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CRISPR-Cas9 in Functional Genomics: Implications for Target Validation in Precision Oncology

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ABSTRACT

CRISPR-Cas9 has revolutionized functional genomics by enabling precise genome editing with unprecedented efficiency. This review focuses on the application of CRISPR-based platforms for target discovery and validation in precision oncology. Key topics include the design of genome-wide CRISPR screens, synthetic lethality identification, and CRISPR interference (CRISPRi) and activation (CRISPRa) methodologies. We highlight current case studies where CRISPR has led to the identification of novel therapeutic targets, discuss technical limitations such as off-target effects, and explore ethical considerations in clinical translation.

Keywords: Cancer Therapy, CRISPR-Cas9, Functional Genomics, Genome Editing, Precision Oncology, Synthetic Lethality

1 Introduction

FUNCTIONAL genomics is the study of genes and their interactions with the environment, often using high-throughput techniques. These technologies can generate large-scale datasets on many gene perturbations and phenotypes. However, many approaches, such as RNA interference, are unsuitable for loss-of-function screens. The emergence of bespoke networks searching for clustering tools yields results in biomedicine and genomics. Established tools have been successfully implemented and extended in different experimental and clinical settings. These network tools represent dedicated datasets. Resequencing technologies generate petabyte-sized data sets containing the particulars of genomic variations. Systems biology approaches identify the possible modifiers of existing phenotype data, which are lacking in standard high-throughput analyses. As the biological understanding of Mendelian traits improves, specific queries based on transcription networks or protein structures will be addressed [1].

On the biotechnology side, emerging technologies raise the prospects of innovative therapeutic approaches. Gene files containing individual whole-genome sequences have been generated, ready for interpretation and diagnosis of rare heritable diseases. This work has been paralleled by efforts to develop non-viral oligonucleotide-based therapies involving various approaches inspired by DNA nanotechnology basics in the spirit of functional genomics. Global databases depicting the Interactome and all mammalian transcriptomes have been produced, changes accessible across the Web, and involved multiple academic institutions and organizations. However, a better understanding of existing data remains a challenge.

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Validation of predicted cross-talks requires access to novel substrates and experimental facilities, often lacking outside the laboratory publishing the data [2].

In cancer systems biology, high-throughput sequencing technologies have recently enabled the discovery of many somatic mutations in human cancers. An example found in breast cancer exemplifies the emergence of new targets, and many of the targeted therapies involved in understanding the action mode of TGFβ in different cancers will represent enormous amounts of data waiting to be integrated. The genes contributing to tumorigenesis and the genes responding to therapy remain a blank slate. Existing networks built upon expression data and priors act as scaffolds on which new data can be mapped to characterize the acquired, endogenous, and induced synthetic lethalities of each drug, including the double deletions and pharmacophore drug discovery perspectives. These networks can also be mined by manually scoring genes' position in the network and cross-talking pathways. Findings are validated in the latest sequencing validation through various technologies, antibody microarrays, and functional assays. Analysis builds on prior knowledge and requires gene profiling approaches. The output of probes is a matrix, obtained with bioinformatic tools performing multidimensional data normalization, hierarchical culture classification, and disease/culture network [3].

2 Overview of CRISPR-Cas9 Technology

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a biological mechanism first found in bacteria that provides immunity from invading foreign phages or plasmids endowed with nucleic acids 1. Cas (CRISPR-associated) genes are a family of evolutionarily related proteins involved in this process. The CRISPR-Cas system is divided into Classes 1 and 2. Class 1 includes family 1 (Cse1 and Cse2) and family 3 (Csm1, Csm2, Csm3, Cmr1, Cmr2, and Cmr4) comprising several subunits and subcomplexes. Class 2 includes family 5 (Cas9, Cpf1, and C2c2) represented by a single protein. The Class 2 type II sgRNAs, that are short RNAs complementary to target DNAs, unite with Cas9. Its HEPN (higher eukaryotes and prokaryotes nucleotide-binding) endonuclease domain generates two nicks of the target DNA in conjunction with RuvC endonuclease activity 2. Thus, Cas9 can bind and nick its double-stranded DNA (dsDNA) target, eliciting cell repair either through Non-Homologous End Joining (NHEJ) or Homologous Directed Repair (HDR) [4].

