Applications of CRISPR technology to lung cancer research

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CRISPR technology has revolutionised lung cancer research. It can be used to model lung cancer in animals, to find new players in cancer initiation and progression, to diagnose earlier, and even to design novel gene therapies. https://bit.ly/3GPjT6o

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Introduction

Clustered regularly interspaced palindromic repeats (CRISPR) technology has revolutionised genome engineering and currently possesses a long list of applications across cell types and organisms. Earlier observations proposed that CRISPR constituted a bacterial adaptive immune system against viruses and plasmids [1]. A landmark work published in 2012 demonstrated that the CRISPR/CRISPR-associated protein 9 (Cas9) system could produce double-stranded breaks (DSB) in prokaryotic DNA when programmed with CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) [2], opening the possibility to make a disruption in a specific place of the DNA sequence and consequently distort the gene product. The crRNA is a 17–20 amino acid sequence complementary to the DNA target sequence that gives the specificity to the CRISPR system. The tracrRNA serves as scaffold for the Cas nuclease (*e.g.* Cas9). The two RNAs exist as separate molecules in nature. The hybridisation of crRNA and tracrRNA forms the guide RNA (gRNA). Further re-engineering (fusion) of these two RNAs into a single-guide RNA (sgRNA), a simpler system to work with, serves the same purpose [2]. Later, it was observed that CRISPR/Cas9 could also edit mammalian DNA, which opened new avenues for precision medicine in humans [3, 4]. DNA editing possibilities are particularly interesting in cancer as genetic and epigenetic aberrations cause malignancies and CRISPR-based genetic interventions could provide new knowledge and potentially cure the disease.

Lung cancer was the second most diagnosed tumour type and the leading cause of cancer death (1.8 million) worldwide in 2020 [5]. It constitutes a global health threat causing an enormous health and economic burden. The most frequent genetic alterations of non-small cell lung cancers (NSCLC), which comprise 85% of all lung cancers, are amplifications in *NKX2-1*, *TERT*, *EGFR*, *MET*, *KRAS*, *ERBB2* and *SOX2*, somatic mutations in *TP53*, *KRAS*, *LKB1*, *EGFR*, *NF1*, *BRAF*, *MET*, *RIT*, *CDKN2A* and *ERBB2*, and genetic fusions in *ALK*, *ROS1*, *RET*, *FGFR* and *NTFK1/2/3* [6–8]. Most of them are also driver mutations [8]. Small cell lung cancers (SCLC), which comprise 10–15% of all lung cancers, are prone to metastasise and possess gene mutations in *TP53* (90%), *RB1* and *KMT2D* [9]. Epigenetic alterations seen in lung cancer include aberrant DNA methylation, histone acetylation, chromatin remodelling, microRNA silencing and other post-transcriptional modifications of RNA by noncoding RNAs [10, 11]. In this sense, CRISPR technology can help in: 1) modelling lung cancer disease based on the knowledge of causative epi/genetic alterations; 2) the identification of novel genes responsible for lung cancer onset, progression and therapy success; 3) the diagnostic pathway; and 4) providing innovative nucleic acid-based therapies (figure 1).

How does CRISPR technology work?

The most widely used CRISPR/Cas system is the type II CRISPR/Cas9 composed by the RNA-guided endonuclease from *Streptococcus pyogenes* (spCas9) that is routed to the target DNA sequence by the RNA molecules crRNA and tracrRNA (figure 1) [2]. Once Cas9 recognises the protospacer adjacent motif (PAM) 5′-NGG-3′ (where "N" can be any nucleotide base) downstream of the complementary target sequence, there is base-pairing between crRNA and the target DNA. PAM is a 2–6 nucleotide sequence

