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REVIEW

A glance at genome editing with CRISPR–Cas9 technology

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Abstract
In recent years, CRISPR–Cas9 technology is widely acknowledged for having major applications in the field of biotechnology for editing genome of any organism to treat a variety of complex diseases and for other purposes. The acronym 'CRISPR–Cas' stands for clustered regularly interspaced short palindromic repeats–CRISPR-associated genes. This genetic organization exists in prokaryotic organisms and aids in the development of adaptive immunity since a protein called Cas9 nuclease cleaves specific target nucleic acid sequences from foreign invaders and destroys them. This mode of action has gained interest of the researchers to understand the insights of CRISPR–Cas9 technology. Here, we review that CRISPR–Cas organization is restricted to two classes and possesses different protein effectors. We also review the architecture of CRISPR loci, mechanism involved in genome editing by CRISPR–Cas9 technology and pathways of repairing double-strand breaks (DSBs) generated during the process of genome editing. This review also presents the strategies to increase the Cas9 specificity and reduce off-target activity to achieve accurate genome editing. Further, this review provides information on CRISPR tools used for genome editing, databases that are required for storing data on loci, strategies for delivering CRISPR–Cas9 to cells under study and applications of CRISPR–Cas9 to various fields. Safety measures are implemented on this technology to avoid misuse or ethical issues. We also discuss about the future aspects and potential applications of CRISPR–Cas9 technology required mainly for the treatment of dreadful diseases, crop improvement as well as genetic improvement in human.

Keywords CRISPR · Cas9 · Target · Off-target · Genome editing

Introduction

'CRISPR–Cas9' stands for 'Clustered Regularly Interspaced Short Palindromic Repeats-Cas CRISPR-associated protein 9'. CRISPR–Cas9 technology has immense potential in genome editing as well as gene therapy for treating diseases related to cancer, infections and genetic disorders (Doudna and Charpentier 2014; Hsu et al. 2014). This technology serves as a framework to carry out genome editing for the purpose of investigating and studying various diseases (Cox et al. 2015; Jinek et al. 2013; Mali et al. 2013). CRISPR–Cas9 organization helps in determining the activity of a gene during any illness state, rectifying a gene that causes harmful mutation and switching off/on cancer causing genes or switching on tumor suppressors (Doudna and Charpentier 2014; Hsu et al. 2014; Charpentier and Marraffini 2014; Wang et al. 2016). This technology possesses enormous capability to cure patients suffering from cancer by increasing the efficacy of immunotherapy and minimizing the price of T cell treatment (Eyquem et al. 2017; Ren et al. 2017; Legut et al. 2018). CRISPR–Cas9 technology can also cure many diseases related to nerve cells such as Duchenne muscular dystrophy, heart related diseases and diseases arising from the breakdown of immune system such as autoimmune disorders (Barrangou and Doudna 2016; Heidenreich and Zhang 2016; Strong and Musunuru 2017; Xiong et al. 2016). As this technology has a wide range of applications in various fields, it becomes essential to have a detailed understanding of this technology and its future aspects.

A wide range of strategies exist in prokaryotes such as archaea and bacteria that provide resistance against invading foreign agents mainly viruses and plasmids. Prokaryotic organisms have the two strategies of immunity, viz. innate immunity and adaptive (acquired) immunity. In innate immunity, prokaryotes recognize the foreign invaders on

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encounter and contribute to first line of defense mechanism whereas adaptive immunity serves as the second line of defense mechanism by providing immunological responses and storing immunogenic memory for their defense during second encounter. Adaptive immunity in archaeal and bacterial genomes is due to the existence of CRISPR–Cas structure because CRISPR along with Cas proteins target foreign mobile genetic elements (MGEs) and hence, they are eradicated eventually (Barrangou et al. 2007; Van Der Oost et al. 2014). CRISPR–Cas organization provides immunogenic memory to bacteria in order to protect themselves from foreign invaders during second exposure (Marraffini and Sontheimer 2010). CRISPR–Cas system exists in prokaryotic genomes, and thereby characterizes about 83% of archaea and 45% of bacteria (Barrangou and Marraffini 2014).

CRISPR loci were reported to be nearly palindromic and were first observed in *iap* gene (gene causing alkaline phosphatase isozyme conversion) of *Escherichia coli* in an intergenic region upstream to the gene (Ishino et al. 1987). These DNA repeats were also studied in many bacterial species like *Mycobacterium tuberculosis* (Groenen et al. 1993), *Streptococcus pyogenes* (Hoe et al. 1999), *Methanocaldococcus jannaschii* (Bult et al. 1996), in archaeal species namely *Haloferax mediterranei*, *Haloferax volcanii* (Mojica et al. 1995), *Thermotoga maritima* (Nelson et al. 1999) and in filamentous cyanobacterium *Anabaena* sp. (Masepohl et al. 1996) as well as in other archaeal and bacterial species. The acronym 'CRISPR' was coined in 2002 (Jansen et al. 2002). In 2005, three autonomous groups reported that CRISPR–Cas organization occurs in prokaryotes (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005) and its contribution to adaptive immunity was proved in 2007 (Barrangou et al. 2007). Several studies on the mechanism of CRISPR–Cas system were experimentally carried out in vitro by infecting different hosts with different kinds of bacteriophages and plasmids (Barrangou et al. 2007; Díez-Villaseñor et al. 2010; Pougach et al. 2010; Westra et al. 2010). It was first reported in *Streptococcus thermophilus* that the bacterium integrated nucleotide sequences termed 'protospacer' of foreign MGEs into its CRISPR locus and developed protection against invaders and these sequences are termed as 'spacers' (Barrangou et al. 2007). As viruses evolve rapidly, the bacteria also need to develop effective defense mechanism against these viruses. Therefore, the *cas* genes of the CRISPR–Cas system evolve with magnificent variations in their gene repertoires and loci structure (Makarova et al. 2011, 2015).