One of the major reasons for the growing popularity of the CRISPR-Cas9 platform is that it allows creating larger fragments of edited genetic material in cells. In addition to the small fragments of altered genetic DNA load a user can design with available sensors, the editing with grafts (insertion of larger DNA) was more complicated and model-dependent. A new generation of Cas9 liposomes presenting nanoparticles approved by regulators is yet another major step forward. This could prove invaluable for a widely personalized “off-the-shelf” transfection of inexpensive high-performance vectors already in clinical use targeted to well-defined mutations. Moreover, it can substantially reduce the costs of cell transfection with CRISPR sgRNAs in future personalized studies maximizing treatment outcomes together with specificity [3].

3 Functional Genomics: Concepts and Applications

Functional genomics is the study of gene function, focusing primarily on understanding the relationship between genes, their products (usually mRNA or proteins), and the organism's phenotype. Over the years, various types of tools including genome perturbations, expression perturbations and other perturbation approaches such as chemical libraries have been developed to manipulate and study the genome. These approaches can broadly be classified into two categories, perturbation tools that alter the copy number of genes and perturbation tools that alter the expression level of genes. The latter category includes highly effective methods such as small interfering RNAs (siRNAs) or Vectors expressing short hairpin RNAs (shRNAs) that leverage the natural RISC machinery inside the cell to degrade or destabilize target mRNAs, and ORFeome-based expression cloning that utilizes viral based vectors to express cDNAs within cells [5]. However, it is worth noting that while those classical methods are powerful, they do not allow for precise and specific fine-tuned analyses. Expression cloning is typically conducted in a gain-of-function or over-expression fashion with poorly controlled expression levels, so that cDNAs often expressed at a high level raising the question of physiological relevance of observed phenotypes. On the other hand, these RNAi-based approaches have relied on technologies providing strong loss of function or knock-down effects with low residual expression levels leading to the need for careful screening design and controls being laid out [5].

Previous technological advances in genomics, particularly the sequencing of genomes and widespread RNA transcript identification and quantitation have facilitated the growth of genomics research. Yet, the full exploitation of genomics into functional systems biology has proven to be challenging. Although a majority of attempts to gain gene function knowledge are mostly indirect inference from sequence-based approaches and exercises, there is still a strong demand to globally identify and validate gene function from a phenotypic view (prospect into a "dark matter" genome). In this regard, a classical line of research through functional genomics would be to design systematic perturbation strategies to study genome-wide gene-to-phenotype relationships [1], as

shown in Table 1.

Table 1. Classical line of research through functional genomics.

Application Area	Description	Impact
Personalized Medicine	Tailoring immunotherapy based on tumor checkpoint profiles	Increases therapy success rates
Biomarker Development	Identifying predictive biomarkers for response	Helps select appropriate patients for therapy
Combination Therapies	Combining checkpoint inhibitors with chemo/radiotherapy	Overcomes resistance, enhances effectiveness
Resistance Mechanism Research	Investigating why some patients do not respond	Drives new drug development
Vaccine Development	Using knowledge of immune evasion to design cancer vaccines	Stimulates broader anti-tumor immunity

4 Role of CRISPR-Cas9 in Functional Genomics

Gene-editing technologies have dramatically changed the face of biological research, making routine tasks such as precise genome editing in bacterial, yeast, Drosophila, mouse, and cell lines possible for many labs. The most prominent and considered a revolutionary discovery in this field is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) or CRISPR-associated 9 (Cas9) technology. What makes this technology revolutionary is its ease of use, efficiency, and specificity. It is much cheaper than traditional methods, and it can be used to target all kinds of genome sequences, mutagenizing, restoring, or tagging almost any gene in any organism. By attaching an activation domain to catalytically inactive Cas9 and expressing this in cells, it is possible to stimulate the expression of a specific endogenous gene. A recent effort from two groups has generated pooled guide RNA (gRNA) libraries for coding and regulatory regions of the human, mouse, and Drosophila genomes with high coverage, thus facilitating functional phenotypic screening with CRISPR/Cas9 for the first time in mammalian cells [1, 6].

Conventional screening methods, such as cDNA/siRNA library screenings, have lacked control over the expression levels of the perturbation. It is difficult to know how effectively each checklist item is tested. Nevertheless, expression cloning based on a strong promoter has proved a useful tool to identify novel genes and validate previously discovered ones. This method detects the relevant gene only if it is overexpressed, and false negatives may be discarded if the search is based on the existence of a large-insert library. Similarly, functional genomic screening would only work if the copy number of each queried clone is either reduced or the encoded protein is lost. Notably, siRNAs targeting a gene may cause either target mRNA degradation or transient protein depletion, providing only short-lasting perturbation [7].