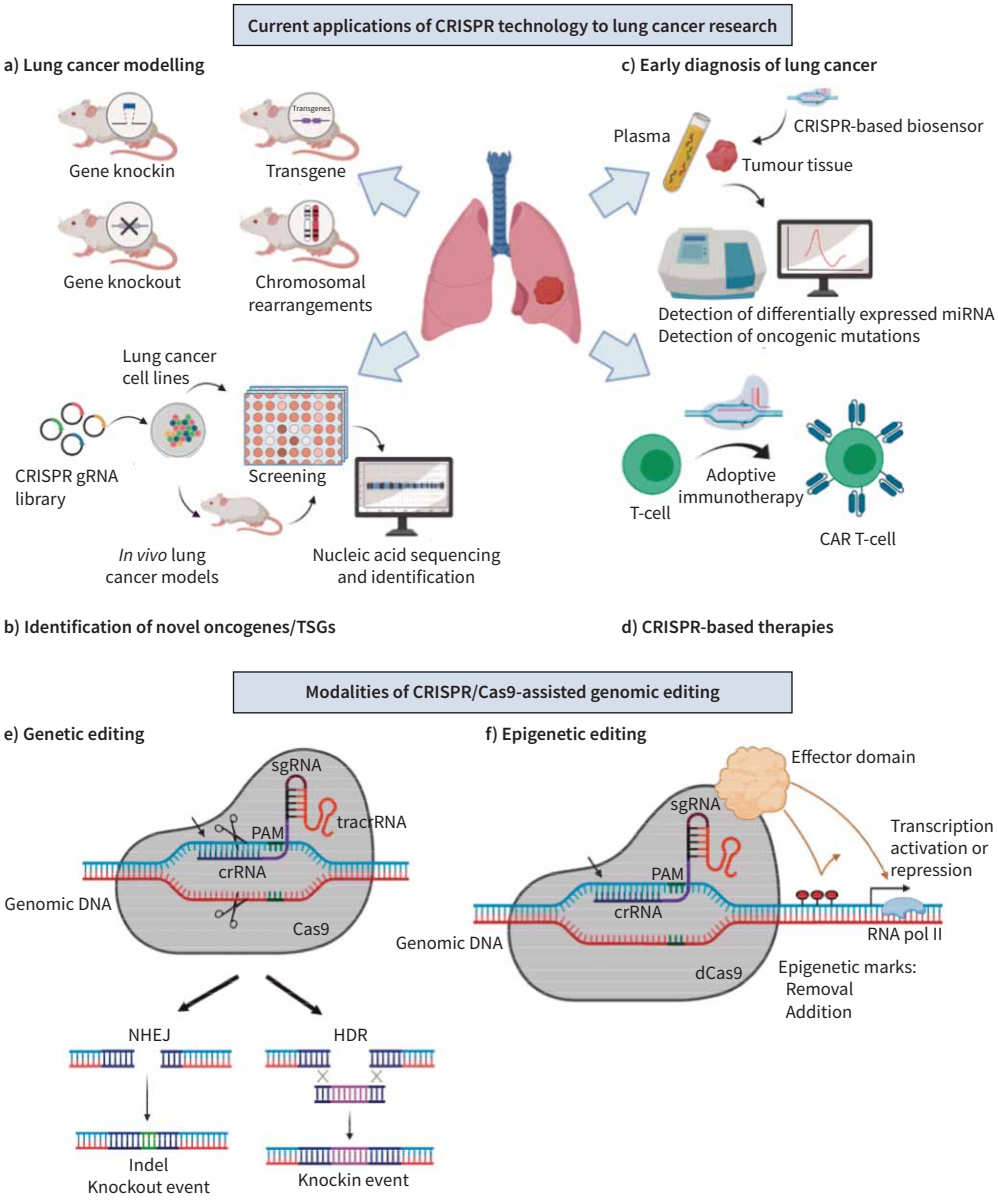

FIGURE 1 Schematic representation of current applications of CRISPR (clustered regularly interspaced palindromic repeats) technology to lung cancer research, and modalities of CRISPR/Cas9-assisted genomic editing. Upper panel represents the four main applications of CRISPR. a) Lung cancer modeling through the generation of knockouts and knockins. The knockins include the production of transgenes and chromosomal rearrangements. b) Identification of oncogenes or tumour suppressor genes through the construction of guide RNA (gRNA) libraries whose target genes are interrogated for changes in lung cancer cell behaviour, and later sequenced and identified. There can be an intermediate step involving animals to assess tumourigenesis *in vivo*. c) Construction of diagnostic tools for early detection of lung cancer. The CRISPR-based biosensor is able to detect and report the presence of lung cancer-related miRNAs or mutations in plasma and tissue, respectively. d) Generation of novel therapies by creating chimeric antigen receptor (CAR) T-cells *ex vivo* through CRISPR. Such cells can be infused again to the lung cancer patient to promote an anti-cancer immune response. Lower panel represents the two main CRISPR modalities. e) Genome editing uses the endonuclease Cas9 that produces a double-strand break (DSB) at the genomic DNA once the single guide (sg)RNA binds the target gene and Cas9 recognises the photospacer adjacent motif (PAM) sequence. When DSB occurs, two possible repair pathways can take place, namely the non-homologous end joining (NHEJ) repair pathway (left) and the homologous-direct repair (HDR) pathway (right). In the NHEJ repair pathway, an indel is introduced due to the error-prone nature of this repair mechanism and, consequently, the target gene loses its function, producing the gene knockout. In the HDR pathway, and in the presence of an external donor DNA, a recombination event

occurs, and the donor DNA gets integrated into the genomic DNA, producing a knockin. f) Epigenome editing uses a Cas9 unable to cut but capable of acting as a vehicle to transport an effector domain at the target gene. There, the effector domain can locally remodel the epigenetic status of the gene leading to its expression activation or repression. dCas9: deactivated Cas9; crRNA: CRISPR RNA; tracrRNA: trans-activating CRISPR RNA; RNA pol II: RNA polymerase II.

