

Exploring the CRISPR/Cas9 System in Targeting Drug Resistant Cancer Stem Cells

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Abstract: The genome-editing technology CRISPR/Cas9 holds great promise for the discovery of therapeutic targets in cancer and other diseases. With the help of this system, DNA can be added or removed from a genome in a sequence-specific manner. In order to increase the efficacy of CRISPR/Cas9 targeting and thereby reduce off-target effects, significant efforts are underway. The CRISPR/Cas9 technology has been employed in cancer biology to do powerful site-specific gene editing, making it more valuable for biological and therapeutic applications. CRISPR technology-based experimental procedures have yielded a very promising method for developing viable cancer therapies that are inexpensive and simple. This review explores various uses of gene-editing tools based on CRISPR in oncology and possible future cancer therapies.

Keywords: CRISPR/Cas9, Cancer, Genome-engineering, Gene therapy, Gene editing-tools.

1. Introduction:

Cancer is a disorder characterized by numerous genetic and epigenetic changes in the oncogenes and tumor suppressors. Mutations in a specific set of genes are one of the significant hallmarks for the transformation of a normal cell to a cancer cell. The proto-oncogenes and tumor suppressor genes play an important role in regulating the cell cycle by checking whether the cell is prepared for cell division or not. Usually, if the cell size or if the DNA is not copied properly, these regulatory genes arrest the cells until the repairs have been done. While in cancer cells, these genes lose their regulation to check for the errors in the cell, the cell would continue to proliferate. Mutations that a cell encounters are dependent upon the gene function. For instance, tumor suppressor genes function to stop the cell cycle if the cell has errors with respect to DNA replication, thereby suppressing the proliferation of damaged cells. Hence the tumor suppressor genes encounter recessive mutations where both alleles of the respective genes get mutated, thereby resulting in loss of function mutations. While proto-oncogenes function to promote the normal growth and division of cells, they encounter dominant mutations in which one allele of the gene is sufficient to alter the gene function, thereby resulting in to gain of function. Since cancer arises from mutations in a set of genes editing the genome might provide greater insight and a unique approach to treat cancer. Novel experimental approaches for modeling the disease to analyze its gene functions are imperative. Recent development in the field of genetic engineering has allowed specific DNA sequences in cell genomes in culture or in animal models to be precisely deleted or otherwise modified to explore the role of

genes involved in cancer initiation, progression, and therapeutic response. Doudna and Charpentier *et al.*, have discovered one such versatile technology that provides the ability to add or extract DNA in a sequence-specific way in the genome are the clustered regularly interspaced short palindrome repeats/CRISPR associated protein 9 (CRISPR/Cas9). They were awarded a Nobel Prize in chemistry for their significant contribution to this technology. Despite several shortcomings, it may be argued that CRISPR/Cas9 is a particularly promising representative of these new approaches, potentially enabling for precise editing of specific gene sequences (Rath, Amlinger, Rath, & Lundgren, 2015)

2. CRISPR/Cas9 System:

CRISPR/Cas9 is a highly flexible and prokaryotic adaptive immune system comprising of a programmable RNA molecule that aids in the targeting of an associated Cas9 endonuclease to specific foreign genetic intruders based on recognized sequences. (Koonin & Makarova, 2013) A Cas9 endonuclease and a single-stranded guide RNA (sgRNA) are two components of the CRISPR-Cas9 system. (Church *et al.*, 2013; Wyman *et al.*, 2013) In a sequence-specific way, the sgRNA directs the Cas9 endonuclease to cleave both DNA strands. At a sequence of 3 base pairs upstream of an "NGG" protospacer adjacent motif (PAM), DNA is cleaved. (Ho *et al.*, 2015) The genome gets repaired by the DNA-DSB repair mechanism after the double-strand break. Targeted genome modifications can be made using the CRISPR/Cas9 system, such as the introduction of small insertions and deletions (indels) mediated by the relatively error-prone non-homologous end-joining (NHEJ) pathway or the pathway of high-fidelity homology-directed repair (HDR). Using a 17–21 nucleotide-targeting sequence, genes of interest can be easily targeted. By phenotype-based screening of genomic changes, a pooled population of sgRNAs can be introduced into Cas9-expressing cells to classify genes that are essential for a specific phenotype. (Sanjana, Shalem, & Zhang, 2014)

