Cancer Gene Therapy (2015) 22, 509–517

www.nature.com/cgt

REVIEW

CRISPR/Cas9: molecular tool for gene therapy to target genome and epigenome in the treatment of lung cancer

M Sachdeva[1], N Sachdeva[2], M Pal[1], N Gupta[1], IA Khan[1], M Majumdar[1] and A Tiwari[1]

Although varied drugs and therapies have been developed for lung cancer treatment, in the past 5 years overall survival rates have not improved much. It has also been reported that lung cancer is diagnosed in most of the patients when it is already in the advanced stages with heterogeneous tumors where single therapy is mostly ineffective. A combination of therapies are being administered and specific genes in specific tissues are targeted while protecting normal cell, but most of the therapies face drawbacks for the development of resistance against them and tumor progression. Therefore, therapeutic implications for various therapies need to be complemented by divergent strategies. This review frames utilization of CRISPR/Cas9 for molecular targeted gene therapy leading to long-term repression and activation or inhibition of molecular targets linked to lung cancer, avoiding the cycles of therapy.

Cancer Gene Therapy (2015) **22,** 509–517; doi:10.1038/cgt.2015.54; published online 23 October 2015

INTRODUCTION

Lung cancer is one of the leading causes of cancer deaths worldwide. It is incurable and possesses varied epidemiology on the basis of gender, geographic regions and age. This is especially true for non-small-cell lung cancer (NSCLC), which accounts for ~80% of all lung cancers and has a 5-year overall survival rate <15%.[1] In addition, ~40% of all patients diagnosed with NSCLC have unresectable stage III disease or medically inoperable disease. Traditional therapeutic strategies, such as surgery, chemotherapy and even radiotherapy, are the main modalities for NSCLC patients, but high systemic toxicity and drug resistance result in poor survival in most cases. Thus, enhancing patient survival by developing new therapeutic methods is urgently needed. The CRISPR/Cas9 system from *Streptococcus pyogenes* was discovered as a genome editing tool for human genome in the year 2012, and advances to optimize CRISPR/Cas9 have paved a way to develop lung cancer treatment. This review suggests the utility of CRISPR/Cas9 for therapeutic application in lung cancer treatments.

LUNG CANCER

There are two major historical groups for lung cancer: small-cell lung cancer (SCLC) and NSCLC. NSCLC is further grouped as adenocarcinoma, squamous cell carcinoma and large-cell undifferentiated carcinomas that cover 80–85% of lung cancers. SCLC is far more lethal than NSCLC, but it covers 15–20% of all lung cancers. Depending on which cells in the lungs are affected by the cancer, SCLC is split into small-cell carcinoma (the most prominent type), mixed small-cell carcinoma and combined small-cell carcinoma. Besides the two major types of lung cancer, there are other types of tumors that can appear in the lungs, and some of these are benign.[2] In lung cancer, the most consistent genetic abnormalities are the loss of the short arm of chromosome 3 and the hyperproliferation of chromosomes 1 and 12. The loss of 3p alleles was observed in >90% of SCLC and ~50% of NSCLC. Some tumor suppressor genes such as *RASSF1A* on 3p21 are absent in all SCLCs and in 65% of NSCLCs. Other candidates at the 3p are *RARB*,[3] *FHIT*,[4] *β-catenin*[5] and *caveolin-1*.[6] Three major types of cancer treatments include local treatment by surgery or radiations, systemic treatment by chemotherapy and a combination of both. Clinically, multimodality therapy is the most common treatment of cancer. In addition, immunological therapy and gene therapy have gained interest in recent years, and their clinical application is in the developmental phase. Although surgery is one of the important therapies for lung cancer patients, a large fraction of patients cannot undergo resection. Therefore, there is an urgent need for therapeutic strategies avoiding curative resection. The currently used chemotherapy for the treatment of lung cancer includes the use of cisplatin- and platinum-based chemotherapy that brings conformational changes, as the intrastrand cross-links hinder DNA replication. Cytotoxic effects include mitochondrial damage, decreased ATPase activity and altered cellular transport mechanisms during the S phase and arrest cell cycle at the G2 phase. Again, the chemotherapeutic drug docetaxel interferes with mitosis and ceases relatively fast in dividing cancer cells. Major drawbacks include cells developing resistance after median period of therapy cycle, and thus the treatment stops. These drugs generally lack specificity and may kill healthy cells too, and hence continuing long treatment is not possible because of the side effects such as loss of hair, nausea, vomiting, gastrointestinal tract disturbances and hematotoxicity. Bypassing the side effects of drugs, personalized therapy directed to a particular molecular target has been developed (Table 1) for NSCLC. For lung adenocarcinoma, various studies are ongoing to discover molecular targets and directed therapies to the targets. A study on 623

[1]School of Biotechnology, Rajiv Gandhi Proudyogiki Vishwavidyalya, Bhopal, India and [2]Padmashree Dr DY Patil Vidyapeeth's Department of Biotechnology and Bioinformatics, Mumbai, Maharashtra, India. Correspondence: M Sachdeva, School of Biotechnology (An Autonomous University Teaching Department), Rajiv Gandhi Proudyogiki Vishwavidyalya, State Technological University of Madhya Pradesh, Airport Road, Bhopal 462033, India.
E-mail: meenusachdeva9sep92@gmail.com
Received 21 July 2015; revised 17 September 2015; accepted 22 September 2015; published online 23 October 2015

Table 1. Molecular targets of lung cancer

Molecular target	*Function in normal cell*	*Function in cancer cell*	*Administered drug*	*Mechanism of action*	*Reason for failure of therapy*	*References*
EGFR	Bound to ligand activates RAS/RAF and PI3K/AKT/mTOR pathways, responsible for cell survival and proliferation	Overexpression	Tyrosine kinase inhibitors such as erlotinib and gefitinib	Blocks kinase activity, docking to ATP-binding site in tyrosine kinase domain and blocks downstream pathways	Cell develops resistance to tyrosine kinase inhibitors after a few cycles of administration	66,67
ROS-1			TAE684 as kinase inhibitor and crizotinib			68
Alk	Not expressed in normal lung cell	Small inversion at 2p, ALK is fused to the N terminus of the EML4 gene. Over nine different fusion variants described	Crizotinib	Acquired resistance within 1 year of therapy		69,70
K-RAS	Binary on/off switches dependent on guanosine diphosphate (GDP)/guanosine triphosphate (GTP)	Mutation at codons 12, 13 or 61 remains switched on, constitutively GTP bound and leads to stimuli insensitive several linked downstream pathways	Lacks clinical utility. MEK and PI3K inhibitors are being administered	Selumetinib or in combination with docetaxel for MEK and for PI3K, NVP-BEZ235 in combination to MEK inhibitor, ARRY-142886, and inhibitors for heat-shock protein-90		71,72
Chimeric RET receptor tyrosine kinase	KIF5B and the RET proto-oncogene	Pericentric inversion of 10p11.22–q11.21, and thus overexpression of chimeric RET receptor tyrosine kinase				73
MET receptor tyrosine kinase	Activation of MET depends on docking of hepatocyte growth factor (HGF)	Overexpressed hepatocyte growth factor	Onartuzumab, alone or in combinations	Competitive inhibitor against HGF	MET amplification causes inactivity of gefitinib during the course of treatment	74,75