5 Precision Oncology: An Overview

Precision oncology strategies rely on the characterization of molecular aspects that modify cancer genesis and propagation. The identification of cancer-driving genes and gene alterations is a valuable starting point in precision oncology 4. Signal transduction alterations, induced either by the aberrancy in the compositional content of protein kinases or in downstream effectors (oncogenes), are thought to be a generalized mechanism of action for invading cancers. The drug-targeting feasibility of this class of proteins is one of the main advantages over others and arose the interest of their pharmaceutical development. Despite remarkable discoveries of new anti-cancer compounds in the past few decades, molecules targeting oncogenes remained elusive because a sequence of highly ordered microstates are embedded in the long folding pathways of large proteins and only the following limited drugs have been further developed. Other secondary alterations of tumor suppressor gene mutations, gene expression dysregulation or aberrant post-translational modifications are also noteworthy subjects for drug developments and they are theoretically more tractable and amenable for drug targeting [2].

A class of advancement statements has been made regarding CRISPR/Cas9 technology. This class of CRISPR/Cas9 innovation aligned with previous statements, is that no matter whether DNA repair could be repaired through NHEJ or HDR, gene knockout should be the best entry point to knock out gene function upon CRISPR/Cas9 introduction because the generation of frameshift of protein coding genes fell under the domain of NHEJ. Small indels were generated at target sites of protein-coding regions and after the translation, those indels were acknowledged as lack of frameshifts of the original normal sequence and would be degraded by the translational machinery. Hence it was concluded that CRISPR-induced knockouts should fulfill data requirements and medical rationale usually better than any approaches tried before [3].

Old conditions on impossible drug targets and number of disallowed patients in drug development have largely been revised with the advance of molecular scouting technologies in basic and clinical oncology. A new generation of broadly programmable CRISPR/Cas9 technology, free from undesirable treatment of premature RNA/antibodies, has begun to receive attention [8].

6 Target Validation in Precision Oncology

The tumor types selected for these studies have been

chosen based on four criteria: Precision oncology approaches for these tumor types are currently being tested in clinical trials, target validation approaches have been utilized widely; Target validation approaches would benefit greatly from the application of the CRISPR platform as revealed in Table 2 described by [9]. Due to their intrinsic tumor biology, model systems that recapitulate the tumor types of interest are available. In discussing how effectively functional genomics can be interpreted to test the promise of new targets for a precision oncology approach, it will be important to acknowledge caveats concerning the interpretation of the data as well as the model systems selected [3, 8].

Table 2. Target validation approaches: Updated insights.

Step	Description	Techniques/ Tools Used
Literature Review	Collection of studies on checkpoint inhibitors and resistance	PubMed, clinical trial databases
Patient Sample Collection	Obtaining tumor biopsies and blood samples	Biopsy techniques, venipuncture
Immunohistochemistry (IHC)	Detection of checkpoint protein expression in tumors	PD-L1 staining, CTLA-4 staining
Flow Cytometry	Analysis of immune cell populations and checkpoint markers	Flow cytometry panels for PD-1, CTLA-4
Genomic and Transcriptomic Analysis	Identification of resistance-related mutations	Whole-exome sequencing, RNA-Seq
Functional Assays	Testing T-cell activity and response to inhibitors	Cytotoxicity assays, cytokine profiling
Data Analysis	Statistical evaluation of response and resistance correlations	SPSS, R programming

7 Mechanisms of CRISPR-Cas9 Action

The active CRISPR-Cas9 nuclease targets genomic sequences of interest specifying one or more short guide RNAs. Although the type II CRISPR mechanisms of action are well understood, new CRISPR-related systems and additional functions of known systems have recently been discovered. To understand Cas9 editing, it is necessary to understand the mechanisms that allow it to identify, bind, and cleave genomic targets. Following Cas9 binding to gRNA, the gRNA-Cas9 complex interacts with double-stranded DNA through a process known as target DNA search [10]. The search occurs in two phases: an initial phase involving Cas9-gRNA independent binding. Cas9 engages in a 1D diffusion along the DNA and sometimes forming nonspecific complexes with the DNA. By using FRET imaging, it is recently revealed that suitable combinations of protein and DNA that are subject to unbinding are necessary for target searching to be rapid. Then, after becoming aware of the target PAM sequence (NGG), Cas9-gRNA shifts to transcriptional activation and direct unwinding of the target DNA [11].