In recent years, Cpf1 nuclease, also known as Cas12a, was identified in *Lachnospiraceae bacterium ND2006* (LbCpf1) and *Acidaminococcus* sp. *BV3L6* (AsCpf1) (Zetsche et al. 2015) and has also paved the way for their potential applicability in the field of genome editing (Zetsche et al. 2015, 2017; Kim et al. 2016a, b; Hur et al. 2016; Kleinstiver et al. 2016; Tang et al. 2017). Cas12a can effectively modify genomes of microorganisms with reduced destructive outcome compared to Cas9 and as a result, the enzyme can be largely implemented in the area of biotechnology (Swarts and Jinek 2018). CRISPR–Cas12a has also major applicability for manipulating genomes of plants (Kim et al. 2017), non-mammalian vertebrates (Moreno-Mateos et al. 2017), mammals (Kim et al. 2016; Hur et al. 2016), yeasts (Świat et al. 2017) and insects (Port and Bullock 2016). Another enzyme called Cas13a can only cleave target sites of RNA sequences. CRISPR–Cas13a can be useful for editing RNA sequences and developing RNA interference in higher class organisms to provide protection from viruses (Aman et al. 2018). This enzyme also helps in understanding the insights of RNA in eukaryotic organisms and treatment of diseases (Abudayyeh et al. 2017). CRISPR–Cas13a is also utilized for diagnostic test for identifying genetic materials within an organism that possess pathogenicity (Knott and Doudna 2018).

Genome engineering was first performed in bacteria (Jiang et al. 2013) and mammalian cells (Mali et al. 2013; Cong et al. 2013) by using CRISPR–Cas9 system. Gene editing technologies that are accomplished based on restriction enzymes, e.g., transcription activator-like effector nucleases (TALENs) and zinc-finger nuclease (ZFNs) depend on binding of protein–DNA (Gaj et al. 2013). The disadvantage of these technologies is that proteins are needed to be designed for each experiment (Barrangou and Doudna 2016). The Cas9 endonuclease from the bacterium, *Streptococcus pyogenes*, termed SpCas9 is widely employed for genome engineering where the gRNA guides the SpCas9 for cleaving the target sites based on DNA–RNA hybridization (Sander and Joung 2014). In addition to genome editing and disease treatment, CRISPR–Cas toolbox has recently emerged to provide various strategies for functional genomics screening, point-of-care diagnosis as well as live-cell imaging (Knott and Doudna 2018). In this review, we explain the mechanism involved in CRISPR–Cas9, its classification, its tools and databases developed for acquiring precise gene editing, increase in Cas9 specificity, off-target activity reduction, its delivery strategies to cultured cells, its applications and future perspectives.

Classification of CRISPR–Cas system

CRISPR–Cas organization occurs in two forms of classes, and is further categorized into six types (I–VI) and 27 subtypes (Makarova et al. 2015; Shmakov et al. 2017; Koonin et al. 2017). Large number of archaea (such as in entire hyperthermophiles) and bacteria are known to consist of Class 1 CRISPR–Cas system in their genomes, whereas the Class 2 system is known to exist in bacteria, but not in

hyperthermophiles (Makarova et al. 2015; Chylinski et al. 2014). Based on the character of nuclease effector, Class 1 system comprises types I, III and IV which have multi-subunit Cas protein effector complexes whereas Class 2 system includes types II, V and VI with single protein effector modules. The nuclease effector proteins are necessary at interference stage (Makarova et al. 2011, 2013; Shmakov et al. 2015). CRISPR–Cas systems targeting the DNA viruses are type I, II and V, whereas type VI targets the RNA viruses. However, type III is both DNA and RNA targeting CRISPR–Cas system, though target for type IV system has not yet been identified (Koonin et al. 2017). Table 1 represents the classification of CRISPR–Cas organization and their few subtypes and effectors.

Structure of CRISPR loci

In CRISPR loci, a series of repeats are present that flank the 'spacer' sequence and this spacer sequence matches with the sequences in virus, plasmid or other pathogen genomic elements (Bolotin et al. 2005; Van der Oost et al. 2009; Horvath and Barrangou 2010; Terns and Terns 2011; Deveau et al. 2010). Generally, an AT-rich leader sequence is located in the upstream position of the CRISPR array (Jansen et al. 2002). On one end of the array, a set of conserved genes coding for varieties of Cas proteins, called CRISPR-associated (*cas*) genes are present (Marraffini and Sontheimer 2010). The structure of CRISPR loci is shown in Fig. 1.

Table 1 CRISPR–Cas system class 1 and 2 with their effectors

Class	Type	Sub-type	Effector	References
1	I	I-C	Cas5, Cas7, Cas8	Koonin et al. (2017)
		I-E	Cas5, Cas6, Cas7	
	III	III-A	Cas5, Cas7, Cas10	
		III-B		
	IV		Csf1, Cas5, Cas7	Makarova et al. (2011)
2	II		Cas9	Heler et al. (2015)
	V	V-A	Cas12a (Cpf1)	Zetsche et al. (2015)
		V-B	Cas12b (C2c1)	Shmakov et al. (2015)
		V-U	C2c4, C2c5; five subgroups (V-U 1–5)	Koonin et al. (2017)
	VI	VI-A	Cas13a (C2c2)	Abudayyeh et al. (2016)
		VI-B	Cas13b (C2c6)	Smargon et al. (2017)
		VI-C	Cas13b (C2c7)	Koonin et al. (2017)

Mechanism involved in CRISPR–Cas system

The mechanism behind CRISPR–Cas organization operates in three noticeable phases: (1) adaptation, (2) expression and maturation, and (3) interference (Amitai and Sorek 2016; Puschnik et al. 2017).

Adaptation of CRISPR–Cas spacer sequences

The adaptation phase occurs in two steps; firstly, Cas proteins of the bacterium identify the invader and acquire specific sequences from foreign nucleic acids and these sequences are termed as 'protospacer' and secondly, the protospacer is incorporated in the extremity of the leader sequence in the CRISPR array as 'spacer' and this causes the first repeat of the CRISPR array to be extended (Pourcel et al. 2005; Yosef et al. 2012; Mojica et al. 2009). These spacers are responsible for creating immunological memory to archaea and bacteria for defense, in case, they encounter the MGEs for the second time (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). Cas1 and Cas2 are essentially involved in this phase (Yosef et al. 2012).