Abbreviations: ATP, adenosine triphosphate; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase.

genes in 188 tumors revealed several new molecular targets in addition to *TP53*, *KRAS*, *STK11*, *EGFR* and *CDKN2A*. The study also revealed several known genes with lower mutation frequencies—*PTEN*, *NRAS*, *ERBB2*, *BRAF* and *PIK3CA*. The newly identified genes include tumor suppressor genes (*NF1*, *RB1*, *ATM* and *APC*) along with tyrosine kinase genes (ephrin receptor genes, *ERBB4*, *KDR*, *FGFR4* and NTRK genes) that may function as proto-oncogenes. Apart from these, alterations in genes from the MAPK, p53, Wnt, cell cycle and mTOR signaling pathways have also been demonstrated. These molecular targets are either mutated and have altered gene expression or they have altered copy number. Inhibitors of the MEK kinase could be tested in tumors with *NF1* mutations, whereas inhibitors of KDR, such as sorafenib and sunitinib, might be tested in tumors with *KDR* mutations.[7] Using information from patient's tumor profile and designing treatment has revolutionized the personalized treatments not only for genomic targets but also for epigenetic targets such as DNA methyltransferases (DNMTs) and histone deacetylase (Table 2). Unfortunately, the currently designed therapies have failed and the reasons for their failure are depicted in Tables 1 and 2. Furthermore, most of the DNMT inhibitors are not stable in neutral aqueous solution, and they readily disintegrate forming more stable and toxic analogs, and their efficacy is likely affected by limited stability and rapid inactivation by cytidine deaminase in the liver,[8] yet the effectiveness of DNMT inhibitors against solid tumors is quiet promising for lung cancer and several malignancies.[2] It has been widely known that epigenetic proteins lack the intrinsic capability to bind to target, and therefore it requires a certain protein to recognize the target. Henceforth, treatment by inhibitors of epigenetic proteins may cause gene expression change at a global level and an array of side effects. Further knowledge about tissue-specific epigenetic phenomena and related gene may help in enhancing the administration of drugs to reset the aberrant epigenome in cancer.[9] Consequently, permanent modulation of the epigenetic protein localized to a given target can be opted. Two ways for permanent genome modulation have until now been reported, that is, either based on the use of nucleic acid or of proteins. Epigenetic editing via nucleic acid-based method uses small interfering RNA (siRNA) molecules delivered to the cell. These siRNAs either target the cytosol or the nucleus. On the basis of their target sites, the mechanism of action differs; within the cytosol siRNAs degrade mRNAs, consequently leading to post-transcriptional silencing of the gene. However, siRNAs within the nucleus can regulate the cognate gene inducing methylation-related changes.[10] For lung cancer, DNMT1 mRNA has been targeted. For instance, in a study on lung cancer cell lines CALU-6 and A459, siRNA, complementary to DNMT1 m-RNA lowered DNMT1 level and thus expressing *RASSF1A*, *CDKN2A* gene. MicroRNAs (miRNAs) are found to be linked to the functioning of DNMTs. This suggests miRNA as a target option for epigenome modulation. In lung cancer, overexpression of miR-29 is linked to downregulated DNMT-3a and 3b, and it was shown to have restored the silenced tumor suppressor genes.[11] Recently,

Table 2. Epigenomic targets for lung cancer and their treatments

Drug	*Targeted gene*	*Clinical study phase*	*Result of experiment*	*Side effects and other considerations*	*Reference*
5-Aza-cytidine	DNMT1	I/II	Downregulated DNMT1 and reversal of methylation of gene	Require low-dose consumption	76
5-Aza-2′-deoxycitidine+ Valproic acid	DNMT1	I	Promising results when administered with HDACi sodium phenyl butyrate		77
MMA	DNMT1		Invasion ability of the cell is reduced		78
NS398			Demethylate SPARC gene		79
FK228		Preclinical	Induce apoptosis	Inhibits a variety of EGFR-related pathways, leading to downregulation of Bcl-2 and Bcl-xL and upregulation of Bax	80
SGI-110+Entinostat		Preclinical	Demethylate >300 gene promoters, epigenetic reprogramming of EZH2 target gene expression. p21 and apoptotic gene BIK induction		8

Abbreviations: DNMT1, DNA methyltransferase; HDACi, histone deacetylase inhibitor.

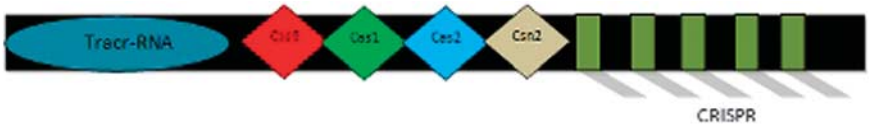

Figure 1. Type II CRISPR/Cas9 gene locus of *Streptococcus pyogenes*. Cas9, CRISPR-associated protein-9; CRISPR, clustered regularly interspaced short palindromic repeat.

downregulation of SIRT6, NAD (+)-dependent class III deacetylase sirtuin, was found in NSCLC linked to proliferation, differentiation and apoptosis of cancer cells. Theoretically, overexpression of SIRT6 or silencing Twist1 is one of the options. SIRT6 is found to inhibit the expression of Twist1.[12] Downregulation by antisense or siRNA works well to restore the reexpression of aberrantly hypermethylated genes, but still there are controversies mainly because of the risks associated with the application to humans, that is, instability, nonspecific effects and complexity in developing a suitable delivery system. Protein-based gene modulation is based on the construct with the DNA sequence recognizing motif, that is, DNA-binding domain fused to the effector domain that targets a particular gene. Initial advancements in the DNA-binding domains used for treatment and research purposes in cancer have two options: ZF (zinc finger) and TALE (transcription activator-like effector) domains. Particularly for epigenetic editing, protein-based modulating tools ZFN and TALENS may not serve as a versatile tool because epigenetically silenced chromatin is usually highly compacted and thus it can be inaccessible to these motif-bound reactivation proteins. Although various studies have used transcription-activating drugs together with the construct, RNA-guided endonuclease may serve as another option.