Cas9 translocation along DNA is shown to occur in discrete steps corresponding to nucleoprotein complex diffusion along the DNA scaffold. After several rounds of NTP hydrolysis, the yoke closes the upper lobe and the binding is coupled to DNA bending. By matching their protospacers, the transcript-derived crRNAs and the trans-encoded tracrRNAs assemble into a crRNA-tracrRNA heteromeric RNA molecule that provides a target-recognition and trans-activation platform for the Cas9 nuclease [12]. After binding, the cr/tracr complex scans nearby double-stranded DNA searching for PAM sequences. Once a target recognition site is found, the distal end of the tracer RNA transcript undergoes a two-step conformational change that brings the Cas9 nuclease into a "ready to cleave" state. A fixed displacement between the two helical parts of the active tracrRNA is monitored by fluorescence resonance energy transfer (FRET) and is believed to trigger the expulsion of the recognition sgRNA tail thus exposing the RuvC and HNH active sites to the substrate DNA [12].

8 Designing CRISPR-Cas9 Experiments for Target Validation

The design process starts by choosing library lengths and sgRNA design criteria. Next, sgRNAs are selected and ranked based on their predicted on-target and off-target activity [6]. Typically, at least two sgRNAs per targeted gene are selected. A plasmid library is generated, constructed using BsaI-based Golden Gate assembly. Next-generation sequencing (NGS) is performed to determine sgRNA representation, which measures sgRNA library quality and preparatory transduction steps. Last, analysis of the output from high-throughput screening determines hit genes. Computational analysis deconvolutes sequencing data for genetic screening and mutational data for pooled analysis of output tissues, which identifies and ranks candidate genes. sgRNA design processes, library construction, and large-scale sequencing are critical to experimental success. High experimental variability necessitates large-scale throughput systems and robust computational analyses [13].

Several methods can generate sgRNA libraries for

CRISPR-Cas9 screening. A common library design is based on a synthetic oligonucleotide (oligo) pool and the pKLV2 vector. Oligos targeting the −275 to −12 region of selected promoter sequences is designed with a HindIII site at the 5′ end and a BsaI site at the 3′ end. A sequence encoding the hU6 promoter, sgRNA scaffold, and SV40 terminator sequence is constructed with a BsaI site at the 5′ end and EcoRI, a recognition site for the A1I restriction enzyme. Annealed oligos are cloned into the linearized vector. The sgRNA library pool is generated via BsaI double digestion, product purification, and subsequent self-ligation [13]. There are two methods for selecting high-quality sgRNAs for experiments. The random biological sgRNA pool is returned in a −1.5 to +1.5 scale R. If the scale is larger than +1.5, the sgRNA with the lowest R score is iteratively removed until no sgRNAs exceed a +1.5 score. A second R score is calculated. If this scale is less than −1.5, a random biological sgRNA close GGCAMGC is returned. If its scale exceeds +1.5, all sgRNAs with a +1.5 to +2.0 are returned in the order of |R| value [14].

9 Case Studies in Precision Oncology Using CRISPR-Cas9

There are two kinds of the libraries used: One is custom-designed, focusing on biologically relevant oncogenes and targets for drug resistance; the other is open access library, and the newest library is already available.

To illustrate the usefulness of CRISPR-Cas9/ORF library combination, several case studies are briefly presented, focusing on the steps for validating a previously uncharacterized p53 target, a new resistance mechanism of BIBF1120 on targeting all three forms of mutant RAS, and target validation of the novel bispecific antibody trifunctional IgG3 against mesothelin, which is a good target for MPM therapy [1] as seen in Table 3. Considering the space limit, these cases highlighted an early application of CRISPR-Cas9 and high-throughput gain-of-function screening in the study of MEK inhibitor resistance, which is the most rewarding work on target validation for precision oncology and is full of useful information of practical value [13].

In these cases, other useful functions of CRISPR-Cas9 are also presented: For example, before embarking on a project needing library construction, researchers may want to test whether certain genes are amendable to CRISPR-mediated knockout [15]. For this purpose, a simple software for guide RNA design is available, which allows the construction of a CRISPR toolkit of Good Laboratory Practice standard, enabling similar functional studies in other labs [6]. Some effective expression vectors of Cas9/sgRNA are also available. A simple manual is provided to facilitate the cloning and validation of small sgRNA plasmids or libraries [16]. Validated sgRNA-expressing plasmids can be easily pooled in vitro for human cell transfection or virus packaging and further application for screening high-throughput libraries or target validation. It is hoped that people from the same exciting field can eventually test even the most challenging and exciting hypotheses for practical biological and medical significance.