Expression and maturation of CRISPR–Cas system

During the expression and maturation phase, the leader sequence situated upstream to the CRISPR loci, acts as a promoter and initiates transcription of the loci, giving rise to long precursor CRISPR RNA or pre-crRNA and subsequently, processing of this pre-crRNA into small and mature units, known as crRNA takes place (Pougach et al. 2010; Yosef et al. 2012; Wei et al. 2015). Representation of crRNA is exhibited by joining of a spacer region (sequence showing complementarity to the foreign nucleic acid) at the 5′ end

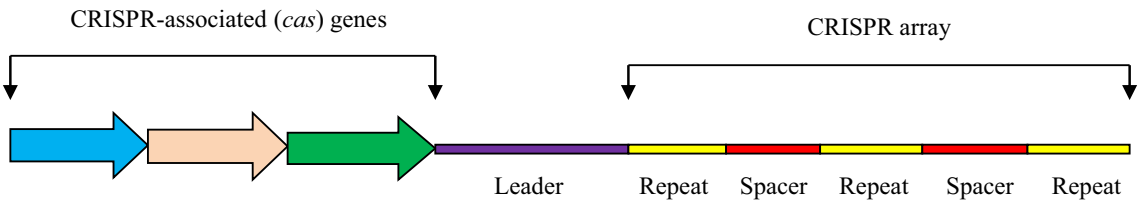

Fig. 1 Structure of CRISPR locus. In CRISPR locus, each 'repeat' sequence is flanked by 'spacer' sequence and these spacers match with the genomic sequences found in virus, plasmid or pathogen. Upstream to CRISPR array, leader sequence and CRISPR-associated (*cas*) genes are located

to repeat sequence at the 3′ end (Garneau et al. 2010; Barrangou 2015).

Interference of CRISPR–Cas system

During the interference phase, Cas–crRNA complex formed as a result of recruitment of Cas proteins to crRNA, detects the foreign MGEs via Watson–Crick base pairing of sequences that is complementary to the crRNA and hence, the targeted element is subjected to cleavage (Amitai and Sorek 2016). Existence of a small conserved sequence (2–5 bp) called protospacer adjacent motif (PAM) juxtaposed to target site in the invading nucleic acid is essential for identification between self and non-self nucleic acids by the Cas–crRNA complex (Mojica et al. 2009; Deveau et al. 2008; Westra et al. 2013).

Structure of Cas9 enzyme

Cas9 enzyme has enormous possibilities in genome engineering (Wilkinson et al. 2019). Cas9 is a DNA endonuclease and it possesses two RNA molecules, i.e., crRNA and transactivating crRNA (tracrRNA). It can detect and degrade any foreign nucleic acids and so, Cas9 is extensively used in the area of biotechnology for genome editing (Mali et al. 2013; Cong et al. 2013). Structurally, Cas9 has two lobes, i.e., nuclease (NUC) lobe and recognition (REC) lobe (Jinek et al. 2014; Nishimasu et al. 2014). NUC lobe comprises two nuclease domains, i.e., HNH and RuvC and a PAM interacting domain (PI) (Nishimasu et al. 2014). REC lobe consists of a Bridge Helix (BH) which is rich in arginine and is divided into three α-helical sub-domains, i.e., REC1, REC2 and REC3 (Wilkinson et al. 2019).

Biology of CRISPR–Cas9 belonging to type II

Type II organization represents the following vital elements: genes, i.e., *cas1*, *cas2* and *cas9*, CRISPR array, as well as a tracrRNA that shows complementarity to the sequence of CRISPR repeat (Chylinski et al. 2014; Deltcheva et al. 2011). During the acquisition of spacers, all the Cas signature proteins are associated (Heler et al. 2015; Wei et al. 2015), whereas in interference stage, only the role of Cas9 is significantly involved (Jinek et al. 2012; Sapranauskas et al. 2011). Class 2 type II organization codes for endonucleases such as Cas9 signature protein and a non-coding RNA called tracrRNA in addition to crRNA (Barrangou and Doudna 2016). Base pairing of crRNA and tracrRNA results into crRNA: tracrRNA hybrid, following which RNase III cleaves the hybrid, thereby, forming a mature dual-RNA hybrid (Deltcheva et al. 2011) and subsequently, recruitment of Cas9 proteins occurs (Jinek et al. 2012). Chimeric single guide RNA (sgRNA) is constructed by hybridizing 5′ end of the tracrRNA with 3′ end of the crRNA, thereby, resulting into single guide RNA, which has potential use in genome engineering as it can degrade any target DNA sequence (Gasiunas et al. 2012). The type II includes two parts, Cas9 and sgRNA (Jinek et al. 2012).

The sgRNA guides the Cas9 endonuclease and recognizes G-rich PAM (i.e., 5′-NGG) and then identifies the target DNA sequence that lies in the upstream position of the PAM sequence and causes melting of the target DNA (Sternberg et al. 2014). As a result, upstream to the PAM, the strands undergo directional separation, i.e., an R-loop is formed and subsequently, sgRNA strand is incorporated and thereby, forms RNA–DNA heteroduplex (Sternberg et al. 2014; Szczelkun et al. 2014; Anders et al. 2014). The duplex is formed by base pairing of the ~ 20 nt spacer sequence of the sgRNA with the protospacer of the target DNA as they are complementary to each other (Gasiunas et al. 2012; Jinek et al. 2012). One of the domains of Cas9 enzyme, HNH cleaves the DNA sequence that shares complementarity to the sequence (target sequence) in guide RNA and the other domain of the enzyme, RuvC cleaves the sequence that shares non-complementarity to the sequence (non-target sequence) in guide RNA (Jinek et al. 2012; Sapranauskas et al. 2011; Gasiunas et al. 2012). The two domains cleave the RNA–DNA hybrid at a site 3 bp upstream to the PAM and the outcome of cleavage is the creation of a double-strand break (DSB) with blunt ends (Gasiunas et al. 2012; Jinek et al. 2012). The three phases of mechanism of CRISPR–Cas9 organization and the process of genome editing by this organization are depicted in Fig. 2.