Many CRISPR / cas9 system variations have been developed till date. (Table 1)

3. CRISPR/Cas9 in cancer therapeutics:

The advancement of CRISPR/Cas9 technology in cancer research has enormous potential to influence the future of oncology. CRISPR/Cas9 technology has revolutionized research into genetic mechanisms that govern cellular differentiation and function.

i. Generation of Cancer Models:

Cancer is caused by mechanisms that are regulated by underlying genes. (Arrowsmith *et al.*, 2015) It is crucial to be able to decode the molecular genetics of disease in order to elucidate these underlying mechanisms. (Nguyen & Tian, 2008) The interaction between the genotype, chemotherapeutic results, and the immune microenvironment is invaluable for dissecting cell lines and animal models. Genetically engineered CRISPR cancer models can now be generated easily, effectively, and inexpensively. (Sayin & Papagiannakopoulos, 2017) Leukemia models have been developed by targeting several inactivated genes in primary hematopoietic stem and progenitor cells (HSPCs) via a lentivirus-delivered Cas9-sgRNA system. (J-COLLINS *et al.*, 2017) Several genes are targeted by the pooled lentiviruses, including Tet2, Runx1, Dnmt3a, Nf1, Ezh2, and Smc3. With a fluorescent marker, various targeted HSPCs that are involved in the production of myeloid malignancy

have been selected. Several other cancer models have been developed using the CRISPR / Cas9 technology.(Sánchez-Rivera & Jacks, 2015)An organoid model of colon cancer was generated by introducing mutations of APC, SMAD4, TP53, KRAS, and/or PIK3CA using CRISPR technology. (Kellis *et al.*, 2014)In uncovering some essential aspects of tumor initiation, maintenance, and progression, genetically modified mouse models(GEMMs)(Frese & Tuveson, 2007)and non-germline GEMMs (nGEMMs)(Heyer, Kwong, Lowe, & Chin, 2010)of cancer have played a critical role. In addition, they have emerged as true models for testing a number of anticancer agents, as well as for uncovering drug resistance mechanisms.(Z. Chen *et al.*, 2012; Engelman *et al.*, 2008)However, it is a slow and costly method to produce GEMMs, involving complex ES cell manipulation and/or pronuclear injection, as well as comprehensive mouse husbandry to acquire animals containing alleles of interest. Cancer nGEMMs will simplify this process by circumventing the need for complex genetic crosses by re-targeting ES cells in sequence.(Heckl *et al.*, 2014)Nevertheless, the inability to introduce several genetic modifications simultaneously in mice or ES cells remains a major obstacle.

In a study byJaenisch *et al.*, they have expanded their CRISPR-Cas9 techniques for rapidly generating mice with conditional Cre/loxP-based alleles and reporter alleles, as well as using sgRNA pairs to generate mice with small deletions.(Yang *et al.*, 2013)These studies have shown the ease with which multiple gain-of-function and loss-of-function mutations can be produced in ES cells or mice, an advance that has opened the door to the production of novel cancer GEMMs and non-germline GEMMs (nGEMMs) with unparalleled speed and accuracy. Indeed, an explosion of new GEMMs and nGEMMs containing uniquely complex genetic alterations is expected to occur, allowing comprehensive analysis of several stages of tumor evolution with unparalleled speed and efficiency. (Figure 2)

The CRISPR-Cas9 system can also be used to refine existing cancer models beyond new model growth. ES cell lines derived from well-studied GEMMs can be readily re-engineered to produce oncogene and tumor suppressor genes with additional constitutive or conditional mutant alleles. (Dow & Lowe, 2012)Candidate cooperating mutations can thus be easily studied, and it is possible to confirm putative synthetic lethal interactions. In addition, this method will allow preclinical studies consisting of cohorts of mice that better reflect the genetic heterogeneity of human cancers. (Figure 2).

ii. **Non-coding RNA editing in human cancers mediated by CRISPR/Cas9:**

Evidence has shown that non-coding RNAs (ncRNAs) are involved in cancer genes' epigenetic regulation and associated pathways. RNAi was commonly used to inhibit the expression of protein-coding genes before the advent of CRISPR/Cas9. However, when using RNAi for the intervention of ncRNAs, it has been proven to be an inefficient method. ncRNAs have been shown to play a critical role in cancers, mainly involving microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). Since about 99% of the human genome lacks protein-coding potential in the ncRNA-related region, and most genetic alterations occur in this vast region, targeting the non-coding area with the CRISPR/Cas9 method is perhaps a feasible approach to cancer therapy.