TYPE II CRISPR/Cas9 SYSTEM

CRISPR/Cas is heritable and part of the adaptive immune system in bacteria and archaea generating response against invading phages and plasmids. Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) protein locus encodes Cas proteins and a repeat-spacer array consisting of interspersed identical repeat sequences and unique invader-targeting spacer sequences. A type II CRISPR/Cas9 system is an adaptive immunity system, in which CRISPR spacers direct to the target, whereas Cas enzymes control spacer acquisition and phage defence.[13] The type II system responsible for cell immunity essentially comprises at least three parts: Cas endonucleases, the mature CRISPR-RNA (crRNA) and trans-activating crRNA (tracrRNA). With several Cas protein loci, only Cas9 is said to have endonuclease activity.[14] (Figure 1) CRISPR/Cas-mediated immune response involves three stages (Figure 2): first is the adaptation phase, in which part of the sequences encoded by the virus or plasmid is integrated to the host chromosome flanking the CRISPR array at one end. This foreign DNA is called *spacer*, and it integrates permanently and becomes memory for the next invasion by the same virus or plasmid; hence, it has a role in adaptive immunity. The second stage is the expression phase, in which transcription of the CRISPR array to pre-crRNA takes place, followed by the third-stage interference where processed pre-crRNAs guide Cas protein(s) to the invading nucleic acid and cleave the cognate for their ultimate destruction.[15] The third stage, interference for type II, is slightly expensive, as it requires RNase III to cleave hybridized crRNA–tracrRNAs, and remove the 5′ end of each spacer, yielding mature crRNAs attached to both tracrRNA and Cas9; crRNA guides cas9 to cleave the target DNA[16] (Figure 3). CRISPR uses Watson and Crick base pairing to bind to the target DNA, and it requires the recognition of a short trinucleotide protospacer adjacent motif (PAM). It ignores the sequence that is fully complementary to crRNA if PAM is absent. PAM acts as a site at which two strands start to separate and PAM proximal region seeds R loop formation between Cas9 and the DNA target.[16] A recent study on *in vitro* characterization of Cas9 binding and cleavage using all-in-one retroviral delivery vectors coexpressing two single-guide RNAs (sgRNAs) and Cas9(D10A) nickase revealed that binding to the target is not enough for nuclease catalytic activity, but the extent of base complementarity between PAM-distal target and 5′ crRNA sequences is what fully determines cleavage activity.[17] For *S. pyogenes*, PAM site consensus sequence was reported to be 5′-NGG-3′. Cas9 has arginine residue conserved at its carboxy terminal, which interacts via a major groove and reads out 5′-NGG-3′, whereas the

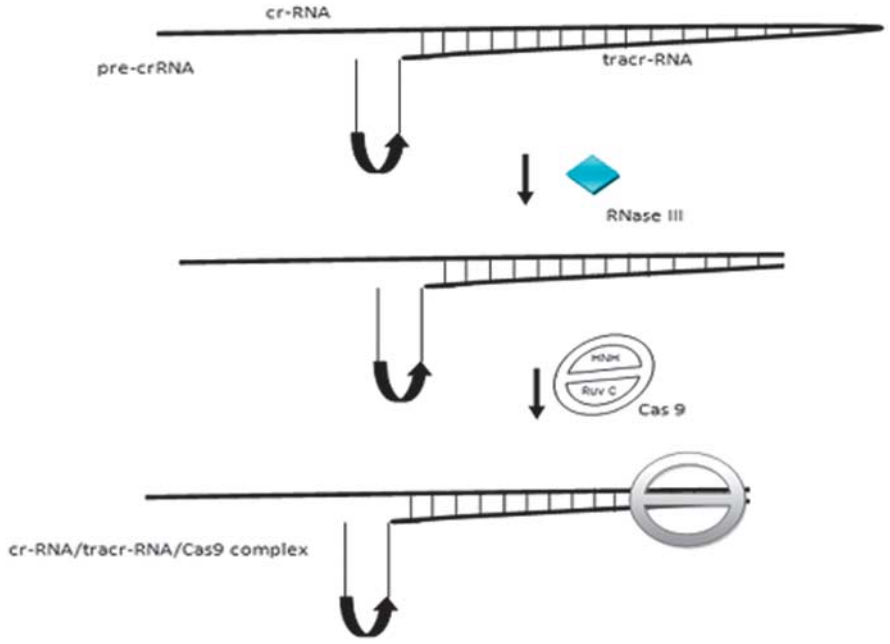

Figure 2. Mechanism of action of type II CRISPR/Cas9. Cas9, CRISPR-associated protein-9; CRISPR, clustered regularly interspaced short palindromic repeat.

complementary strand that lies in the minor groove interacts with the phosphodiester group at the +1 position in the target DNA strand causing the regional strand separation upstream of the PAM.[18] Cas9 consists of two distinct endonuclease domains, that is, HNH and RuvC/RNase H-like domains, both catalyzing the cleavage of the target DNA strand.[15,19–22] Cas9 is a multidomain enzyme[23] that uses an HNH nuclease domain to cleave the target strand (defined as complementary to the spacer sequence of crRNA) and a RuvC-like domain to cleave the nontarget strand, enabling the conversion of the double-stranded DNA cleaving Cas9 into a nickase by selective motif inactivation.[24] Cas endonucleases usually disrupt the particular virus or plasmid creating double-strand break (DSB) and repair ends via non-homologous end joining, generating insertions or deletions (indels) and leading to frameshifts.[25] The type II bacterial system from *S. pyogenes*, as depicted in Figure 1, cleaves DNA *in vitro* simplified through the fusion of the crRNA and tracrRNA via hairpin linker region termed as guide RNA scaffold.[26] In *in vitro* conditions, site-specific disruption can be done by designing a construct comprising promoter-driven Cas9 and guide RNA scaffold to which a customized target sequence of 20 nucleotides in length including 5′-NGG-3′ as PAM within the sequence can be inserted. This target sequence guides cas9 to specific sites within the genome (Figure 4). The CRISPR/Cas9 systems need to be carefully designed to avoid potential off-target cleavage sites, including those with mismatches to the 12 bases proximal to the guide strand PAM. In addition, because non-Watson–Crick base pairing is known to occur,[27] it is possible that CRISPR/ Cas9 systems have more off-target activities compared with corresponding ZFNs and TALENs.[28]