Pathways of DNA double-strand break (DSB) repair

Genome editing by CRISPR–Cas9 system primarily involves DNA DSB to be generated at the target gene locus (Carroll 2011). The target DNA strands cleaved by two nuclease domains of Cas9 results in the creation of DSBs at the sequences contained in crRNA (Nishimasu et al. 2014; Jinek et al. 2012). Repairing of DSBs is facilitated by either of the two pathways such as homology-directed repair (HDR) or non-homologous end-joining (NHEJ) (Sander and Joung 2014; Ghezraoui et al. 2014). NHEJ involves short insertions and/or deletions (termed as indels) to be incorporated and these cause disruption of the target locus due to shifting of the translational reading frame and consequently, NHEJ becomes a fallible pathway (Lieber et al. 2003). However, DSB repairing by HDR pathway occurs by external delivery of donor template DNA that possesses homology to the target locus, which

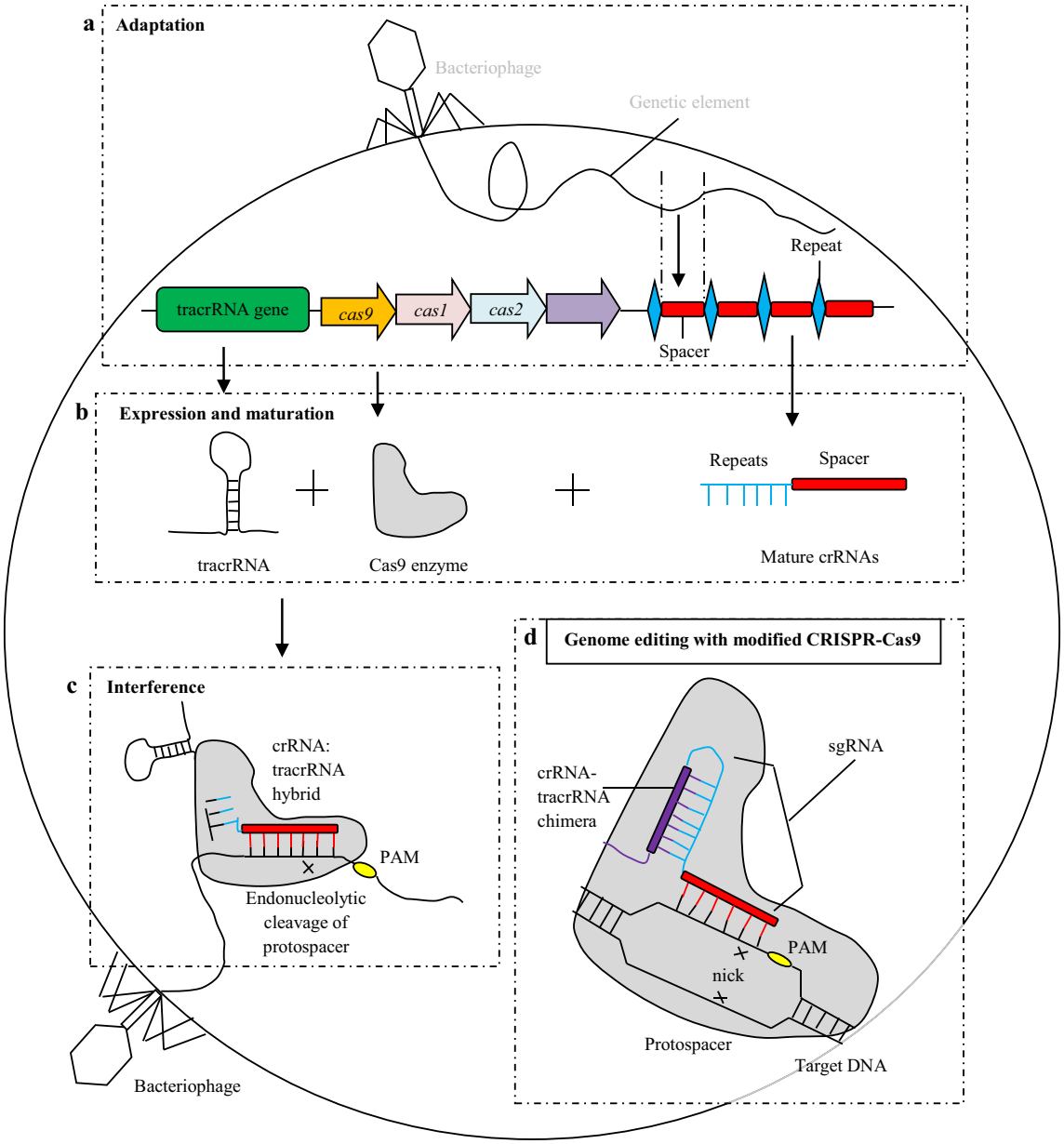

Fig. 2 Mechanism of natural CRISPR–Cas9 system existing in prokaryotes and modified CRISPR–Cas9 technology used for genome editing. **a–c** There are three phases of mechanism of naturally existing CRISPR–Cas9 system: **a** adaptation: bacteria acquire specific genomic sequences termed 'protospacer' from phages and incorporate them in CRISPR array as 'spacer'. **b** Expression and maturation: leader sequence situated in CRISPR loci initiates transcription of the loci, leading to the production of tracrRNA, Cas9 enzyme and crRNA. **c** Interference: base pairing of crRNA and tracrRNA takes place which results into crRNA: tracrRNA hybrid and subsequently, recruitment of Cas9 proteins occurs. The hybrid leads the Cas9 to cleave the protospacer and degrade it. The protospacer of the bacteriophage is identified as it is complementary to the spacer of crRNA and as a result, base pairing takes place between them. **d** In genome editing, chimeric single guide RNA (sgRNA) is constructed by hybridization of tracrRNA and crRNA. sgRNA identifies target DNA sequences that lie upstream to protospacer adjacent motif (PAM). Strands upstream to PAM undergo directional separation and subsequently, sgRNA strand is incorporated and form RNA–DNA heteroduplex as a result of base paring between sgRNA and protospacer of the target DNA as they are complementary to each other. The domains of Cas9 cleave the target and non-target DNA sequences and generate double-strand breaks (DSBs)

then hybridizes and results in accurate mutations or incorporation of sequences of interest (Sander and Joung 2014). Figure 3 represents the pathways involved in DSB repair.