that works as a check control for the Cas nuclease to cut. It allows the Cas nuclease to distinguish between the target DNA (contains PAM) and the bacterial DNA, in order to avoid an undesirable cut. Then, Cas9 cleaves 3–4 nucleotides upstream from the PAM, producing a DSB [12]. DSBs are preferentially repaired by the non-homologous end joining (NHEJ) repair pathway, which is error-prone and forms an indel (a genetic mutation characterised by a small insertion or deletion of nucleotides), leading to the target gene knockout [3]. An alternative repair pathway is the homology-directed repair (HDR) pathway which has higher fidelity but is only functional during S/G2 cell cycle phases. The reason is that HDR needs a homologous template strand, and such a strand is only available when sister chromatids are present (*i.e.* during the S/G2 phase). The presence of an available DNA homologous donor at the proximities of the DSB allows the generation of knock-in events (*e.g.* correction of mutations), although still with limited efficiency [3]. Apart from the original Cas9, there exist a varied range of Cas enzymes with optimised functionality and different capabilities, such as the smaller Cas9 from *Staphylococcus aureus* (SaCas9) [13]. Being smaller is an advantage for its easier packaging into adeno-associated viruses, for example. Another Cas variant is the Cas12a (or Cpf1) included in the type V CRISPR/Cas12a system that does not need tracrRNA and uses a T-rich PAM sequence [14]. Not needing tracRNA adds simplicity to the system, while the ability to recognise a T-rich PAM sequence is advantageous when intending to cut T-rich DNA sequences. Moreover, Cas13 belongs to the type VI CRISPR/Cas13 from the bacterium *Leptotrichia shahii* and cleaves single-stranded RNA [15]. As Cas13 cannot edit the genome, its effects are largely reversible, thus avoiding the off-target effects derived from the formation of indels through the NHEJ repair pathway. Another advantage is that Cas13 can be used in non-dividing cells because RNA does not require HDR. An interesting modality of Cas enzyme is the dead Cas9 (dCas9) which lacks endonuclease activity due to two point mutations at the RuvC1 and HNH nuclease domains (figure 1) [16]. This system enables DNA base substitution, epigenetic editing and gene tagging depending on the effector domain dCas9 is fused to.