CRISPR/Cas9 SYSTEM AS A GENOME MODULATION TOOL IN HUMANS

The discovery that type II CRISPR/Cas system from *Streptococcus thermophilus* can mimic in *Escherichia coli*[29] was a breakthrough for using the system either from *S. thermophilus* or *S. pyogenes in vitro*.[30,31] Jinek *et al.* developed sgRNA for *S. pyogenes* system that complements the crRNA and tracrRNA in original type II system.[26] This system was capable of DNA binding and cleaving the target as directed by sgRNA, that is, the fused crRNA–tracrRNA, bypassing the processing part.[32] Another strategy to utilize this system has been developed, which is referred to as homology-directed repair, in addition to DSB followed by nonhomologous end joining that can only disrupt the gene either by insertions, deletions or frameshifting the transcript.[33,34] Precise base-pair mutations into the Tet1 and Tet2 genes was done through homology-directed repair-mediated coinjection of single-stranded mutant DNA oligos, sgRNAs and Cas9 mRNA.[35] This system is highly efficient to target multiple genes in one go. CRISPR/Cas9 mediated simultaneous disruption of five genes (Tet1, 2, 3, Sry, Uty–8 alleles) in mouse embryonic stem cells by co-injection of Cas9 mRNA and sgRNAs targeting Tet1 and Tet2 into zygotes. This generated mice with biallelic mutations in both genes with an efficiency of 80. This technique cuts short the traditional recombination and intercrosses in mice to generate multiple mutations, thus accelerating *in vivo* studies of functionally redundant genes and epistatic gene interactions.[36] However, for multiple gene modulations, co-transfection of multiple plasmids showed variable gene expression levels. The reasons for this observation could be difference in copy number and transient gene activation leading to variations in the desired outcome. A lentiviral construct with Cas9 or a dCas9 variant, a reporter gene and up to four sgRNAs, each from independent Pol III promoters, was designed majorly for human cells. This system was efficient to facilitate multiplex gene editing.[37] Not just multiple gene targeting, the efficiency of gRNA to detect single-nucleotide polymorphism enables allele-specific gene modulation. In one study, the specific gRNA for the F344-albino mutation (gRNA:Tyr^{c}) only targeted the F344-allele, whereas the gRNA for DA-wild-type (gRNA:Tyr^{C}) only targeted the DA-allele, thereby changing the dominant coat-color phenotype of the F1 pups. This study also revealed interallelic gene conversion as inferred from albino coat-color rats that carry homozygous alleles of F344 QUOTE. CRISPR/Cas9 proves to be a highly efficient tool for allele-specific gene modulation by either silencing the gene or crossing homologous chromosomes to repair the dominant disease-related allele.[38] It has also been proven to be a mammalian genetic

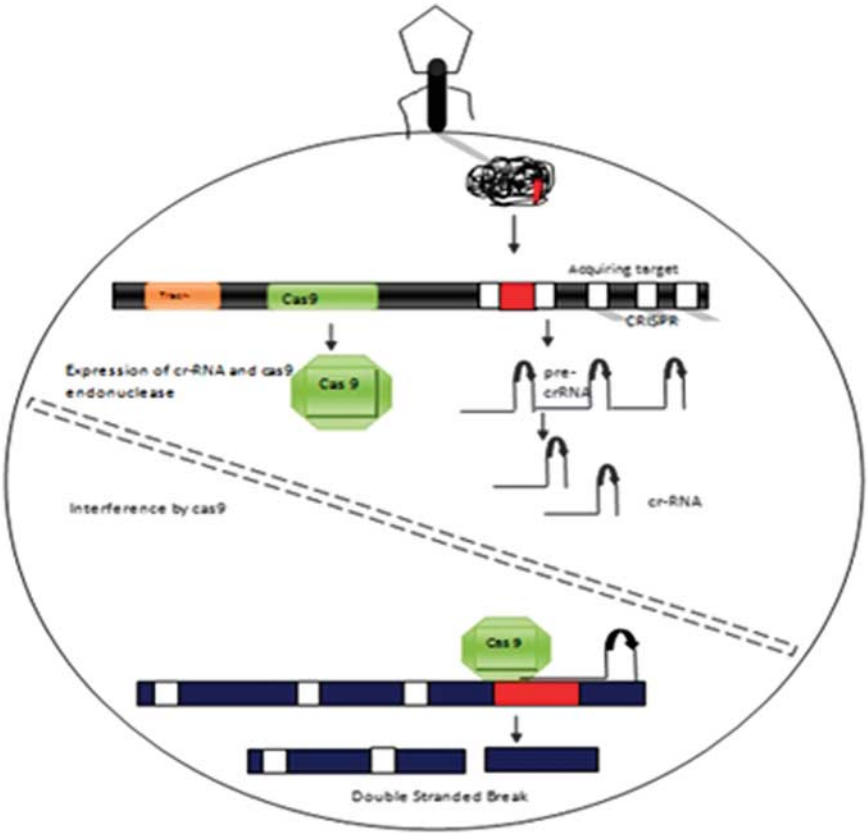

Figure 3. Processing of CRISPR-RNA (crRNA). CRISPR, clustered regularly interspaced short palindromic repeat.