On-target activity and off-target activity

The comprehensive Cas9 specificity is subjected to the bases within the ~ 20 nucleotide sequence of the sgRNA, but at the

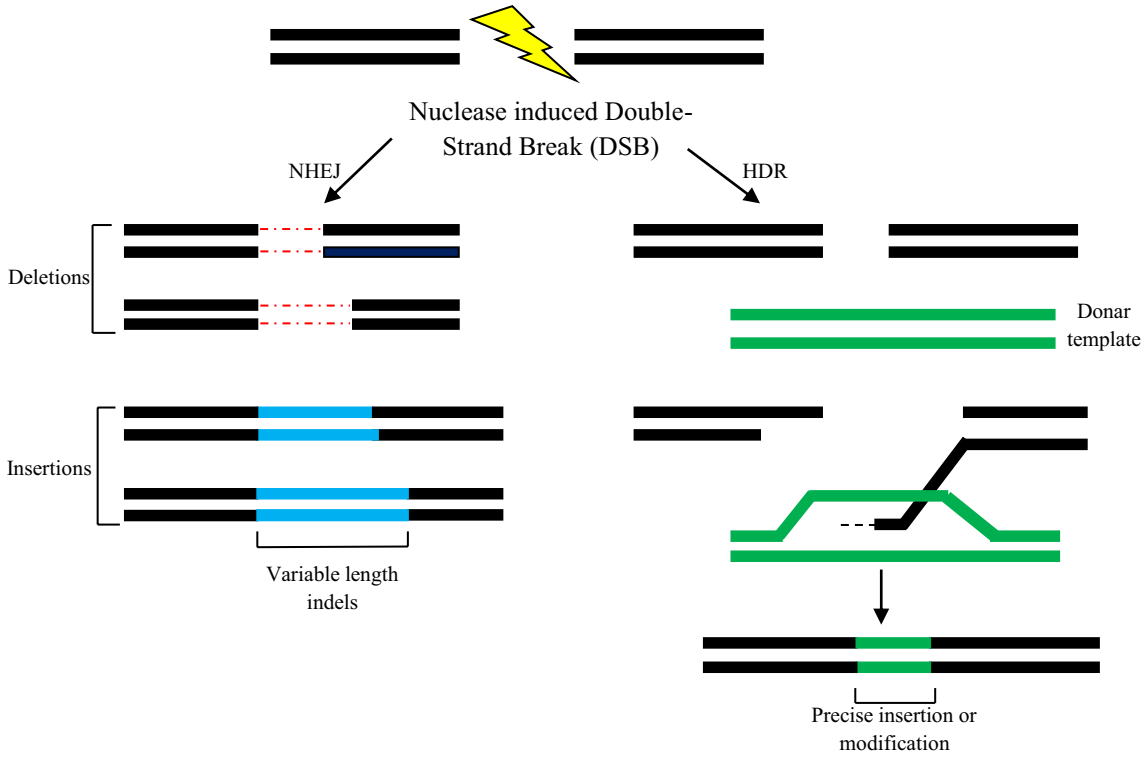

Fig. 3 Pathways of DSB (double-strand break) repair. DSBs generated during genome editing can be repaired by either of the two pathways, i.e., non-homologous end-joining (NHEJ) and homology-directed repair (HDR). At DSB sites, NHEJ includes short insertions and/or deletions (termed as indels) while HDR includes accurate insertions or mutation using donor template

time of hybridization of sgRNA and target DNA, multiple mismatches occur and Cas9 has the capability to tolerate about five such mismatches (Jiang et al. 2013; Cong et al. 2013; Fu et al. 2013; Hsu et al. 2013). These mismatches are the result of off-target sites (sites different from target sites in terms of few bases) contained within the target DNA that remains temporarily bounded to the sgRNA sequence (Wu et al. 2014). Studies reported that Cas9 also facilitates the binding of sgRNA with off-target sites and consequently, Cas9 cleaves these sites to form DSBs (Wu et al. 2014; Ran et al. 2013). In order to carry out genome editing, a Cas9 should cleave the target DNA sequence precisely (Hsu et al. 2013), and the off-target effect has to be minimized essentially.

Improving Cas9 specificity and reducing off-target activity

Upon inactivation of either HNH or RuvC nuclease domains of Cas9, Cas9 is converted into DNA nickase called Cas9 nickase (Cas9n) which cleaves the target DNA into single-strand break (SSB) instead of DSB (Sapranauskas et al. 2011; Gasiunas et al. 2012; Jinek et al. 2012). Cas9n has increased specificity for target sites and the repairing of SSB occurs by high-fidelity base excision repair (BER) (Dianov and Hübscher 2013). Using of 'paired nickase' was found to increase Cas9 specificity where two gRNA paired with Cas9n cleaved the target sites and generated DSB (Ran et al. 2013; Mali et al. 2013; Cho et al. 2014). Hybridization between inactive or dead Cas9 (dCas9) and FokI nuclease has also enhanced target sites cleavage (Guilinger et al. 2014; Tsai et al. 2014). Further, dCas9 can be used for silencing an undesirable or diseased gene as well as for activating a favorable gene (Qi et al. 2013; Gilbert et al. 2013; Perez-Pinera et al. 2013).

Inactivation of Cas9 immediately after target site cleavage was found to reduce off-target sites effect. A strategy termed as self-limiting circuit for enhanced safety and specificity (SLiCES) was developed for elimination of Cas9 action from cells (Petris et al. 2017). Anti-CRISPR proteins found in phages to escape from CRISPR–Cas immunity were also used for destroying Cas9 enzyme (Pawluk et al. 2016). Moreover, genetically engineered SpCas9 nucleases such as high-fidelity Cas9 (Cas9-HF1), hyper-accurate Cas9 (HypaCas9) as well as enhanced specificity Cas9 (eSpCas9) were created to facilitate genome editing (Kleinstiver et al. 2016; Slaymaker et al. 2016; Chen et al. 2017).