Table 3. Designing CRISPR-Cas9 experiments for target validation.

Checkpoint Target	Clinical Response Rate	Common Resistance Mechanisms	Key Findings
PD-1/PD-L1	20–40%	Loss of antigen presentation, T-cell exclusion	PD-L1 expression correlates with better response
CTLA-4	10–20%	Upregulation of alternative checkpoints (e.g., TIM-3)	Combination therapy improves outcomes
Combined PD-1 + CTLA-4	40–60%	Immune-related adverse events, adaptive resistance	Higher efficacy, higher toxicity
Checkpoint Target	Clinical Response Rate	Common Resistance Mechanisms	Key Findings

10 Challenges in CRISPR-Cas9 Target Validation

The CRISPR-Cas9 technology allows multiplexed targeting of genomic loci and is a powerful tool for screening in functional genomics. CRISPR screens have much in common with their RNAi predecessors but many technical differences, such as vehicle preparations, clonal isolation of cells, and sequencing. A simple workflow schematic is presented as a ready metric for new users. Instead of focusing on one or two “star genes”, broad and unbiased genome-wide screens can be performed. Off-target burden and toxicity in lentiviral and short hairpin schemes need to be weighed against specificities and genome coverage in gRNA design [6, 17]. Cells can be screened in the pool or as clonal pops but extraction of quality data may be more effortful using bottlenecked pools. New software is needed to analyze, visualize and filter out noise from spurious hits. Exhaustive analyses of datasets with several rounds of filtering allow for biological insights that are not apparent at first [18].

285,000 gRNA and 160,000 compound unique spectrum numbers offer unprecedented coverage in time frames log10 smaller than “big” modelling infrastructures, which interrogate merely one cancer type. Over 10,000 gRNA are assembled prior to any engagement to arrays or beads resulting in a 100-140 billion query screen [19]. Most smaller gRNA libraries contain only 60,000–90,000 gRNA,

while CRISPR libraries span ~200,000 gRNA, covering in theory ~90% of the human genome. It should however be borne in mind that gRNA settings generate oligomeric species that hybridize to up 10 separate captured targets. This relatively slow and wasteful polymerase chain reaction is solved by multiplexing 15 or more species in separate tubes [6].

Compound library design and screening considerations are still being evaluated. Bank size, batch-wise solubility, polyfunctionality, and activity validation of compounds are issues. Initial tests involve pure compound read outs but intermolecular interactions with cell constituents and early-on toxicity can significantly heighten compound screening times and costs [20].

11 Ethical Considerations in CRISPR Research

Rapid development of innovative gene-editing tools has opened the door for a new era of precision medicine in cancer treatment. Based on methods manipulating the RNA-guided endonuclease history of nature, these tools allow correcting mutations, knocking out tumor drivers, and creating DNA double-strand breaks at pre-selected genomic sites in diverse cell types. Early preclinical studies improved safety and efficiency of gene delivery, while current works focus on disrupting or repairing mutated oncogenes in patient-derived tumor models, developing genetic susceptibility gene screening methods, and assessing of long-term consequences and off-target effects [21].

Phenomenon-based genome-scale functional genomic studies have been developed utilizing CRISPR/Cas9 platforms to establish and improve gene libraries for gene knockout, library fabrication protocols, CRISPR system optimization for genome engineering, and deep sequencing and analysis pipelines for mutation-based screening deconvolution. Active research has been ongoing to unravel cancer vulnerabilities revealed by gain-of-function mutations in tumor suppressors, aberrant dependencies resulted from genome alteration and structural variants, dormant oncogenic mutation perturbations collaborative activation strategies, and function-based CRISPR/Cas9 CAMP models [17].

Two main types of CRISPR/Cas9 systems have been engineered as exciting CRISPR/Cas8, Cas12, and Cas13 proteins have been developed to overcome the restriction issues imposed by differing genome editing characteristics of Cas9 orthologs. Simple and efficient CRISPR/Cas9 systems have enabled the broad application at the subgenomic level of genome editing and up-and downstream functional genomic studies. Active works have been ongoing to improve specificity and delivery methods of CRISPR/Cas9 systems. Applications of CRISPR/Cas9 in functional genomic studies in precision oncology include discovering actionable cancer genomic alterations and tumor vulnerabilities in currently target-untaggable genes. As engineering, detection, and therapeutic tools in precision medicine, CRISPR/Cas9 systems hold great promise and hope for valuating new drug targets, tailoring treatment plans, and monitoring treatment response in real-time [22].