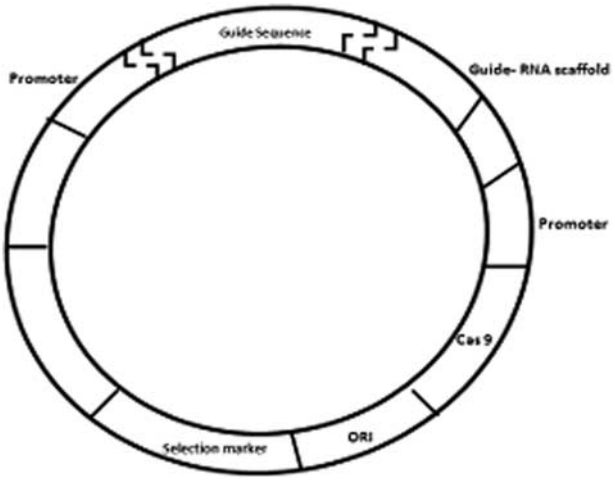

Figure 4. Type II CRISPR/Cas9 construct for experimental procedures. Cas9, CRISPR-associated protein-9; CRISPR, clustered regularly interspaced short palindromic repeat.

testing and screening tool. Virtually, because of the frequency of the PAM motif in mammalian genomes and the small oligo, the sgRNA design may enable the generation of complex pools of targeted whole-genome sgRNA libraries analogous to shRNA libraries prerequisite for any large-scale multiplex screen. Although CRISPR-associated RNA-guided nucleases are highly efficient genome editing tools, they serve off-target effects. It has been found that RNA-guided nucleases can induce mutations at sites that differ by as many as five nucleotides from the intended target. To overcome this problem, truncated gRNA has been used, and it is reported that shorter regions of target complementarity < 20 nucleotides in length can decrease the undesired mutagenesis at some off-target sites by 5000-fold or more without sacrificing on-target genome editing efficiencies.[39] Cas9 endonuclease has been engineered to check its other potentialities. The Cas9 is converted to nickase by changing D10A in the RuvC1 domain or H840A in HNH-like domains in Cas9[40–42] that facilitates homology-directed repair in mammalian genomes with reduced mutagenic activity. Nickase Cas9 is further explored for improving the target specificity of Cas9. Combined with two complementary offset sgRNAs, Cas9 was found to generate indels, thereby reducing unwanted cleavage because base excision repair mechanism mends individual off-target single-stranded nicks with high fidelity.[43]

ENGINEERING dCas9

Inactivated cas9 (dCas9) has been engineered to control the expression of endogenous genes termed CRISPRi/a systems. This system enables the localization of effector domains to repress or activate gene transcription without modifying DNA.[31,39] dCas9 was fused with a C-terminal VP64 acidic transactivation domain, transfected in human embryonic kidney 293T (HEK293T) cells with four gRNAs designed to target the promoter of *IL1RN* gene, leading to substantial induction of *IL1RN*.[44] The DNA-binding activity of a Cas9 catalytic inactive mutant has been exploited to engineer RNA-programmable transcriptional silencing and activating devices.[31,45] Previously unmodified dCas9 rendered a modest block in transcription in mammalian cells, limiting the utility of the system as a tool for programmed knockdown of genes; however,

recent advances, that is, codon-optimized sp CRISPR/Cas9, have paved the way for human cell gene knockdown or transcriptional regulation. Catalytically inactivated Cas9 (dCas9) directed to the coding region of a gene blocks RNA polymerase binding or elongation, thus repressing the transcription in bacteria. Eukaryotic genome is highly complex; genes are regulated at the DNA level by cognation of activating and repressive transcription factors acting at DNA regulatory elements, whereas at the chromatin level they are regulated by epigenetic modifications such as histone or DNA, acetylation and methylation, deacetylation and demetylation. dCas9 was proven to be efficient to target eukaryotic genome even without any human-oriented optimization. Recently, dCas9 was developed for the human cell with codon optimization and construct carrying human codon-optimized dCas9 fused to two copies of a nuclear localization sequence, a hemagglutinin tag and blue fluorescent protein fused to a certain effector domain that could be either repressive or activating. For instance, using a dCas9-fused KRAB domain shows a fivefold decrease in green fluorescent protein signal, whereas cells expressing dCas9 alone, dCas9-CS or dCas9-WRPW show a twofold decrease in green fluorescent protein signal. The other group proved that dCas9 alone (that is, without use of the KRAB domain) is capable of repression of up to 86% for endogenous human genes efficiently with specific and correct sgRNA target sites.[46] In addition, CRISPR-Cas9-based acetyltransferase consisting of the nuclease-null dCas9 protein was fused to the catalytic core of the human acetyltransferase p300. The fusion protein catalyzes acetylation of histone H3 lysine 27 at its target sites, leading to robust transcriptional activation of target genes from promoters and both proximal and distal enhancers. Gene activation by the targeted acetyltransferase was highly specific across the genome.[47] dCas9 has been used for simultaneous induction of multiple endogenous genes with defined stoichiometry. In the CRISPR-on system, a novel class of artificial transcription factors was designed and checked for activation of the endogenous *IL1RN*, *SOX2* and *OCT4* genes simultaneously.[48] CRISPR/Cas9 system is far more flexible and useful than short hairpin RNA (shRNA) for knockout screens[49,50] for the study of phenotypes that require complete inactivation of genes. Currently in mammalian cells, RNA interference (RNAi) is the predominant method for genome-wide loss-of-function screening; CRISPRi may serve more purposes as the utility of RNAi ceases because of its inherent incompleteness of protein depletion and confounding off-target effects.[51] Cas9/sgRNAs can target elements across the entire genome, as the targeting specificity is conferred by short guide sequences that can be easily generated at a large scale by array-based oligonucleotide library synthesis. It can also target promoters, enhancers, introns and intergenic regions, whereas RNAi is just limited to transcripts. dCas9 can be sequestered to functional domains[39,52] to broaden the repertoire of perturbation modalities, including genome-scale gain-of-function screening using Cas9 activators and epigenetic modifiers. The lentiviral delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeting 18 080 genes with 64 751 unique guide sequences enabled both negative and positive selection screening in human cells. GeCKO (genome-scale CRISPR-Cas9 knockout) library was created and used to identify genes that were essential for cell viability in cancer and pluripotent stem cells. Furthermore, in a melanoma model, genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF, were found including previously validated genes *NF1* and *MED12*, as well as novel hits *NF2*, *CUL3*, *TADA2B* and *TADA1*.[53] RNAi is already being used for therapeutic purposes, and these results make way for entry of CRISPR/Cas9 technology in therapeutic applications.[39] The ability to precisely regulate any gene as it occurs naturally in the genome provides a means to address a variety of diseases and disorders.[54] CRISPRi can be used for multiplexed control of endogenous genes by dCas9 fusion protein and stably repressing genes comparable to gene silencing efficiency typically achieved by RNA interference while minimally affecting transcription of nontargeted genes.[39] CRIPSRi has proved its worth not only in controlling gene expression but also in sequence-specific inhibition of miRNA. Three stable NIH3T3 reporter cell lines were reported, each of which expressed a doxycycline-inducible shRNA and a Venus-sensor fusion protein containing the target shRNA region to enhance measurement sensitivity. crRNAs were designed to separately recognize the linker between the loop and seed region of shRNAs 1–3 in the three vectors (Crispsh1–3). After transient transfection of Crispsh1, 2 and 3 separately into the corresponding reporter cell lines, a significant enhancement of fluorescence signal was detected, indicating that the inhibition of the targeted shRNAs might result from specific DSBs in the target regions.[55] Virtually, CRISPR/Cas9 may target genes that are molecular targets or epigenomic targets for lung cancer, paving the way for gene therapies and bypassing drawbacks of molecular targeted drugs and epigenetic inhibitors. Although until now this is in infancy, engineering CRISPR/Cas9 to target gene for therapeutic purpose if proved successful can pave the way to a new era of gene therapy with permanent cure.