The sgRNA libraries and genome screening

With the help of genome-wide sgRNA libraries, genome screening can be achieved efficiently for determining and analyzing functional genes involved in a phenotype of interest (Gilbert et al. 2014; Shalem et al. 2015; Chen et al. 2015; Kampmann et al. 2014, 2015; Gilles and Averof 2014; Malina et al. 2014; Li et al. 2014; Wang et al. 2014; Koike-Yusa et al. 2014). Lentiviral genome-wide sgRNA library has found an extensive use for genome screening or mutagenesis screening because screening by CRISPR–Cas system is comparatively better than that by RNA interference (RNAi) (Koike-Yusa et al. 2014).

A study reported that the artificially created CRISPR–Cas9 paired gRNA (pgRNA) library induced the deletion of large genomic fragments and facilitated the detection of a long non-coding RNA (lncRNA) in cancer cells (Zhu et al. 2016). CRISPR–Cas9 mutant library created in rice is extensively useful in genome screening since functional genes as well as phenotype mutants can be determined, and hence paves the way to crop improvement (Meng et al. 2017).

CRISPR tools

Genome scan is carried out using a variety of bioinformatic tools as these tools function to detect specific target sites as well as off-target sites and design sgRNA for achieving high specificity for cleaving the target site. Bioinformatic tools are listed in Table 2.

Database for CRISPR–Cas system

Databases have been developed to provide information on *cas* genes and CRISPR loci. Table 3 provides the list of useful databases.

Strategies for delivering CRISPR

Researchers have experimentally proved that the delivery of Cas9 and gRNA can be carried out by nucleofection (Mali et al. 2013; Fu et al. 2013), lipofectamine-mediated transfection (Mali et al. 2013; Cong et al. 2013; Fu et al. 2013; Li et al. 2013), polyethylenimine-mediated transfection (Zuckermann et al. 2015) as well as electroporation (Ding et al. 2013; Straub et al. 2014). At present, the most reliable approach for CRISPR–Cas component delivery to cultured cells is by the use of viral vectors.

Lentivirus

Delivery of CRISPR system had been successfully achieved in model organisms by lentivirus vectors for inducing cancers such as brain (Zuckermann et al. 2015), colon (Roper et al. 2017; O'Rourke et al. 2017), lung (Sánchez-Rivera et al. 2014; Rogers et al. 2017, 2018; Walter et al. 2017), pancreatic cancer (Chiou et al. 2015) and breast (Annunziato et al. 2016) cancer. These findings have provided significant insights for understanding cancer in general.

Adenovirus-associated virus (AAV)

Till date, the most effective virus vector is AAV as it provides a long-term expression of CRISPR system and is safe for use because of its non-pathogenic nature (Burger et al. 2005; Taymans et al. 2007). Genome editing by these viral vectors is possible because AAV has many serotypes for inducing cancer in model organisms and this is useful for genome editing (Platt et al. 2014; Chow et al. 2017; Yin et al. 2017; Winters et al. 2017).

Adenovirus

Adenovirus has also been successful in delivery CRISPR–Cas9 in vivo (Wang et al. 2015). However, the use of this virus has now been restricted because the virus possesses immunogenic and adjuvant property (Nelson et al. 2017).

Applications of CRISPR–Cas technology

Gene therapy

Genome editing experiments based on CRISPR system have demonstrated that this technology has enormous potentiality in the field of gene therapy in order to modify or eliminate disease genes (Firth et al. 2015; Wu et al. 2013; Long et al. 2014, 2016; Osborn et al. 2014; Nelson et al. 2016; Tabebordbar et al. 2016). A study demonstrated that by the use of organoids of cultured intestinal stem cells, cystic fibrosis was treated in vitro by homologous recombination of *CFTR* (cystic fibrosis transmembrane conductance regulator) locus, a gene loci that cause the disease (Schwank et al. 2013). CRISPR–Cas based genome editing has successfully been carried out in diseases such as Fanconi anemia (Osborn et al. 2014), and crystalline gamma c (*Crygc*) associated cataract (Wu et al. 2013).

Neuroscience

Studies demonstrated that CRISP-Cas9 system has potential for treating the neurodegenerative disorders like Duchenne muscular dystrophy and for this purpose, AAV was

Table 2 List of CRISPR tools

Name of tools	Tool type	Website	Purpose
CRISPResso2	Computational tool	http://crispresso.pinellolab.partners.org/	Analyzes the results obtained from the experiments of genome editing; examines as well as determines the differences between experiments performed, requires less time for programming (Clement et al. 2019)
CRISPR-ERA (editing, repression and activation)	Computational tool	http://crispr-era.stanford.edu/	Detects sgRNA binding sites by scanning the genome; determines sgRNA binding specificity as well as efficiency (Doudna and Charpentier 2014; Cong et al. 2013; Ran et al. 2013; Qi et al. 2013; Gilbert et al. 2014); used for genome imaging (Chen et al. 2013)
WU-CRISPR	Computational tool	http://crispr.wustl.edu/	Selects genomic gRNA for Cas9; develops the competence of design of CRISPR assay (Wong et al. 2015)
CRISPR-P	Web tool	http://crispr.hzau.edu.cn/CRISPR/	Selects target sites within desired DNA which has high specificity for Cas9; aids in the prediction of off-target loci; determines restriction sites as well as off-target sites (Lei et al. 2014)
CRISPR-P 2.0	Web tool	http://cbi.hzau.edu.cn/CRISPR2/	Predicts on-target and off-target effect of sgRNA on the target DNA (Liu et al. 2017); determines microhomology score and secondary structure of sgRNA (Bae et al. 2014); helps in visualization of GC content with the sgRNA (Liang et al. 2016; Ren et al. 2014)
CRISPRseek	Web tool	http://www.bioconductor.org	It is a bioconductor package; designs gRNA specificity for target sites in a genome; studies the off-target sites (Zhu et al. 2014)
COSMID (CRISPR off-target sites with mismatches, insertions, and deletions)	Web tool	http://crispr.bme.gatech.edu	Recognizes potential off-target sites throughout the genome; determines the mismatch bases as well as the excluded or incorporated bases (Cradick et al. 2014)
CHOPCHOP v2	Web tool	http://chopchop.cbu.uib.no	Designs sgRNA; predicts potential off-target sites; aids in targeting a wide range of sequences of desired genome by sgRNA (Labun et al. 2016)
Cas-Designer	Web tool	http://rgenome.net/cas-designer	Recognizes target sites contained in a genome of interest which is then cleaved by Cas9; gene knockout can be achieved by selection of suitable target site; from the supplied sequences of DNA, record for all desirable gRNA sequences as well as off-target sites can be determined (Park et al. 2015)
E-CRISP	Web tool	http://www.e-crisp.org/	Designs sequences of gRNA; determines target sites that show complementarity to the gRNA and subsequently, cleaving of the dsDNA by Cas9 endonuclease takes place (Heigwer et al. 2014)