a. ncRNA editing:

Multiple genetic and epigenetic changes, including a greater prevalence of oncogene mutations and/or tumor suppressors, define cancer. Evidence has shown that non-coding RNAs (ncRNAs) are involved in the epigenetic regulation and associated pathways of cancer genes. RNAi was commonly used to inhibit the expression of protein-coding genes prior to the advent of CRISPR / Cas9.(Fatica & Bozzoni, 2014) However, when using RNAi for the intervention of ncRNAs, it has been proven to be an inefficient method. (Fatica & Bozzoni, 2014)ncRNAs have been shown to play a critical role in cancers, mainly involving microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). Since about 99% of the human genome lacks protein-coding potential in the ncRNA-related region,(Fatica & Bozzoni, 2014; Lander *et al.*, 2001) and most of the genetic alterations occur in this vast region, targeting the non-coding area with the CRISPR / Cas9 method, is perhaps a feasible approach to cancer therapy.

b. miRNA editing:

Till now, many studies have shown that the CRISPR/Cas9 system is an excellent choice for editing/regulating the ncRNA-related genome. miRNAs have been extensively researched in the CRISPR / Cas9-related region as one of the key ncRNAs. Cloning CRISPR/Cas9 constructs with sgRNAs explicitly targeting the biogenesis processing sites of interested miRNAs, Chang *et al.*, showed that changes to the structure of primary miRNAs produced by CRISPR/Cas9 could lead to the down regulation of both in vivo and in vitro of these mature miRNAs. (Chang *et al.*, 2016)In addition, this study also showed that CRISPR / Cas9 could clearly reduce the off-target effects for miRNAs with highly conserved sequences across the same family, properly designed for the sgRNAs. Zhou and colleagues used the CRISPR/Cas9 method in another study to successfully knock out miR-3188 in cell lines of hepatocellular carcinoma (HCC) and found that miR-3188 KO effectively suppressed cell development, invasion, and migration and inhibited the growth of xenografts in nude mice.(Zhou, Deng, Liang, Jaoude, & Liu, 2017)Yue's group stated that lentiviral CRISPR / Cas9 vectors were highly successful in inserting indels (insertions and deletions) into the miRNA sequences of the precursor. They successfully disrupted miR-21 expression using this approach and found that pre-miR-21 sequence disruption results in decreased cell proliferation, migration, and invasion of ovarian cancer cells.(Huo *et al.*, 2017)

c. lncRNA editing:

LncRNAs have been confirmed to have been successfully edited/regulated by the CRISPR / Cas9 system in addition to miRNAs. UCA1 (urothelial carcinoma-associated 1), an upregulated lncRNA in bladder cancer, could be targeted and effectively inhibited both in vivo and in vitro(Zhen *et al.*, 2017)by specifically engineered CRISPR/Cas9 gRNAs, indicating that lncRNA expression can be modulated by making use of CRISPR/Cas9 system and can be further used as an approach for therapeutic to clinical cancer therapy.

One of the drawbacks of applying the CRISPR/Cas9 method to non-coding genes is that a certain non-coding gene may not actually produce functional loss from tiny indels. (Ho *et al.*, 2015) implemented a specific selection method to solve this challenge, HR (homologous recombination), to incorporate marker genes into the

genome, and UCA1 and lncRNA-21A and AK023948 were successfully knocked out in HCT-116 and MCF-7 cells, respectively, with the aid of the CRISPR-Cas9 system. In addition, CRISPR-Disp (CRISP-Disp) was developed by (Shechner *et al.*), a targeted localization approach that uses Cas9 to deploy large RNA cargos to DNA loci. They found that functional RNA domains up to a length of at least 4.8 kb could be inserted at various points in CRISPR gRNA, enabling the formation of Cas9 complexes with natural lncRNAs. (Shechner, Hacisuleyman, Younger, & Rinn, 2015)