VARIED UTILIZATION OF CRISPR/Cas9 IN CANCER STUDY AND TREATMENTS

The CRISPR/Cas9 system is a breakthrough in the study of cancer genes in mouse models. Xue *et al.*[56] used hydrodynamic injection to deliver a CRISPR plasmid DNA expressing Cas9 and sgRNAs to the liver that directly targeted the tumor suppressor genes PTen and p53, alone and in combination, and found results phenocopying deletion of the gene using Cre–LoxP technology, proving its feasibility of use for efficient correction of a genetic disease. In a study on lung adenocarcinoma, adeno-associated virus was used to model the dynamics of *KRAS, p53* and *LKB1,* and it successfully generated loss-of-function mutations in p53 and Lkb1, as well as homology-directed repair-mediated $KRAS_{G12D}$ mutations forming tumors of adenocarcinoma pathology.[57] Expanding the utility, CRISPR/Cas9 can be used to assess novel and putative targets and their functional significance. *SF3B1* provided the first causal link between SF3B1 hot-spot mutations and splicing alterations. SF3B1-mutant cells are not dependent upon the mutated allele for *in vitro* growth, but instead depend upon the function of the remaining wild-type alleles. This was made possible by the Degron-KI system (CRISPR/Cas9-mediated knock-in of inducible degron tags).[58] For most kinds of chromosome structural aberrations that were hard to mimic, CRISPR/Cas9 proved its efficacy to model them successfully. Targeted DNA fragment inversions and duplications of DNA fragments ranging in size from a few tens of bp to hundreds of kb can be easily achieved in human and mouse genomes by CRISPR with two sgRNAs. In addition, DNA fragment duplications and deletions are generated by CRISPR through trans-allelic recombination between the Cas9-induced DSBs on two homologous chromosomes (chromatids) and have paved a way to study millions of regulatory DNA elements, as well as vast numbers of gene clusters.[59] Chromosomal rearrangements that lead to expression of therapeutically actionable gene fusions is common in pathogenesis of human cancers. Modeling such genetic events in mice has proven challenging and requires complex manipulation of the germline. The CRISPR/Cas9 system efficiently induced specific chromosomal rearrangements *in vivo* using viral-mediated delivery to somatic cells of adult animals. A mouse model of Eml4-Alk-driven lung cancer was generated successfully that harbored tumor with Eml4-Alk inversion, expressing the Eml4-Alk fusion gene, displaying histopathological and molecular features typical of ALK(+) human NSCLCs, and responded to treatment with ALK inhibitors.[60] Tackling multiple genes together using CRISPR/cas9 has led to synthesizing AND gate genetic circuit for the identification of

cancer cells and stringent hold of specificity and efficacy of cancer gene therapy. The circuits integrate cellular information from two promoters as inputs and activate the output gene only when both inputs are active in the tested cell lines. Circuits have been created for genes such as *hBAX*, *p21* and *E-cadherin*. The circuits effectively inhibit bladder cancer cell growth, induce apoptosis and decrease cell motility by regulating the corresponding gene. Thus, CRISPR/Cas9 has proved its worth for synthetic biology.[61] Recent advances made in CRISPR/Cas made efficient inhibition of miRNA. Modified CRISPR interference system prevents the expression of both monocistronic miRNAs and polycistronic miRNA clusters. This system serves several advantages over chemically modified complementary antisense oligonucleotides that need expensive modifications and made loss-of-function miRNA mutations much simpler than homologous recombination. Zhao *et al.*[55] established three exogenous shRNA 1–3 stably in NIH3T3 reporter cell lines. crRNAs were designed to separately recognize the linker between the loop and seed region of shRNAs1–3 in the three vectors (Crispsh1–3) that inhibited the targeted shRNAs. DrugTargetSeqR combines high-throughput sequencing, computational mutation discovery and CRISPR/Cas9-based genome editing. Physiological targets of drugs can be identified using CRISPR/Cas9-mediated genome editing in combination with high-throughput sequencing and computational mutations. To find the target for a drug, the target protein is mutated, which confers resistance to the drug in cells, as well as suppresses drug activity in a biochemical assay. For instance, using the technique DrugTargetSeqR, ispinesib, an inhibitor of kinesin-5, is reported to be an anticancer agent.[62] Selinexor, an oral bioavailable inhibitor of exportin-1 (CRM1/XPO1), is a potential anticancer drug currently under evaluation in human clinical trials, and it was recently validated using CRISPR/cas9 for drug target. A homozygous mutation C528S in the XPO1 gene made cells resistant to selinexor (KPT-330) and thus validates XPO1 as the prime target of selinexor in cells and identified the selectivity of this drug toward the cysteine 528 residue of XPO1.[63] Furthermore, CRISPR-Cas9 allows comprehensive identification of protein domains that sustain cancer cells and are potential targets for drugs and therapy. This is done by selecting exons encoding protein functional domain. A screen of 192 chromatin regulatory domains in murine acute myeloid leukemia cells identified six known drug targets and 19 additional dependencies.[64] Most of all, the CRISPR/Cas9 system is a perfect replacement for almost all long experimental procedures for cancer gene therapies. CRISPR/Cas9 targeting the promoter of human papillomavirus (HPV) 16 E6/E7 and targeting E6 and E7 transcript revealed that CRISPR/Cas9 targeting promoter, as well as targeting E6 and E7, resulted in the accumulation of p53 and p21 proteins, thus reducing the proliferation of cervical cancer cells *in vitro* and *in vivo* and remarkably inhibiting tumorigenesis in mice. This paved a way for application of CRISPR/Cas9 to target HR-HPV key oncogenes in cervical and other HPV-associated cancer therapies.[65]