Table 2 (continued)

Name of tools	Tool type	Website	Purpose
CRISPR MultiTargeter	Web tool	http://www.multicrispr.net	Identifies potential target sites for sgRNA; recognizes target sites complementary as well as non-complementary to the supplied DNA sequences (Prykhozhij et al. 2015)
CRISPy	Web tool	http://staff.biosustain.dtu.dk/laeb/crispy/	Selects sgRNA target sequences of desired DNA; helps in viewing the image of the target DNA; stores data regarding off-target sites (Ronda et al. 2014)
CRISPy-web	Web tool	http://crispy.secondarymetabolites.org/	Predicts target sites; designs sgRNA that can target the DNA of interest in supplied sequence of genome (Blin et al. 2016)
EuPaGDT (eukaryotic pathogen gRNA design tool)	Web tool	http://grna.ctegd.uga.edu	Designing gRNA CRISPR–Cas system against pathogenic eukaryotic organism; helps in gRNA library construction (Peng and Tarleton 2015)
CCTop (CRISPR/Cas9 target online prediction)	Web tool	http://crispr.cos.uni-heidelberg.de	Identifies target sites for sgRNA; useful for gene inactivation; widely used for HDR as well as NHEJ for repairing DSBs (Stemmer et al. 2015)

Table 3 List of CRISPR databases

Databases	Website	Purpose
CRISPRI	http://crispi.genouest.org/	Stores the entire set of repertoires of *cas* genes; searches for sequences that correspond with spacers; locates CRISPR loci in a genome of interest, graphical tools are available in this database (Rousseau et al. 2009)
CRISPRdb	https://crispr.i2bc.paris-saclay.fr/crispr/	Determines the framework of CRISPR structure such as the arrangement of repeats and spacers and accordingly, structures can be studied; tools are available in this database (Grissa et al. 2007)
CRISPRz	http://research.nhgri.nih.gov/CRISPRz/	Stores the CRISPR target sequences that have been approved through experiments performed from published genomes and zebrafish genome (Varshney et al. 2015)
GenomeCRISPPR	http://genomecrispr.org	Analyzes the results estimated from experiments through screening of CRISPR–Cas during genome editing (Rauscher et al. 2016)
WGE (Wellcome Sanger Institute Genome Editing) database	http://www.sanger.ac.uk/htgt/wge	In a genome of interest, CRISPR target sequences can be determined and featured through this database; assigned for computing data for off-target sites; stores ideal target sequences from any genome of an organism (Hodgkins et al. 2015)
PICKLES (pooled in vitro CRISPR knockout library essentiality screens)	http://pickles.hart-lab.org	Stores the gene essentiality profiles of any gene of interest, mostly genes that are carcinogenic (Lenoir et al. 2017)
CRISPRminer	http://www.microbiome-bigdata.com/CRISPRminer	Stores data such as CRISPR–Cas system divisions, illustration of CRISPR–Cas system in archaea and bacteria, assemblage of self-target and putative self-target sites, interpretation of anti-CRISPR proteins that have been practically determined by Zhang et al. (2018)

used for delivering CRISPR–Cas9 to a model organism (Mendell and Rodino-Klapac 2016). Huntington disease, a neurodegenerative disease, is caused by the presence of mutant allele *HTT* and by applying CRISPR–Cas9 system, the allele was silenced or inactivated (Shin et al. 2016; Xu et al. 2017; Monteys et al. 2017). Disease causing gene in neurons can be identified from the cultures of induced pluripotent stem cells (iPSCs) by using CRISPR–Cas9 system (Polstein and Gersbach 2015; Zetsche et al. 2015).

Agriculture

In plant genomes the most extensively used CRISPR systems are CRISPR–Cas9 and CRISPR-Cpf1 (Zetsche et al. 2015; Jinek et al. 2012). This technology has been applied for editing the genomes in plants such as wheat (Shan et al. 2013), tobacco (Li et al. 2013), sweet orange (Jia and Wang 2014), rice (Shan et al. 2013; Miao et al. 2013; Xie and Yang 2013) and *Arabidopsis* (Li et al. 2013; Feng et al. 2014; Yin et al. 2017). The latest adapted two-step CRISPR–Cas9 technology permits genome editing to occur scarlessly. Scientists can rely on this stepwise method for performing successful genome editing with the purpose of analyzing results of phenotypic changes and correlation between genotype and phenotype. The approach can be useful for crop improvement (Elison and Acar 2018).