Modelling lung cancer genetically

CRISPR technology has successfully modelled lung adenocarcinoma *in vivo* through somatic editing. Notably, lung tissue is relatively accessible by CRISPR constructs delivered *via* viruses or DNA transfection. For instance, Platt *et al.* [17] generated Cre-dependent Cas9 knockin mice and administered intratracheally an adenovirus vector containing sgRNA guides to target *TP53*, *LKB1* and *KRAS*, and a construct containing a $Kras^{G12D}$ HDR donor. Such a delivery formed several tumours, ranging from alveolar carcinomas to invasive adenocarcinomas in less than 2 months. Similarly, Sánchez-Rivera *et al.* [18] delivered lentiviruses with a construct containing sgRNA against *PTEN*, *Nkx2-1* and *Apc* as well as Cas9 and Cre intratracheally into LoxP $Kras^{G12D/+}$ and $p53^{flox/flox}$ transgenic mice to model lung cancer disease. To model SCLC, a similar strategy was followed but with two constructs, one containing Cas9 and Csy4 (a RNA endonuclease) and the other containing sgRNA against p107 and p130, and Cre, which were delivered intratracheally into transgenic $Trp53^{flox/flox}$ $Rb1^{flox/flox}$ mice [19]. One of the advantages of CRISPR *versus* traditional techniques to produce cancer transgenic organisms is that CRISPR closely mimics the stochastic tumour formation as gene editing only occurs in a fraction of somatic cells [20]. Additionally, it can be easily adapted to other organisms, having been applied, for instance, in pigs [21]. The CRISPR/Cas9 system also served for modelling oncogenic chromosomal rearrangements found in lung cancer. This has been difficult in the past with other techniques, due to the non-physiological fusion expression and the presence of wild-type alleles. A gene fusion is a hybrid gene formed by two independent genes that come together because of a chromosomal rearrangement (*e.g.* chromosomal translocation). In this regard, one study reported the generation of the chromosomal rearrangement *EML4-ALK* (found in 5–7% of NSCLC) in adult mice using an adenoviral shuttle vector containing Cas9 and the two sgRNA targeting *EML4* and *ALK*, which was delivered through the trachea [20]. This technology has also served to elucidate the cell of origin of these tumours [22]. Another oncogenic genomic rearrangement generated in the laboratory using CRISPR/Cas9 is the *Rlf-Mycl* fusion, present in up to 7% of SCLC subtype A [23].

Identification of novel genes responsible for lung cancer onset, progression and targeted therapy success

The expression of genes can be modified with CRISPR to elucidate their putative oncogenic or tumour-suppressive role or to identify unknown genes crucial for the initiation, progression, therapy

resistance, synthetic lethal interactions, oncogenic addiction and immunotherapy efficacy in lung cancer. To this end, researchers have produced single gene knockouts or epimutants to high-throughput CRISPR screens through large-scale sgRNA synthesis and library generation. Herein, we will focus on whole genome-scale screens. Chen *et al.* [24] utilised a loss-of-function CRISPR/Cas9-induced mutagenic screen using 67 405 sgRNAs to identify novel suppressor genes of NSCLC metastasis. Genes were pulled out by deep sgRNA sequencing of the metastatic lesions. Regarding the identification of oncogenes, Han *et al.* [25] generated CRISPR/Cas9 screens in two-dimensions, three-dimensional spheroids and tumour xenografts to find novel driver genes in lung cancer, and they discovered carboxypeptidase D as an important oncotarget. Genome-wide loss-of-function CRISPR/Cas9 screens have also been deployed *in vitro* for the identification of genes responsible for drug resistance and their driver mechanisms. For example, several genes involved in resistance to epidermal growth factor receptor tyrosine inhibitors have been identified in PC-9 human lung adenocarcinoma cells and the responsible mechanism seems to be endoplasmic reticulum stress [26]. Moreover, the same type of screen has allowed the discovery of synthetic lethal interactions between two oncogenic signalling pathways in lung cancer. In particular, loss of *SHOC2*, a scaffold protein made of leucin-rich repeats that participates in the activation of mitogen-activated protein kinase (MAPK), has been shown to cooperate with MAPK kinase (MAPKK or MEK) inhibitors in the context of *KRAS*-mutated cell types such as the $KRAS^{G12C}$ lung cancer cell lines A549 and NCI-H23 [27]. Concerning oncogenic addiction, Romero *et al.* [28] discovered *solute carrier family 33 member 1* (*Slc33a1*) as a therapeutically actionable target. *Kelch-like ECH Associated-Protein 1* (*KEAP1*), a gene that harbours loss-of-function mutations in 20–30% of lung adenocarcinomas, has a strong dependency on the previous one. This finding was possible with the utilisation of a druggable genome CRISPR/Cas9 screen in *Keap1*-mutant cells. Regarding immunotherapy, with an epigenetic-focused CRISPR/Cas9 screen targeting 524 epigenetic regulators, researchers have unveiled *Asf1a*, a H3-H4 chaperone, whose loss confers sensitisation to anti-programmed death-1 (PD-1) therapy to a $Kras^{G12D}/Trp53^{-/-}$ transgenic model of lung adenocarcinoma [29].