FUTURE DIRECTIONS

Although varied drugs and therapies have been developed for lung cancer treatment, over the past 5 years overall survival rates have not improved much. In addition, in most of the patients, lung cancer is diagnosed already in advanced stages with heterogeneous tumors, where single therapy is mostly ineffective. A combination of therapies is being administered and specific genes are targeted in specific tissues while protecting normal cell, but most of the therapies face drawback for the development of resistance against them and tumor progression. Therefore, a therapeutic implication for various therapies needs to be complemented by divergent strategies. Virtually, cas9 can target any gene in a tissue-selective manner (tissue-specific promoters in plasmid or viral construct) either inhibiting, repressing, activating, translocating, inverting or duplicating them, and hence CRISPR/Cas may serve as a boon for lung cancer therapeutics. It may repress overexpressed epidermal growth factor receptor, overcoming the main setback of tyrosine kinase inhibitor or Alk-activated pathway that are still in infancy. K-RAS is one of the primary targets for gene therapy using RNAi; CRISPRi is a perfect candidate to overtake with increased number of advantages over RNAi, such as it does not compete with endogenous machinery such as miRNA expression or function. In addition, CRISPR/Cas9 acts at the DNA level, and therefore it opens ways to target transcripts such as noncoding RNAs, miRNAs, antisense transcripts, nuclear-localized RNAs and polymerase III transcripts and possesses a much larger targetable sequence space, as promoters and, in theory, introns also.[39] Recent studies on CRISPR/Cas9 to target multiple genes may pave a path to treat multiple mutation in a heterogeneous mass of tumor in NSCLC. Regulating hepatocyte growth factor in Met-derived NSCLC may be done through CRISPR/Cas9. Not just genes marked for molecular targets for particular cancer, CRISPR/Cas9 may be used to modulate epigenetic enzyme. CRISPR/cas9 proves to be a better option to target either DNMT or histone deacetylase for lung cancer treatment, overcoming an array of side effects that follow DNMTi utilization. Engineered dCas9 can recruit the effector domain attached to it, and it can precisely recruit any of the major chromatin-remodeling complexes such as histone acetylases and deacetylases, methylases and demethylases, DNA methylases and demethylases and so on, opening new ways to examine the nature of epigenetic control and modulate expression states. All of these recent advances and those to come in developing and optimizing Cas9-based systems for genome and epigenome editing should propel the technology toward therapeutic applications, opening the door to treating a wide variety of human diseases. Further development of such approaches could give rise to a completely new pharmacological treatment class that might contribute to erasing of disease-associated genomics and epigenomic signatures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; **65**: 5–29.
2 Tang M, Xu W, Wang Q, Xiao W, Xu R. Potential of DNMT and its epigenetic regulation for lung cancer therapy. *Curr Genom* 2009; **10**: 336.
3 Xu XL, Wu LC, Du F, Davis A, Peyton M, Tomizawa Y *et al.* Inactivation of human SRBC, located within the 11p15. 5-p15. 4 tumor suppressor region, in breast and lung cancers. *Cancer Res* 2001; **61**: 7943–7949.
4 Pekarsky Y, Zanesi N, Palamarchuk A, Huebner K, Croce CM. FHIT: from gene discovery to cancer treatment and prevention. *Lancet Oncol* 2002; **3**: 748–754.
5 Kremer M, Quintanilla-Martinez L, Fuchs M, Gamboa-Dominguez A, Haye S, Kalthoff H *et al.* Influence of tumor-associated E-cadherin mutations on tumorigenicity and metastasis. *Carcinogenesis* 2003; **24**: 1879–1886.
6 Sloan EK, Stanley KL, Anderson RL. Caveolin-1 inhibits breast cancer growth and metastasis. *Oncogene* 2004; **23**: 7893–7897.
7 Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K *et al.* Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 2008; **455**: 1069–1075.
8 Tellez CS, Grimes MJ, Picchi MA, Liu Y, March TH, Reed MD *et al.* SGI-110 and entinostat therapy reduces lung tumor burden and reprograms the epigenome. *Int Jo Cancer* 2014; **135**: 2223–2231.
9 Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev Cell* 2010; **19**: 698–711.
10 Malecová B, Morris KV. Transcriptional gene silencing mediated by noncoding RNAs. *Curr Opin Mol Ther* 2010; **12**: 214.
11 Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E *et al.* MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci USA* 2007; **104**: 15805–15810.