iii. Precision medicine and immunotherapy

Multiple drug resistance (MDR) in cancer hinders therapeutic drugs from passing through the plasma membrane, decreasing the efficiency of traditional cancer therapy. The occurrence of such diseases frequently necessitates the introduction of more intrusive treatment alternatives, such as radiation therapy, surgery, and targeted chemotherapy, depending on the stage of cancer development. (Huang, Ju, Chang, Muralidhar Reddy, & Velmurugan, 2017) Cancer develops as a result of the accumulation of mutations, which raises the possibility of using CRISPR/Cas9 to target causative mutations in malignant cells. (Rodríguez-Rodríguez, Ramírez-Solís, Garza-Elizondo, Garza-Rodríguez, & Barrera-Saldaña, 2019) CRISPR/Cas9, when utilized as a diagnostic tool in treatment-resistant malignancies, enabled the discovery of new therapeutic targets, such as cancer multidrug resistance (MDR) inhibitors (Y. Chen & Zhang, 2018). Furthermore, genetic polymorphisms are used to predict patient outcomes throughout treatment)

Chemotherapeutic drugs have been shown to cause off-target mutations, with acquired drug resistance most typically caused by a mutation that replaces threonine with methionine at position 790 of exon 20 (T790M). (Ko, Paucar, & Halmos, 2017) Recently, research was undertaken to investigate cancer resistance to ispinesib, a kinesin-5 inhibitor. (Koch, 2017) Isolated clones of ispinesib-resistant cells showed a 70–300 fold decrease in sensitivity. (Kasap, Elemento, & Kapoor, 2014) Monopolar mitotic spindle development triggered normal bipolar mitotic spindle formation in ispinesib-sensitive cells, suggesting that ispinesib resistance in mutant clones is independent of spindle assembly (Sturgill, Norris, Guo, & Ohi, 2016). The effects of ispinesib were then examined on wild-type HeLa cells that died after being exposed, with Cas9 modified HeLa cells demonstrating a 150-fold increase in resistance. (Tian *et al.*, 2019). Although the studies on the use of CRISPR/Cas9 is much lesser in the context of precision medicine and immunotherapy, the existing results indicate that it could be a potential solution to overcome roadblocks in cancer treatment such as MDR.

As cancer immunotherapy, notably T-cell immunotherapy, is regarded as one of the most significant discoveries in modern biomedical research, however, gene therapy has the potential to improve a variety of features of this approach. CRISPR/Cas9 based methods like knockout of Human leukocyte antigen (HLA), aiming to avoid immune rejection and PD-1 knockout in T-cells aiming to avoid apoptosis and prevent cancer cells from immune-surveillance, are promising methods in the future (Hong, 2018; Maeder & Gersbach, 2016)

iv. Gene Synergistic analysis

A successful technique for the detection of synergistic gene interactions that could be used to block drug resistance is also provided by the CRISPR/ Cas9 method. Using a dual sgRNA library system to screen for combinatorial genes and identify pairs of synthetic lethal drug targets, a CRISPR-based double knockout (CDKO) system in K562 leukemia cells was developed. Phenotype measurement and gene analysis, along with deep sequencing, have identified interactions between synergistic drug targets, such as BCL2L1 and MCL1.(Han *et al.*, 2017)In order to test combinatorial gene function, another simple and effective method called CombiGEM (combinatorial genetics en masse)-CRISPR was also developed.(Wong *et al.*, 2016)This is analogous to the CDKO method, in which two pooled libraries of sgRNA have been integrated into one vector. Some genetic hits (e.g., KDM6B + BRD4) were discovered with this method. Compared to a reported small-molecule inhibitor, the depletion of these genes using the CombiGEM method has shown greater synergistic efficacy against ovarian cancer cell proliferation. The CRISPR technique costs less when compared to drug inhibition. (Larson *et al.*, 2013)

v. Gene Diagnosis

For cancer prevention, genetic diagnostics to identify sensitive genes are important.(Sobey, 2015)A CRISPR-based diagnostic method called SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) has been developed. However, a low-frequency mutation is not easily determined by sequencing. (J-COLLINS *et al.*, 2017)Cas13a, an RNA-guided RNase, is a key factor in this method inducing robust non-specific single-stranded DNA (ssDNA) trans-cleavage as a collateral effect.(Abudayyeh *et al.*, 2017)The reporter signal, which is released following RNA cleavage, is another important factor. For the identification of two cancer mutants, BRAF V600E and EGFR L858R, this procedure has been used and appears to be a highly sensitive detection technique. A similar technique known as DETECTR (DNA endonuclease-targeted trans reporter of CRISPR) has been developed (J. S. Chen *et al.*, 2018)Another Cas family member, Cas12a, is used in this scheme and behaves similarly to Cas13a. For amplifying micro-samples, an additional enzyme, RPA (recombinase polymerase amplification), is used. (Chertow, 2018)