12 Regulatory Framework for CRISPR Applications

The past 3 years have brought the CRISPR rEvolution to small molecules. A transformative way to identify and validate small molecule targets for drug development would empower biologists and chemists to collaboratively devise new therapies with fewer side effects, and advance precision medicine to the next level. With a plethora of luminous new tools for target discovery and validation ready for application, it is now up to biomedical investigators to take the next steps needed to bring new biological marvels into rigorous laboratory tests that probe new biology and transform genomics broadly. Basic biologists, genome editors, and biochemists are uniquely poised to commendably fill the ensuing gaps in supporting the development of small molecules that will be instrumental in winning the global war on diseases [23].

The vast functional genomic potential of CRISPR was immediately evident, given its unique features that distinguish follow on technologies. CRISPR has the potential to launch another comprehensive era of genome-wide exploration of biology leading to transformative small molecules. Bayesian modeling will embrace the wealth of single cell measurement-of-effects data of all inputs, intermediates, and outputs from perturbation to phenotype to more comprehensively interpret biological outcomes in relation to their input targets. Outputs can be mined for exploitable predicted vulnerabilities as part of drug discovery projects [17]. The generation of relevant-weather target discovery platforms needs to be addressed in parallel with the corresponding discovery chemical flood prep in order to capitalize on the exponential interest and funding being directed to evolving CRISPR technologies [24].

13 Future Directions in CRISPR-Cas9 Research

The CRISPR-Cas9 system, a widely-used tool for editing genes and gene regulation, comes with much sophistication. The target is first identified by a guide RNA, then the double stranded DNA is cleaved by the Cas9 protein. Newer tools have adapted the CRISPR-Cas9 system for genome-wide screens 8. These screens perform phenotypic selection and quantification of sequence-modifying CRISPR-Cas9 plasmids, RNA guides, and proteins on a library wide scale. The libraries yield new target pathways and proteins of interest for downstream validation [6]. Existing combinatorial genomic libraries can be leveraged in conjunction with CRISPR-Cas9 screening and other higher-throughput methods for target selection and detection. Various simplified methods are available to ease the screening for target genes that mediate phenotypic

modulation [19]. Non-target-associated control systems can be made available to further validate target perturbation and compound activity. Target imaging methods scout multiple assays to evaluate the success of phenotypic modulation. More forgiving selection designs enable further integration of target screening in new high-throughput assays [25]. The premise for precision oncology is to identify and select targets based on their functional role in cancer. Potential approaches to take full advantage of CRISPR-Cas9 capabilities include a greater understanding of the regulatory genetic architecture of cancers, larger perspectives of gene functions in complex settings, more focus on therapeutic target engagements in vivo, improved quantifications of targets and their modulation factors, and narrower selections of targets effects around key factors [26].

14 Comparative Analysis with Other Genome Editing Technologies

Though the power of CRISPR-Cas9 editing has made it the dominant form of genome editing, alternative techniques continue to be developed in parallel [22]. Furthermore, multiplexed 'pan-genome' strategies are being developed to take the fight to cancer when no obvious targets are open to small molecule inhibitors or antibody therapies [27]. A common entry point to the relatively new CRISPR-Cas9 world depends on the simple promoterless Cas9 transfection into cells engineered to express the requisite guide RNAs via widely available systems [28]. In this 'broad strokes'-initial approach to CRISPR-Cas9 powered phenotype screening, on- and off-targeting rate and specificity, sequencing of off-targeting regions, length of repair fragments in day-by-day passages or clonal analysis of cultivated cells were diminishingly explored in the earliest reports. Given the possibility of single nucleotide insertions or deletions with such blunt ends, gene targeting—generation of specific modifications at the genomic locus of interest relying on homologous recombination of an ectopic DNA template—was not expected. The homologous donor was hoped to be integrated via natural NHEJ. Hence, heterotypic donor templating with long homology arms was thought to splice gene fragments into target genes. Confirmation of genetically engineered targets relies upon sequence coverage or RT-PCR of the spliced regions if functional validation assays are permitted [29]. Currently, there are many different delivery methods of CRISPR/Cas9. There are two short RNA molecules used in the CRISPR/Cas9 system: the CRISPR RNA and trans-activating crRNA. The crRNA and tracRNA are assembled together or combined with synthetic single-guide RNA to target all types of mammalian genes [11]. Under the guidance of crRNA and tracRNA, the Cas9 protein is able to bind to the target DNA of the gene of interest. Then, the binding will lead to the formation of the DNA–Cas9 complex. There are also many methods for ensuring that the dox–Cas9 gene is turned on in both endogenous and exogenous constructs. Overall, there are many methods with different efficiencies to deliver CRISPR/Cas9 systems [30].