12 Han Z, Liu L, Liu Y, Li S. Sirtuin SIRT6 suppresses cell proliferation through inhibition of Twist1 expression in non-small cell lung cancer. *Int J Clin Exp Pathol* 2014; **7**: 4774.
13 Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007; **315**: 1709–1712.
14 Garneau JE, Dupuis M-È, Villion M, Romero DA, Barrangou R, Boyaval P *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 2010; **468**: 67–71.
15 Chylinski K, Le Rhun A, Charpentier E. The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. *RNA Biol* 2013; **10**: 726–737.
16 Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 2014; **507**: 62–67.
17 Cencic R, Miura H, Malina A, Robert F, Ethier S, Schmeing TM *et al.* Protospacer adjacent motif (PAM)-distal sequences engage CRISPR Cas9 DNA target cleavage. *PLoS One* 2014; **9**: e109213.
18 Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 2014; **513**: 569–573.
19 Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput Biol* 2005; **1**: e60.
20 Fenner MW. CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea. *Lawrence Berkeley Natl Lab* 2007; **6**: 181–186.
21 Barrangou R, Horvath P. CRISPR: new horizons in phage resistance and strain identification. *Annu Rev Food Sci Technol* 2012; **3**: 143–162.
22 Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA* 2012; **109**: E2579–E2586.
23 Sorek R, Kunin V, Hugenholtz P. CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat Rev Microbiol* 2008; **6**: 181–186.
24 Fonfara I, Le Rhun A, Chylinski K, Makarova KS, Lécrivain A-L, Bzdrenga J *et al.* Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Res* 2014; **42**: 2577–2590.
25 Hou Z, Zhang Y, Propson NE, Howden SE, Chu L-F, Sontheimer EJ *et al.* Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci USA* 2013; **110**: 15644–15649.
26 Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816–821.
27 Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol* 2013; **31**: 233–239.
28 Cradick TJ, Fine EJ, Antico CJ, Bao G. CRISPR/Cas9 systems targeting beta-globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res* 2013; **41**: 9584–9592.
29 Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. *EMBO J* 2011; **30**: 1335–1342.
30 Fineran PC, Charpentier E. Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. *Virology* 2012; **434**: 202–209.
31 Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP *et al.* Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013; **152**: 1173–1183.
32 Westra ER, Semenova E, Datsenko KA, Jackson RN, Wiedenheft B, Severinov K *et al.* Type IE CRISPR-cas systems discriminate target from non-target DNA through base pairing-independent PAM recognition. *PLoS Genet* 2013; **9**: e1003742.
33 Cho SW, Kim S, Kim JM, Kim J-S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 2013; **31**: 230–232.
34 Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell* 2013; **12**: 393.
35 Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S *et al.* Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* 2013; **13**: 659–662.
36 Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **153**: 910–918.
37 Kabadi AM, Ousterout DG, Hilton IB, Gersbach CA. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res* 2014; **42**: e147.
38 Yoshimi K, Kaneko T, Voigt B, Mashimo T. Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR–Cas platform. *Nat Commun* 2014; **5**: 4240.
39 Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013; **154**: 442–451.
40 Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 2013; **31**: 691–693.
41 Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z *et al.* Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 686–688.
42 Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 2014; **32**: 347–355.
43 Ran F, Hsu PD, Lin C-Y, Gootenberg JS, Konermann S, Trevino AE *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; **154**: 1380–1389.
44 Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR *et al.* RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods* 2013; **10**: 973–976.
45 Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res* 2013; **41**: 7429–7437.
46 Lawhorn IE, Ferreira JP, Wang CL. Evaluation of sgRNA target sites for CRISPR-mediated repression of TP53. *PLoS One* 2014; **9**: e113232.
47 Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE *et al.* Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* 2015; **33**: 510–517.
48 Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW *et al.* Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res* 2013; **23**: 1163–1171.
49 Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M *et al.* A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 2004; **428**: 431–437.
50 Luo B, Cheung HW, Subramanian A, Sharifnia T, Okamoto M, Yang X *et al.* Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci USA* 2008; **105**: 20380–20385.
51 Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L *et al.* Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* 2006; **12**: 1179–1187.
52 Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* 2013; **31**: 833–838.
53 Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS *et al.* Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 2014; **343**: 84–87.
54 Gersbach CA, Perez-Pinera P. Activating human genes with zinc finger proteins, transcription activator-like effectors and CRISPR/Cas9 for gene therapy and regenerative medicine. *Expert Opin Ther Targets* 2014; **18**: 835–839.
55 Zhao Y, Dai Z, Liang Y, Yin M, Ma K, He M *et al.* Sequence-specific inhibition of microRNA via CRISPR/CRISPRi system. *Sci Rep* 2014; **4**: 3943.
56 Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS *et al.* CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* 2014; **514**: 380–384.
57 Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR *et al.* CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 2014; **159**: 440–455.
58 Zhou Q, Derti A, Ruddy D, Rakiec D, Kao I, Lira M *et al.* A chemical genetics approach for the functional assessment of novel cancer genes. *Cancer Res* 2015; **75**: 1949–1958.
59 Li J, Shou J, Guo Y, Tang Y, Wu Y, Jia Z *et al.* Efficient inversions and duplications of mammalian regulatory DNA elements and gene clusters by CRISPR/Cas9. *J Mol Cell Biol* 2015; **7**: 284–298.
60 Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han Y-C *et al.* In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature* 2014; **516**: 423–427.
61 Liu Y, Zeng Y, Liu L, Zhuang C, Fu X, Huang W *et al.* Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. *Nat Commun* 2014; **5**: 5393.
62 Kasap C, Elemento O, Kapoor TM. DrugTargetSeqR: a genomics-and CRISPR-Cas9–based method to analyze drug targets. *Nat Chem Biol* 2014; **10**: 626–628.
63 Neggers JE, Vercruysse T, Jacquemyn M, Vanstreels E, Baloglu E, Shacham S *et al.* Identifying drug-target selectivity of small-molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing. *Chem Biol* 2015; **22**: 107–116.
64 Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol* 2015; **33**: 661–667.

65 Zhen S, Hua L, Takahashi Y, Narita S, Liu Y-H, Li Y. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. *Biochem Biophys Res Commun* 2014; **450**: 1422–1426.
66 Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–1500.
67 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–2139.
68 McDermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S *et al.* Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. *Cancer Res* 2008; **68**: 3389–3395.
69 Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL *et al.* Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* 1994; **263**: 1281–1284.
70 Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T *et al.* EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010; **363**: 1734–1739.
71 Roberts P, Der C. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 2007; **26**: 3291–3310.
72 Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R *et al.* Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med* 2008; **14**: 1351–1356.
73 Ju YS, Lee W-C, Shin J-Y, Lee S, Bleazard T, Won J-K *et al.* A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. *Genome Res* 2012; **22**: 436–445.
74 Birchmeier C, Birchmeier W, Gherardi E, Woude GFV. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003; **4**: 915–925.
75 Ma PC, Jagadeeswaran R, Jagadeesh S, Tretiakova MS, Nallasura V, Fox EA *et al.* Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non–small cell lung cancer. *Cancer Res* 2005; **65**: 1479–1488.
76 Belinsky SA, Klinge DM, Stidley CA, Issa J-P, Herman JG, March TH *et al.* Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res* 2003; **63**: 7089–7093.
77 Chu B, Karpenko M, Liu Z, Aimiuwu J, Villalona-Calero M, Chan K *et al.* Phase I study of 5-aza-2′-deoxycytidine in combination with valproic acid in non-small-cell lung cancer. *Cancer Chemother Pharmacol* 2013; **71**: 115–121.
78 Lin R-K, Wang Y-C. A DNA methyltransferase I inhibitor mithramycin A in cancer cells: a pilot study. *Bioformosa* 2007; **42**: 55–62.
79 Pan M-R, Chang H-C, Chuang L-Y, Hung W-C. The nonsteroidal anti-inflammatory drug NS398 reactivates SPARC expression via promoter demethylation to attenuate invasiveness of lung cancer cells. *Exp Biol Med* 2008; **233**: 456–462.
80 Yu X-D, Wang S-Y, Chen GA, Hou C-M, Zhao M, Hong JA *et al.* Apoptosis induced by depsipeptide FK228 coincides with inhibition of survival signaling in lung cancer cells. *Cancer J* 2007; **13**: 105–113.