5.1.1. Retron recombineering

Retron recombineering is a genome engineering technique that uses in-vivo synthesized DNA to modify target gene sequences without generating double-strand breaks [248,249]. Recombineering incorporates either single-stranded or double-stranded DNA into the target genome by homologous recombination using phage-derived proteins [250–253]. Generally, exogenous DNA is synthesized *in vitro* and delivered to the target cell for recombination. Recently, retro elements were shown to be capable of synthesizing multicopy single-stranded DNA (msDNA) inside cells using reverse transcriptase (RT) and non-coding RNA (ncRNA) that is delivered to the cells using a retron plasmid [254,255]. As shown in Fig. 4 retron plasmid consists of sequences for msdRNA (msr), msDNA (msd) and the gene for reverse transcriptase in a single operon. Inside the cells, RT produces an unusual RNA-DNA molecule known as msDNA. msDNA consists of a single strand of structured RNA (msr)connected to a strand of DNA (msd) via a 2′-5′ linkage which connects the 5′end of DNA to the 2′ position of internal guanosine in the msr [256]. RT-derived donor DNA is incorporated into the replicating DNA

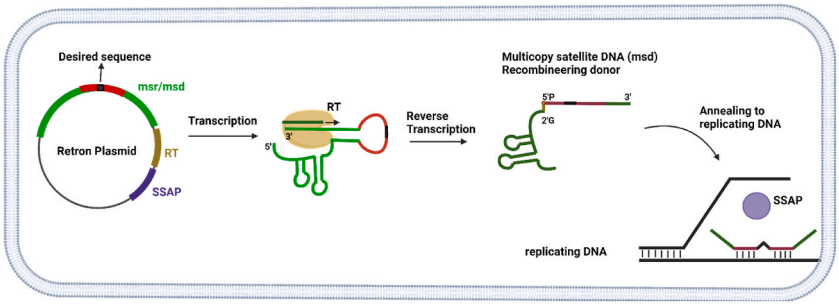

Fig. 4. Schematic representation of the process of retron recombineering inside a cell. Retron plasmids encode sequences of msr/msd sequence with specific mutations, reverse transcriptase (RT) and single strand annealing proteins (SSAP). Inside the cells these plasmids produce multicopy satellite DNA (msd) donor template using reverse transcriptase. These msd donor template with specific mutations anneal to the replicating DNA at the target DNA with sequence complimentary to the msd donor template.

using single-stranded annealing protein (SSAP) [248]. Studies have demonstrated the potential of bacterial retron for the synthesis of ssDNA in yeast [257], and mammalian cell lines, such as mouse NIH3T3 cells [254], very recently in K562 and HEK293T [258]. This technique carries out simultaneous editing of multiple sites in single genome and the barcoding of the mutations for large scale screening applications [249]. Although retron recombineering ensures the availability of adequate amount of template DNA in the target cells, continuous production of donor template by the cells across generations can be a major limitation of this technique in some organisms [248].

5.1.2. Argonaute proteins (NgAgo)

In 2016, F Gao et al. reported DNA-guided DNA endonuclease activity of the Argonaute protein family, Natronobacterium gregoryi Argonaute (NgAgo) [259]. NgAgo uses a 5′- phosphorylated single-stranded guide DNA (5′P-ss gDNA) of 24 nt to create site-specific DSBs (Fig. 6B). These proteins do not require PAM sequence as does Cas9 proteins. Later the authors retracted the publication because of the continued inability of the research community to reproduce the gene editing activity as per their protocol [260] and many others reported the inability of genome editing activity of NgAgo in human cells [261,262]. Later, in 2021, Lee et al. reported the DNA nicking activity of prokaryotic Argonautes (pAgos) [263]. They reported that NgAgo from the halophilic archaea exhibit poor expression and protein folding under normal salt conditions. They also showed that NgAgo with canonical PIWI domain and a previously unrecognized single strand binding domain repA participate in DNA cleavage activity. Many prokaryotic Argonaute proteins target RNA and decrease mRNA levels without generating detectable DNA-double strand breaks [264–266]. Thus it is important to choose the Argonautes proteins with optimal activity under physiological conditions according to the target (DNA or RNA) for specific gene editing applications [267].