vi. Validating the Target

It is a time-consuming and laborious method to reveal the mechanism of action for small-molecule drugs. With a CRISPR-Cas-based genetic screening system (CRISPRres) containing large sgRNA libraries, it is now possible to accurately classify new drug targets.(Neggers *et al.*, 2018)If the molecular binding site is reduced or mutated, resistance is generally gained by cancer cells, but sgRNA sequencing may clearly identify the molecular target. CRISPR was used as the primary target of KPT-9274, an anticancer agent, to successfully recognize nicotinamide phosphoribosyltransferase. Based on CRISPR / Cas9 technology, another team set up a framework called DrugTargetseqR to classify direct physiologic targets by mutating potential targets for drug resistance.(Kasap *et al.*, 2014)In this analysis, kinesin-5 was confirmed by the mutation of kinesin-5 D130V or A133P in HeLa cells as the direct target of ispinesib. Targeting the exons that encode functional

protein domains rather than the 5' exons is reportedly a better way to identify drug targets using the CRISPR system.(Shechner *et al.*, 2015)New insights into target validation studies could perhaps be provided by combining CRISPR genome editing with deep sequencing or cellular biophysical assays.(Arrowsmith *et al.*, 2015; Shi *et al.*, 2015)

4. Anticancer applications of CRISPR system in clinical trials:

The CRISPR / Cas9 framework may also theoretically be used clinically to target cancer-causing genes, based on promising results of preclinical studies. For the negative regulation of the immune system, specifically of T-cells, the PD-1 protein and programmed cell death ligands (PD-Ls) are essential. By evading the immune system, their attenuation of the immune response helps tumor cells survive.(Fife & Pauken, 2011).Pembrolizumab, a PD-1 monoclonal antibody, confirmed that blocking the immune system of PD-1 and PD-L1 could substantially improve the overall survival rate in patients with cancer.PD-1 is, therefore, an appealing target for immunotherapy, and PD-1 inhibitors have been approved by the U.S. Administration of Food and Drugs (FDA) for cancer immunotherapy. CRISPR / Cas9's generation of chimeric antigen receptor (CAR) T-cells is another ex vivo method in clinical trials. The first-in-human trial to examine the effect of HLA-A*0201 restricted NY-ESO-1 redirected engineered T-cells in a wide range of cancer types, including relapsed refractory multiple myeloma (MM), melanoma, synovial sarcoma, and myxoid/round cell liposarcoma, was coordinated by researchers from the University of Pennsylvania (Rodríguez-Rodríguez *et al.*, 2019). While there may be positive results from CRISPR/Cas9 clinical trials, further work is required to ensure that CRISPR / Cas9 is a safe and reliable method for the treatment of human cancers.

5. CRISPR system in targeting Cancer stem cells (CSCs):

Cells within tumors that can self-renew, differentiate and develop tumorigenicity when transplanted into an animal host are known as cancer stem cells (CSCs). A variety of cell surface markers, including CD44, CD24, and CD133, are frequently employed to identify and enrich CSCs. CSC features are controlled by a regulatory network comprised of microRNAs and Wnt/-catenin, Notch, and Hedgehog signaling pathways. Emerging evidence supports the clinical importance of CSCs, revealing that CSCs are resistant to standard chemotherapy and radiation treatment and that CSCs are very likely to be the source of cancer metastases.(*Cancer Stem Cells 2012_ Enhanced Reader.Pdf*, n.d.)The inability to identify or track cancer stem cells groups in an intact environment has impeded the study of stem cell hierarchies in human malignancies. Using CRISPR/Cas9 technology, an approach for integrating reporter cassettes at specified marker genes was developed to address this constraint.(*Cancer Stem Cells 2012_ Enhanced Reader.Pdf*, n.d.)