15 Integration of CRISPR-Cas9 in Clinical Trials

Over the last two decades, CRISPR as a genome-editing tool has emerged as a fast, cost-effective, and precise method to probe gene function and generate animal models. More recently, the CRISPR-Cas9 system has been integrated into live human cell cultures. Encapsulation of Cas proteins, along with the protospacer-adjacent motif (PAM) recognizing crRNA sequences, into purified bacterial vesicles with lipid-based transfection agents allowed for the replication of cell culture experiments in vivo [23]. While initial discoveries of PglC proteins and polysialic acid capsules were made in the bacterium N. meningitidis using CRISPR-Cas9 genetics, mediated delivery of CRISPR substrates paves the way for assessing the functions of these virulence factors in mouse models or via the targeting of other protein substrates [30].

One company claims a critical role in the acute transient silencing of the mutant huntingtin protein in a neurodegenerative disease model [31]. In addition to these medically and biotechnically relevant applications, methods for applying CRISPR-Cas9 to create new genome-harboring genetic circuits in bacteria, yeast, and eukaryotic cell cultures have transcended basic research in several ways. Nanowire-producing microbes could be exploited to make new nanowire-based biosensors in in vivo bioreactors [22]. Even the possibility of the large-scale production of complex cells with overt biotechnological applications should be of concern to regulators and ethicists. Therefore, approaches that anticipate and mitigate dangers with adverse consequences are desirable [32].

16 Patient-Centric Approaches in Precision Oncology

Probing cancer genomes: possibilities, pitfalls, and perspectives CRISPR-based genome-scale approaches urge a revision of the model of cancer biology, which assumes genetic differences are a main driving force for tumorigenesis, in an age of personalized medicine. The genetic basis of cancer pathology is encoded in the DNA sequence. Elliptic point mutations, large-scale structural variants, and epigenetic changes are examples of DNA mutations affecting tumor cells' growth, survival, and drug resistance capabilities. Deep sequencing of whole-exome and whole-genome tumor samples has identified >3 million mutations in common cancers, pointing out an extensive genotypic diversity among tumors [33]. This diversity saturates unspecific drug targets such as signal transduction pathways. Drug development must adapt to the rapidly growing, highly diverse knowledge of cancer epigenomes. Recent approaches developed the first

generations of genome-scale selectable systems.

Genome-scale CRISPR-Cas9 libraries enable the targeted introduction of insertion/deletion mutations at more than ~85% of human coding exons. Such libraries can saturate genomic target states in a single lentiviral transduction step. Robust selection conditions allow for targeted profiling early drug targets. CRISPR-based libraries for activation and inhibition of human gene expression provide alternative approaches to target agnostic screening for druggable targets [17].

CRISPR-Cas9s are envisioned as a fast system with greater enzymatic efficiency, simplicity, and versatility for genome editing. Advances in guide RNA design, delivery, and online prediction models permit the custom generation of rigorously tested gRNAs aimed at a variety of target genes, ranging from a few single-gene to whole-genome libraries. Rapid declines in the price of massively parallel deep sequencing, bioinformatics, and computing power allow the parallel screening of a virtually limitless number of gene candidates and reagents [27].

17 Bioinformatics Tools for CRISPR Data Analysis

CRISPR/Cas system, most advanced genomic engineering and functional genomics tool, is composed of RNA molecules along with a Cas protein. Cas proteins have an intrinsic nuclease activity for programmable genome editing and can modify DNA at specific sites, which is used for a host of applications involving gene editing, transcriptome control, epigenome manipulation [23]. In order to achieve a target function on genomic loci using CRISPR/Cas system, it is important to introduce a specific single-guide RNA (sgRNA) precisely, which can be obtained by designing a short target specific sequence, along with an appropriate Cas protein [6].