5.1.3. Omega and Fanzor

Feng Zhang and his team for the first time identified a group of transposons-encoded RNA-guided endonucleases, named OMEGA (Obligate Mobile Element Guided Activity) and Fanzor that functions similarly to Cas9 and Cas12 endonucleases [268]. IscB, IsrB and TnpB proteins are collectively referred to as OMEGA proteins that are reprogrammable RNA–guided DNA endonucleases associated with IS200/IS605 family of transposable elements found in prokaryotes. These proteins coupled with a noncoding RNA termed omegaRNA (ωRNA), form ribonucleoprotein complex and perform RNA-guided cleavage of dsDNA [269,270]. These OMEGA proteins with smaller sizes can be further re-engineered and optimized for genome engineering applications in eukaryotic cells.

Fanzor (Fz) was reported as eukaryotic programmable RNA-guided endonucleases by Feng Zhang and his team in June 2023 [271]. A representative image of Fanzor protein is shown in Fig. 6C. Earlier in 2013 Bao W et al. reported two TnpB-like proteins (Fanzor1 and Fanzor 2) as eukaryotic TnpB-IS200-IS605-like proteins encoded by transposon elements [272]. The recent development of TnpB proteins of OMEGA system in prokaryotes as RNA-guided DNA endonucleases led the focus on finding a functional understanding of TnpB-like eukaryotic transposon-encoded Fanzor proteins. This research focus led to the understanding of Fz proteins as eukaryotic alternatives of CRISPR or OMEGA systems. Fz1 protein from the fungus *Spizellomyces punctatus* (SpuFz1) exhibited up to 18.4 % indel activity in the human genome [271]. Further, guide RNA engineering and screening of more Fzs might improve the genome engineering potential of Fzs. Detailed characterization and optimization of these proteins with minimal size are suitable candidates for genome engineering in humans. The smaller size of these proteins is the major advantage for AAV-based and nanoparticle-based delivery for human genome engineering applications.

5.1.4. Meganucleases (MegNs)

Meganucleases or homing endonucleases (HEs) are naturally occurring endonucleases found as part of the mobile genetic elements [269]. Engineered synthetic meganucleases are built by fusing nonspecific DNA cleavage domain and zinc-finger domain that can bind longer sequences in the target DNA to produce double-strand breaks, which are repaired mostly via homologous recombination (HR) [270]. Mainly five different families of meganuclease with discrete DNA binding mechanisms are identified and studied in detail [269, 271]. Meganucleases bind to longer target sequences, hence their off-target effects are very rare which makes them the perfect tool for genome engineering [272].). Based on a natural meganuclease, Precision Biosciences has developed ARCUS nucleases for clinical gene editing purposes and used them to produce several allogenic CAR-T cell products that are genetically modified to improve in vivo T cell expansion and also to evade recognition by host immune cells [273]. These products named PBCAR0191 (NCT03666000) and PBCAR19B (NCT04649112) are being evaluated in patients for the treatment of both non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia. Protein engineering of meganucleases for each of the new target sequences requires significant expertise and resources [274].

5.1.5. Zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs)

Before CRISPR/Cas9 was invented, the most widely investigated targeted nucleases for genome engineering were ZFNs and TALENs. ZFNs are engineered endonucleases built by combining custom-designed DNA binding domains of transcription factor zinc finger proteins (ZFPs) with the nuclease domain of *Fok1* restriction enzyme [273]. An array of Cys2-His2 fingers in the ZFP monomers recognize and bind discrete bases in the DNA, and are identified by different methods including phage display [274]. These fingers with specific triplet codon binding capacity are assembled to form a multi-finger protein of three to four fingers that are unique for each target sequence [275]. Correct orientation and spacing of adjacent *Fok*1 leads to dimerization of *Fok*1 and catalytic cleavage of double-stranded DNA within the spacer region of two oppositely bound ZFN monomers [276]. The potential of ZFNs for accurate gene editing was utilized the for the clinical treatment of glioblastoma (GBM) patients who are treated with IL13Rα2-targeted CAR + cytotoxic T-lymphocytes (CTLs) that are genetically modified by knocking out glucocorticoid receptor (GR) [277]. The major

limitation of ZFNs in practical application is the construction of zinc finger arrays for each of the targets and the difficulty in creating zinc fingers that can recognize all possible triplet codons of DNA [278]. Another drawback is the lack of specificity, leading to potential off-target effects [279,280].