Autophagy activation has also been linked to chemotherapy resistance, according to recent research. The autophagic activity of CSC isolated from EOC ascitic effusions was therefore studied both *in vitro* and *in vivo*. CSC in the ovaries, detected by CD44/CD117 co-expression, had higher basal autophagy than their non-stem equivalents. Chloroquine or CRISPR/Cas9 ATG5 knockdown inhibited CSC features like viability and spheroidal structure formation *in vitro*, as well as tumorigenic potential when applied to mice.(Pagotto *et al.*, 2017)

Since the CRISPR-Cas9 gene-editing system modifies gene expression at the DNA level in human cancer cell lines, it is extremely beneficial in cancer cell biology research. The CRISPR-Cas9 system is very selective and stable in its gene-targeting abilities. The transitory inhibitory effect of siRNA limited our prior findings that CD133 was essential for cell proliferation and biological activities in colon cancer cells. Despite the fact that CD133 deletion cells were unable to totally prevent tumorigenic properties, they had a significant impact on cell motility and invasion abilities. West Blot was used to assess the epithelial-mesenchymal transition-protein expression. In CD133knockout cells, vimentin expression was clearly diminished. Through the reduction of cancer stem cell features, CRISPR/Cas9-mediated CD133knockout can be an effective treatment for CD133+ colon cancer. (Li *et al.*, 2019) Ovarian cancer cells express Nanog in response to the AR signalling axis, and the interaction of Nanog with AR signalling might trigger or contribute to OCSC regulation, according to another study. As a result of activating the Nanog promoter, androgen may increase stemness properties in ovarian cancer cells. Various cell kinds can be edited with this tool. A green fluorescent protein (GFP) marker was introduced into Nanog using the CRISPR/Cas9 technology. The GFP marker can directly and precisely reflect Nanog expression using the CRISPR/Cas9 system. They created a durable and dependable cell marker model based on CRISPR/Cas9 technology, which eliminates the genomic instability caused by the incorporation of indirect genetic markers via virus vectors, as well as the disadvantages of markers that fade with time. (Ling *et al.*, 2018)

6. Limitations of CRISPR/Cas9:

Using CRISPR/Cas9 to alter genes *in vitro* and *in vivo* is a powerful tool for researching disease mutations, according to researchers. However, it is not error-free, with roughly 15% of the time the system failing. For therapeutic and clinical applications, the high incidence of off-target activity ($\geq 50\%$)—mutations generated by RGEN (RNA-guided endonuclease) at sites other than the intended target site—is of particular concern. (Analysis of Off-Target Effects of CRISPR_Cas-Derived RNA-Guided Endonucleases 2021.Pdf, n.d.; Off-Target Effects in CRISPR_Cas9-Mediated Genome Engineering 2018.Pdf, n.d.; Target Specificity of the CRISPR-Cas9 System 2013.Pdf, n.d.)

A study (Asai *et al.*, 2019) revealed that due to the limited targeting efficiency of transcriptionally inactive genes, additional treatments such as valproic acid might be required to improve knockout efficiency, in particular for CSC marker genes. Also, the off-target consequences of the CRISPR nuclease's specificity are largely regulated by a seed sequence within 12 nucleotides of the PAM since mutations introduced into this region hampers selective cleavage (Kim *et al.*, 2012). Another recent study using long-read sequencing and long-range PCR genotyping discovered that Cas9-induced DNA breaks result in significant deletions and genetic changes. In addition, the scientists noted that both lesions and the crossover event that results from them occur far from the original target spot. Using CRISPR/Cas9 to cause on-target damage, this discovery raises the possibility of activating dormant oncogenes, inactivating tumor-suppressor genes, and triggering other diseases. Sequencing a few clones and screening for undesirable genomic alterations before bringing them back for expansion will help avoid CRISPR/off-target Cas9's consequences. (Kosicki, Tomberg, & Bradley, 2019)