Microbiology

CRISPR–Cas9 system has also been successful in mutating or deleting genes in yeasts (Enkler et al. 2016; Vyas et al. 2015; Min et al. 2016; Grahl et al. 2017), molds (Fuller et al. 2015) and filamentous fungi (Liu et al. 2015, 2017) which may have industrial importance as well as pathogenicity. The genomes of industrially important bacteria such as *Clostridium* spp. (Huang et al. 2016; Wang et al. 2016; Nagaraju et al. 2016) and *Streptomyces* spp. (Cobb et al. 2014) were edited by this technology for efficient production of biofuels, anticancer agents and antibiotics. Yeast such as *Saccharomyces cerevisiae* possesses HDR repair pathway, which permits it to undergo genome editing accurately. CRISPR–Cas9 technology has been applied to these eukaryotic organisms for manipulating their strains so that they could be used in the field of synthetic biology and metabolic engineering. Based on this technology, genetic interaction screens could be accomplished for creating diversified mutant yeast strains (Adames et al. 2019). In *S. cerevisiae*, genetic manipulations can be achieved by associating the function of selectable marker integration and genome engineering potential of CRISPR–Cas9 organization. Using this application, researchers can investigate yeast cells for understanding the activity of any gene or promoter as well as for developing strains that possess replacements of gene or promoter (Soreanu et al. 2018).

Antiviral therapy

Earlier studies reported that with the help of CRISPR–Cas technology, diseases caused by the viruses such as hepatitis B (Dong et al. 2015; Kennedy et al. 2015; Kennedy and Cullen 2015; Liu et al. 2015; Ramanan et al. 2015; Wang et al. 2015; Lin et al. 2014; Zhen et al. 2015), papillomavirus (Kennedy et al. 2014; Hu et al. 2014a), herpes (Wang and Quake 2014), Porcine endogenous retroviruses (PERVs) (Yang et al. 2015) and Human Immunodeficiency Virus-1 (HIV-1) (Hu et al. 2014b; Li et al. 2015; Wang et al. 2014; Ye et al. 2014; Zhang and Sodroski 2015; Hou et al. 2015) were treated effectively.

Drug discovery and targets

CRISPR–Cas9 technology has great potential in the field of drug discovery and for generation of therapeutic drugs for treating heritable diseases (Fellmann et al. 2017). Genomic screens can be performed by CRISPR–Cas9 system to detect mutated genes that have become drug resistant (Wang et al. 2014; Koike-Yusa et al. 2014; Shalem et al. 2014; Zhou et al. 2014). Genomic screening by CRISPR–Cas9 has also been employed for analyzing the activity of drugs on infectious agents, cancer cells as well as proteins or genes involved (Deans et al. 2016; Marceau et al. 2016).

Antimicrobials

Sequence-specific antimicrobials act specifically to target any pathogenic microbe as well as sgRNA can be designed to target microbes on broad-scale (Barrangou and Doudna 2016). Studies reported that antimicrobial treatment-mediated CRISPR–Cas9 is exceptional when compared with traditional antimicrobials as well as antibiotics (Beisel et al. 2014). The main challenge of CRISPR-based antimicrobial lies in the advancement of delivery strategies of CRISPR–Cas9 system (Barrangou and Doudna 2016).

Cancer

Somatic genome editing mediated by CRISPR–Cas9 system has paved a way in cancer modeling and in the development of model organisms suffering from hematopoietic malignant tumors (Heckl et al. 2014; Chen et al. 2014). CRISPR system can also be used for cancer detection during initial stages of

the disease in an individual because Cas13a has the ability to distinguish mutation that can lead to cancer (Gootenberg et al. 2017). Multiplexed CRISPR–Cas9 genome editing has great potential for analyzing susceptibility in cancer causing cells (Sánchez-Rivera and Jacks 2015). CRISPR system provides immense opportunity in cancer immunotherapy by compressing negative factors and embellishing effectives (Yin et al. 2019).

Other applications

In addition to genome editing, technology based on CRISPR–Cas9 system has recently been applied for live-cell chromatin imaging, chromatin topology manipulations, genome regulation, RNA targeting and epigenome editing (Adli 2018). Transposable elements (TEs) have contributed greatly during genome evolution. A gene of interest can be incorporated within the genome of any organism by altering the activity of these elements. CRISPR–Cas9 technology can be employed for making the best use of TEs in order to regulate transcription process in the organism (Vaschetto 2018).

Discussion and future perspectives

Here, we present deep understanding of the biology of CRISPR–Cas9 system in genome editing. The increase in cleavage specificity of Cas9 and the reduction of off-target activity of this enzyme enable to recognize particular target DNA sequences and then alter or manipulate genome correctly. CRISPR databases and tools provide information and proper facility for altering, manipulating or visualizing genomes to perform correct genome editing experiments. We show that CRISPR–Cas9 technology has been applied to various fields including disease treatment related to genetic disorders or pathogens, agriculture, genetic engineering, clinical applications. However, many challenges are still in the way and need to be overcome. Further studies on CRISPR–Cas9 organization and potential applications of this technology will aid in overcoming these challenges, thereby, leading to better and healthy lives of humans in the society by curing complex diseases and improving crop field.

Improvement of CRISPR tools is essential so that off-target cleavage activity by Cas9 can be reduced effectively. Delivery strategies of CRISPR–Cas9 system into cells of higher class organisms such as mammals and plants precisely are crucially important for improvement. Identification of drug targets by the use of CRISPR–Cas9 technology will aid in the development of new drugs for the emerging dreadful diseases. Insertion of altered TEs by this technology can lead to crop improvement and creation of new ornamental plants. As CRISPR–Cas system is found in bacteria, it undergoes evolution rapidly and thereby, may give rise to new *cas* genes which will encode new proteins and thus, these proteins may have potential for genome editing or other applications in near future. Recently, Cas12a has been known to have great application in genome editing. CRISPR technology has also been applied to treat cancer and moreover, Cas13a can detect mutations that can lead to cancer. This technology is on the brink of treating various cancer and related diseases. Experiments for this CRISPR–Cas9 technology are mostly performed in vitro in model organisms and stem cells such as human pluripotent stem cells (hPSCs). Gene editing by this technology may be performed in human embryonic stem cells (ESCs) in vitro for correcting mutations, but research in ESCs raises many ethical issues. However, genome editing of ESCs may have the potential to give rise to organisms possessing excellent desirable qualities. Safety measures are to be taken while using this technology to prevent its misuse or reduce the risk of negative impact of genome editing.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest in this work.

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