Later TALENs got much attention as targeted nucleases due to their ease of design process compared to ZFNs and higher DNA cleavage capacity [281,282]. TALENs are designed by the fusion of transcription activator-like effector (TALE) with catalytic domains of the *Fok*1 restriction enzyme [283]. DNA binding domains of TALE proteins possess tandem repeats of monomers, each of which binds one nucleotide at the target sequence [284]. Like ZFNs, TALENs also work as dimers, in which each monomer is designed to bind opposite DNA strands separated by ~12–25 bp spacer sequence. Upon DNA binding, *Fok*1 domains dimerize and create double-strand breaks in the spacer sequence [285]. Details of the clinical trials involving allogeneic T cells engineered using TALENs are discussed in the review by CA Harris, 2020 [286].

Both ZFNs and TALENs require labour-intensive custom designing and construction of nucleases for each target with superior on-target cleavage specificity and minimal off-target effects. The need for custom-designed nucleases for each target is laborious, costly and also limits its use for multi-target gene editing.

5.2. Novel CRISPR/Cas9-modified gene editing tools

5.2.1. *CRISPEY (Cas9 retron precISe Parallel Editing via homologY)*

CRISPEY is a combined tool of two techniques: CRISPR/Cas9 and bacterial retrons developed by Hunter B. Fraser and his team at Stanford University [257]. CRISPEY makes use of retrons to in vivo synthesize donor templates for CRISPR/Cas9-based HDR gene editing. Normally CRISPR-based high-throughput screening uses a library of guide RNA to generate DSBs that can be repaired by either NHEJ or HDR pathways. To produce specific variants, a library of donor DNAs carrying the required mutations along with the corresponding guide RNAs needs to be delivered to the cells. This random delivery does not ensure the delivery of the corresponding sgRNA/donor DNA pair to the same cells [257]. CRISPEY can ensure the presence of specific gRNA and corresponding donor DNA in the same cells by delivering an engineered single multi-cistronic vector encoding sequences of msr-msd, RT, and SpCas9/sgRNA as shown in Fig. 5. From a multicistronic plasmid, a gRNA-msr/msd donor RNA and mRNA of Cas9 and reverse transcriptase in produced inside the cells. guide RNA and RT-derived ssDNA are linked together at the 3′end to form an RNP complex with Cas9 to carry out specific gene editing. The potential of CRISPEY in human cell lines is demonstrated and achieved an HDR occurrence of up to 11.4 % [255].Even though CRISPEY has demonstrated to improve HDR efficiency in some cases, it has also shown reduced cell viability due to increase in retron activity post transfection, resulted in lower HDR efficiency. Further refinement is required to improve msDNA synthesis without cytotoxicity of target cells to improve HDR efficiency of CRISPEY [257]. Further refinement to improve msDNA synthesis and HDR efficiency of CRISPEY promises a great tool for precise genome editing and other applications [258]. Achieving effective in vivo delivery of larger plasmids carrying genes for both retrons and CRISPR will the most formidable challenge here.

5.2.2. *Cas-CLOVER*

Cas-CLOVER is a high-fidelity RNA-guided site-specific dimeric nuclease developed to reduce the off-target effects associated with Cas9 nuclease [287]. Cas-CLOVER is engineered by the fusion of the nuclease domain of Clostridium Clo051 type II restriction endonuclease with dead SpCas9 (dCas9) protein via a G4S linker and two SV40 nuclear localization signal (NLS) at both amino and