The humoral and cell-mediated adaptive immune response to bacterial Cas9 is another possible explanation for the failure of CRISPR/ Cas9 mediated editing *in vivo*. Mutagenesis is performed directly at the organ site, which overcomes some of the constraints of transplant-based *in vivo* CRISPR screening. (*Cancer Genetics_ CRISPR Screens Go in Vivo _ Enhanced Reader.Pdf*, n.d.; Chow & Chen, 2018; Ghosh, Venkataramani, Nandi, & Bhattacharjee, 2019) Direct *in vivo* CRISPR screening methods, on the other hand, include constraints such as 1. unknown cell-cell interactions in the host tissue, 2. limited viral transduction efficiency, and 3. immunological rejection. (Chow & Chen, 2018) Recent research has shown that Cas9's continuous attachment to DSBs prevents repair proteins from accessing the break site, lowering its effectiveness. As a result, the Cas9-DSB complex is the rate-limiting step during *in vivo* genome editing. (*Enhanced Bacterial Immunity and Mammalian Genome Editing via RNA-Polymerase-Mediated Dislodging of Cas9 from Double-Strand DNA Breaks 2018.Pdf*, n.d.) According to (*CRISPR-Cas9 Mediated Efficient PD-1 Disruption on Human Primary T Cells from Cancer Patients 2015.Pdf*, n.d.), template-bound Cas9 can be dislodged by the translocating RNA polymerase only when the polymerase approaches the DSB from a specific orientation.

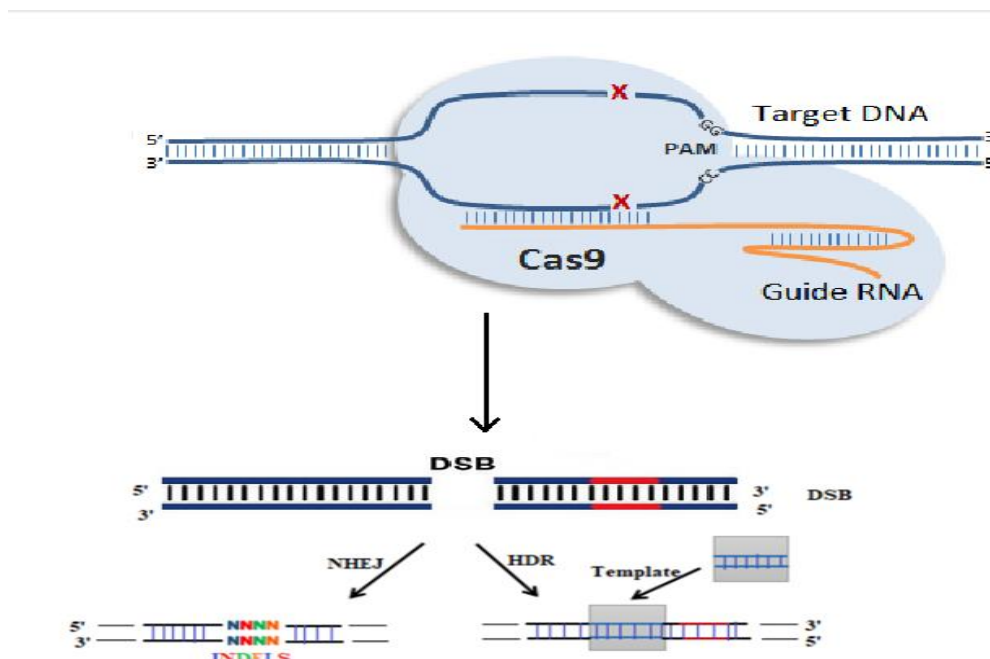


Fig 1: A plasmid-based process and the Cas9 protein complex with sgRNA or RNP are typical methods of delivering the CRISPR system. The Cas9 protein produces a DSB around the PAM site after the sgRNA binds to the target site of the genomic DNA. Random indels or exact modifications introduced by the NHEJ or HDR pathway into the genomic DNA.