Diagnosis of lung cancer

CRISPR technology could also serve for the early detection of lung cancer. Qiu *et al.* [30] have developed and demonstrated the utility of a CRISPR/Cas9-based test with horseradish peroxidase-based detection of let-7a miRNA in plasma which was able to discriminate between NSCLC and healthy patients with high sensitivity and single-base specificity on the miRNA detected. Another test deploying CRISPR/Cas13a coupled to an electrochemical biosensor allowed the detection of six miRNAs in plasma, and differentiated between healthy, benign lung disease and early-stage NSCLC with a sensitivity and specificity of 90% and 95.2%, respectively [31]. Regarding the detection of mutated DNA, Zhou *et al.* [32] developed a test, composed of CRISPR/12a associated with a fluorescent reporter, able to detect $KRAS^{G12C}$ in lung tissues with higher sensitivity (by 10-fold) than PCR and with high specificity.

Gene therapy for lung cancer treatment

The only CRISPR-based gene therapy for lung cancer that has been applied clinically consists of the use of engineered T cells. In 2016, Chinese scientists commenced the world's first clinical trial of CRISPR/Cas9 in patients with metastatic refractory NSCLC (ClinicalTrials.gov NCT02793856; phase I) [33]. The trial, where PD-1 was inhibited, was generally safe. The rationale of targeting PD-1 in NSCLC is that these tumours overexpress the immunosuppressive checkpoint PD-1 that confers them the ability to evade immune system-mediated detection and clearance. T-edited cells persisted for a long time in the circulation, but there was a lack of objective responses probably due to the lower editing efficiency of the methods used in the trial compared with latest editing techniques [34]. However, it is still unclear whether CRISPR-mediated PD-1 T cell knockout has greater advantages than anti-PD-1 or PD-ligand 1 (PD-L1) antibodies [33].

Future perspectives

It is envisaged that novel methodologies will be applied in conjunction with CRISPR technology to improve or complement its applicability. For instance, machine learning will be useful for the prediction of novel genetic vulnerabilities in lung cancer and for a sgRNA improved design with higher specificity and less off-target gene recognition across different lung cancer cell lines. Single-cell (sc) sequencing coupled with CRISPR technology can infer important genetic and transcriptomic changes for lung cancer progression at the single-cell resolution. Recently, researchers reconstructed the mutational metastatic voyage of thousands of *KRAS*-mutated A549 tumour cells from a primary orthotopic xenograft using CRISPR/Cas9 mutagenesis and scRNA sequencing [35]. In the future, there will be a more generalised utilisation of genome-wide high-throughput CRISPR screens generated *in vivo* for the identification of novel oncogenes and tumour suppressor genes whose alterations are responsible for lung cancer initiation and progression. We are aware of the increasing restrictions imposed on the use of animals for research, an

issue which has been politically discussed in the European parliament [36]. The deployment of animals, CRISPR technology and viruses constitute essential pillars for the advancement of cutting-edge lung cancer research. Whilst considering all the ethical aspects is important, further discussion involving stakeholders, policy-makers and their advisors is highly warranted. Moreover, the therapeutic implementation of CRISPR in the clinic to allow direct gene editing in the lungs of cancer patients will become a reality. However, such exciting approaches are hampered by the limitations of current *in vivo* delivery methods (*i.e.* adenoviruses, nanoparticles). In this regard, more efficient, selective, and less toxic delivery vehicles and materials will be developed, which will push the clinical translatability of CRISPR technology to unimaginable levels.

Conflict of interest: The authors declare that there are no conflicts of interest.

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