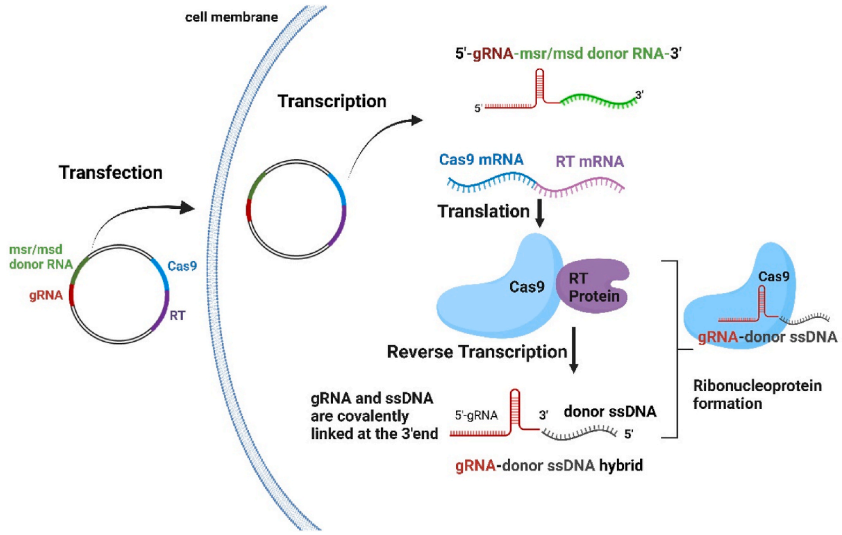

Fig. 5. Schematic representation of the mechanism of CRISPEY. CRISPEY donor plasmid contains gRNA-msr/msd donor template sequence, and sequence for Cas9-reverse transcriptase (RT). Inside the cells, RT produce gRNA – donor ssDNA, which complex with the cas9 enzyme and perform gene editing (knocking in) at the target sequence.

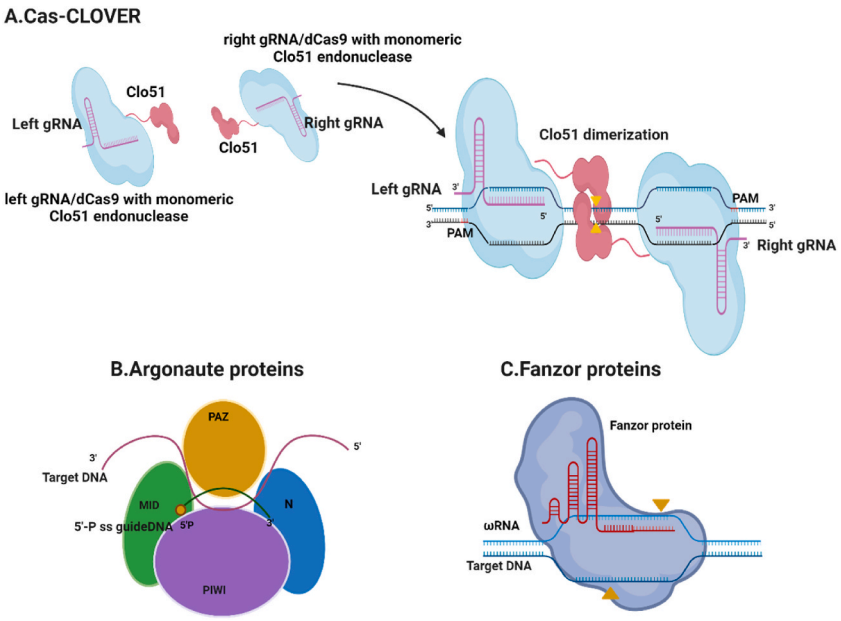

Fig. 6. Representative images of A) Cas-CLOVER: mechanism of gRNA/dCas9 guided dimerization of monomeric Clo51 endonuclease at the target dsDNA. Fusion of Cas9 – monomeric Clo51 protein with guide RNA complex binds to the left and right side of the target sequence. Binding of left and right gRNA/Cas9/Clo51 complex result in dimerization of Clo51 endonuclease. Dimerized Clo51 cleaves the double stranded DNA (dsDNA) at the centre and produce DSBs. B) Argonaute protein. Argonaute proteins is multimeric protein with PAZ, PIWI, N and MID domains. They use a single stranded DNA with 5′-phosphate group as the guide DNA and produce DSBs at the target site. and C) Fanzor proteins use a guide RNA known as omega RNA (ωRNA) to produce staggered double strand break at the target DNA.