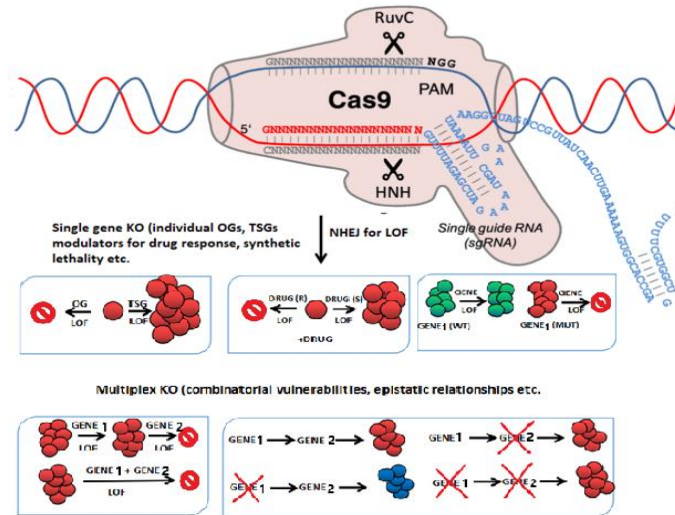


Fig 2: CRISPR-mediated embryonic stem (ES) cell genome engineering or genetically modified mouse model (GEMM)-derived ES cells may be used to rapidly produce novel cancer GEMMs or (nGEMMs) that harbour multiple genetic alterations, such as constitutive or conditional knockout and knock-in alleles, endogenous synthetic tags or reporters, non-coding SNPs and combination of all of these through genome engineering mediated by multiplex CRISPR or re-engineering of ES cells.

Table1: CRSIPR/Cas9 variations:3

Variations	Characteristics	Effects	Merits	Demerits	Applications
CRISPR/Cas9	sgRNA; Cas9 (WT)	Double strand break at target site	Flexible; effective; easily available	Off-target; PAM limited; different altered alleles	Developing research model; functional gene study; identification of drug target
CRISPRi	dCas9; repressor peptide; sgRNA	Blocking transcription elongation or knockdown transcripts	Inducible; reversible; off-target effects and toxicity is low	PAM limited; bidirectional promoters bear off-target effects	Genome and transcriptome based research; knockdown and overexpression of lncRNA
CRISPRa	dCas9; activator peptide; sgRNA	Transcription is increased		PAM limited; complications related to delivery of multiple components	
CRSIPR/Cas9 Nickase	Mutant Cas9 H840A or D10A; sgRNA	Single-strand break	Convenient; efficient; versatile; accurate, scalable; robust	PAM limited; 2 sgRNA for KO	epigenetic modifications can be manipulated; Simultaneous activation and repression

7. Conclusion and Future prospects:

In cancer biology, we envision a new era in which genome engineering based on CRISPR will serve as an important channel between the bench and the bedside. The successful deployment of advanced genetic profiling technologies to comprehensively characterize the tumor of a patient generates detailed roadmaps to instruct the development of tailored experimental systems based on cells or entire animals. These systems can serve as customized platforms for researchers to detect genotype-specific vulnerabilities and synthetic lethal interactions easily and systematically through single or multi-plex CRISPR and small molecule-based approaches.(Matano *et al.*, 2015)

Although there are current technological limitations challenging the use of CRISPR-Cas9 for targeting genes in human cancer patients as a therapeutic technique, including limiting factors like Cas9-DSB complex, Immunity against Cas9 protein, On-target and off-target effects of CRISPR/Cas9 editing, the prospects of this gene therapy are nonetheless very exciting. In order to increase targeting efficiency and minimize off-target impact, improved strategies will be needed. Recent research has shown the ability of this technology to permanently correct in vivo genetic mutations in mouse models of an inherited genetic condition via HDR in the adult liver, successfully alleviating aspects of the disorder.(Zamore, Tuschl, Sharp, & Bartel, 2000)Future developments in this technology would therefore allow therapeutic genetic correction of single or multiple driver mutations to improve the efficiency of editing and distribution of CRISPR-Cas9 components using both viral and non-viral delivery vehicles. The CRISPR-Cas9 technique could be used for precise ex vivo engineering of immune cells for immunotherapeutic applications in addition to permanently correcting cancer-associated mutations. For example, for the production of novel chimeric antigen receptor (CAR)-modified T cells, the CRISPR-Cas9 method could be used to precisely insert the CAR into a safe harbour locus.(Sadelain, Papapetrou, & Bushman, 2012)

The area of genome engineering has rapidly undergone a scientific revolution that promises to change almost every aspect of basic biological and biomedical science since the Doudna and Charpentier groups showed the potential of the CRISPR-Cas9 system as a powerful RNA-programmed genome editing tool.(Jinek *et al.*, 2012)Applying this technology to many areas of cancer biology, ranging from basic science to clinical and translational applications, presents many exciting possibilities to better understand and eventually cure this devastating disease.

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