carboxy terminus of the fusion protein. Monomeric Cas-CLOVER does not exhibit any nuclease activity. RNA-guided recognition of two adjacent target sequences leads to dimerization of the Clo051 monomeric nuclease domain. As shown in Fig. 6A, dimeric Cas-CLOVER binds to target sequences of two half-sites and creates indels by making DSBs at the spacer sequence between the two half-sites. This requirement of recognition of target sequence by dual guide RNA achieves the lowest off-target activity [288]. Cas-CLOVER demonstrated its ability to target mutagenesis in human T cells [287], iPSCs, and CHO [288] cells as well as in plants like tobacco [289] and banana [290]. Poseida Therapeutics initiated the first-in-human clinical trial in 2022 with Cas-CLOVER-engineered p-MUC1C-ALL01 allogenic CAR-T cells in the treatment of subjects with advanced or metastatic solid tumors (NCT05239143). The study evaluates the potential of Cas-CLOVER to achieve targeted inactivation of the T cell receptor beta constant (TRBC) and B2M loci in BCMA-directed CAR-T cells [247].

5.2.3. CasMINI

The larger size of CRISPR/Cas enzymes, Cas9 and Cas12a (1000–1500 amino acids) restricts efficient in vivo delivery, which hampers clinical applications of CRISPR/Cas9 technology. Viral vectors like adeno-associated virus (AAV) vectors have limited packing capacity. The delivery of plasmid encodes both Cas9 enzyme and guide RNA in a single vector in AAV vectors is a great challenge [291].

Recently several compact cas proteins have been identified and characterized. Cas proteins classified under class 2, type V-F (Cas12f) CRISPR nucleases have less than half the size of wild-type Cas9 and Cas12a enzymes. These proteins are made of 400–700 amino acids with a single RuvC nuclease domain [292,293]. Both Kim et al. and Xu et independently reported the miniature Cas system (CasMINI) engineered from the type V-F Cas12f and explored its potential for precise gene editing in mammalian systems [75,294]. CasMINI exhibits transcriptional activation by fusing with transcriptional activator, base editing activity by using dCasMINI- mediated adenine base editor (dCasMINI-ABE), and nuclease activity for efficient genome editing [75,294]. CasMINI can be an ideal protein for both in vivo and ex vivo cell engineering and gene editing applications.

Ma D et al. engineered a compact version of the Cas9 protein from Staphylococcus aureus (mini-SaCas9) by removing the nuclease domain and retaining the DNA binding domain [295]. Wu Z et al. reported a miniature Cas9 from *Acidibacillus sulfuroxidans* (AsCas12f1) of only 422 amino acids which are capable of making DSBs in both bacterial and human genomes [296]. CasX is another compact cas protein identified by Liu et al. that can be used for site-specific gene editing in human cells [297]. Aharrar A. et al., demonstrated three novel miniature Cas12f nucleases, Al1Cas12f1 and Al2Cas12f1 from the genus *Alistipes* and Ti1Cas12f1 from the genus *Tidjanibacter* composed of 486–488 amino acids [298].

Detailed characterization and optimization are required for it to be used for mammalian genome engineering. Compact cas proteins with precise gene editing potential can be easily delivered via both viral and non-viral vectors for wider genome engineering applications. These compact cas proteins with nanoparticle delivery systems can improve the in vivo delivery efficiency and hence improve

the therapeutic effect of CRIPSR/Cas9 genome engineering.

6. Conclusion

The invention of CRISPR/Cas9 in 2012 as a gene editing tool in mammalian cells brought revolutionary changes in biomedical research. The first decade of CRISPR/Cas9 has shown rapid advancements with many clinical trials being conducted for cancer and other genetic diseases. Clinical trials of CRISPR/Cas9 are mostly carried out for non-solid, haematological malignancies and hereditary diseases like SCD, ATTR, etc. showing great clinical outcomes so far. A multidimensional and systemic analysis of the clinical outcome and its potential side effects in the long-term duration is crucial to comment on its efficacy and safety at this moment.

Though CRISPR/Cas9 is well established and widely used for various gene editing purposes, it still possesses a significant gap in large-scale clinical applications. The lack of a proper delivery vehicle significantly affects gene editing efficiency. The larger size of the Cas9 protein significantly affects the delivery efficiency even with viral vectors and other non-viral methods. Expression of gene editing components in non-target tissue/cells leads to off-target effects is another limitation that significantly affects the clinical application of CRISPR/Cas9. Thus it is very important to address these limitations to improve the clinical application of CRISPR/Cas9 gene editing. Recently scientists have come up with many advanced versions of Cas proteins with half the size of Cas9 to overcome the delivery challenges. Recent findings of miniature cas proteins are giving hope for improving the delivery efficiency with both viral and nanoparticle-based delivery methods. Similarly, new alternative gene editing proteins like Omega, Fanzor, and Argonaute proteins with compact size can be optimized to improve gene editing efficiency in mammalian systems. The recent progress in modified Cas proteins like Cas-CLOVER can eliminate the chances of off-target effects and hence overcome the limitations of off-target effects.

Another major limitation is the lack of a suitable biocompatible delivery system with higher delivery and gene editing efficiency. Current clinical studies widely depend on viral vectors due to their high transfection efficiency. However, immunogenicity is a major concern associated with viral vectors. An incident of death of a patient who received CRISPR/Cas9-based gene editing therapy for muscular dystrophy due to innate immune response associated with adeno-associated viral (AAV) vectors [299] are case we need to consider while selecting the suitable delivery vehicle for clinical applications. Apart from immunogenicity, viral vectors have other limitations such as chances of integrated mutagenesis, lower packing capacity, high cost of production, etc. Current clinical trials for cancer therapy are mostly carried out in the ex-vivo mode of gene editing by editing CAR-T cells for haematological malignancies, which partly avoids the challenges associated with immunogenicity related to viral vectors. However, to fully utilize the potential of CRISPR/Cas9, it has to be delivered in vivo to treat diseases like solid tumors of glioblastoma, HCC, etc. that require in-situ gene editing.

A combination of CRISPR/Cas9 and nanoparticles can have a tremendous impact on anti-cancer therapy. Research on the development of biocompatible and target-specific nanoparticles with higher delivery efficiency and less toxicity for direct applications in humans especially for the treatment of cancer is progressing. Lipid nanoparticles have already entered clinical trials for CRISPR/Cas9-based gene editing in the liver (160,161), giving hope for more nanoparticle-based gene editing for cancer therapy as well. Recently reported nanoparticles highlight the targeting potential of nanoparticles for specific gene editing in cancer cells such as targeted delivery of the CRISPR/Cas9 using targeting molecules like Angiopep-2, and hyaluronic acid, providing great hope in minimizing the off-target mutations associated with in vivo delivery of CRISPR/Cas9. Another significant advantage of the nanocarriers is the stimuli-responsive (pH, ROS, hypoxia) release of the CRISPR/Cas9 components inside the tumor microenvironment that ensures controlled gene editing. Multifunctional nanoparticles provide simultaneous targeting capacity, codelivery of chemotherapeutics (like small molecule drugs), and other components such as photodynamic, photothermal, and immune activators.

In conclusion, progress in biological alterations to improve the specificity and efficacy of gene editing of CRISPR/Cas and delivery vehicles with high targetability and higher gene editing efficiency will significantly uplift the in vivo application of the nanoparticle-based CRISPR/Cas9 gene editing in cancer as well as other diseases.

CRediT authorship contribution statement

Najma Nujoom: Writing – original draft, Visualization. **Manzoor Koyakutty:** Writing – review & editing, Conceptualization. **Lalitha Biswas:** Writing – review & editing. **Thangarajan Rajkumar:** Writing – review & editing, Conceptualization. **Shantikumar V. Nair:** Writing – review & editing, Validation, Conceptualization.

Data and code availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Shantikumar Nair reports was provided by Amrita Vishwa Vidyapeetham Amrita Centre for Nanosciences and Molecular Medicine. Shantikumar Nair reports a relationship with Amrita Vishwa Vidyapeetham Amrita Centre for Nanosciences and Molecular Medicine that includes: employment. If there are other authors, they 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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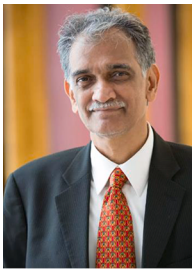

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