For DESeq2-based analysis of CRISPR screen dataset, sgRNA-to-gene mapping, CRISPR/Cas9 design, sgRNA plasmid assembly and expression prediction ranked by various sequence features can be called using user defined sgRNA candidates or characterized on well-known sgRNAs in the databases. It is user-friendly, can build R outputs for post-screen analysis, and is one of the most advanced software for CRISPR data analysis [34]. The development of small Csy4 proteins for CRISPR-Cas 9 and Cas12a multiplexed detection of RNA viruses, including SARS-CoV2, is reviewed. The methodologies for multiplexed RNA targeting, single-fusion probe sensing, and transistor-upregulated signal amplification in the detection of RNA viruses are discussed [35].

CRISPR/Cas9-based adenoviral vectors to efficiently knock down target genes in various human or murine cancer cell lines, which provide an effective method for the regulation of gene expression in cancer cell line models and for further discovery of desired anticancer targets are reported. Potential limitations of CRISPR/Cas9 system are also discussed [27]. CRISPR 3D is software that combines CRISPR screening data, transcriptomic profiles, and drug sensitivity data. It provides deeper exploration of the signature genes and mechanisms among many small molecules in cancers and systematic strategies for the discovery of drug combinations [36].

18 Collaboration Between Academia and Industry

International collaboration in the field of bioengineering has resulted in many successes [37]. Many top bioengineering institutions work together on topics such as genomics and biotechnology to ensure that success spreads worldwide. Cultured meat has many problems to solve, and these collaborations between research institutions and universities around the world may mean that those problems can be solved quicker. Emerging fields like gene editing take time to build the academic support to have an impact, and the globalized knowledge sharing means it is less likely that one society outgrows another and the gap continues to grow [38]. Academia and industry collaboration is a commonplace method of innovation. Many long-standing research institutions funded by taxpayers allow industry access to researchers and their skillsets [37]. The funding allows researchers to have equipment and hire staff that would otherwise be unobtainable, and industry are able to develop better products and services. Academic and industrial collaboration is expected, and academics are then expected to seek funding to further their research and progress their field [39]. Companies that dedicate themselves to providing applications of university-provided research typically to allow success [40]. Collaborations between disciplines have been common since scientific study began. Funders commonly require funds to be shared between disciplines for the grant to be considered. There are therefore many reasons why researchers have elected to consult researchers with vastly different backgrounds on how to build a synchronous lab or overcome obstacles to effective research [41].

19 Impact of CRISPR-Cas9 on Cancer Therapeutics

The application of CRISPR/Cas9 in the battle against cancer begins with the identification of realistic targets, development of tools for in vivo delivery, and establishment of efficient systems for monitoring the results of genetic editing beyond simple segregation [27]. Genetic screens are important experimental strategies for the systematic investigation of many biological processes governing basic functions of cells or tissues, as well a pathological situation such as cancer [17]. High-throughput technologies validate clues revealed by bioinformatics of cancer genomics, but need innovative approaches to generate and maintain the cells of interest [12]. For this, upon witnessing the modularity and flexibility of CRISPR/Cas9 technology for rapid engineering of robust knockout (KO) cells tailored to specific molecular

mechanisms of interest, genetic screens in human cells using the CRISPR-Cas9 system were first termed CRISPR screens [17]. CRISPR screens can comprise either a pooled or tiled format. While the former focuses on KO of many genes significantly reducing the fitness of cancer cells, the latter evaluates the impact of a wide range of single agent treatments [19]. Genome-scale CRISPR dropout screens in human myeloma cells were conducted to identify genetic vulnerabilities and therapeutic targets [42]. Examining the effects of modulation of the formation of the Myc–Mnt/MAX heterocomplex on proliferation and transcription in growth factor-sufficient primary human NSCLC-derived cell lines using dCas9-KRAB. CRISPR-mediated frigging of Myo3B induces an adaptative response conferring a strong proliferative and migratory advantage in melanoma by down regulating the LMTK3 pathway [42]. C6 cells moded by CRISPR/Cas9 knock out PCBP2, and the results were validated biologically [12]. CRISPR screens were used to identify factors controlling sensitivity to the multiple myeloma drug thalidomide (42). PSMC6, a protein within the 26S proteasome in charge of unfolding ubiquitinated substrates, is highly expressed in cancer cells, thus making the proteasome a target for the development of anti-cancer drugs [17].

20 Patient Outcomes and CRISPR-Cas9 Applications

Since the discovery of oncogenes in the 1970s, functional genomic approaches enabling genome-wide identification of cancer genes in mammalian systems have been actively pursued [43]. The goal of this research pathway is to accurately identify cancer genes and associated therapeutic vulnerabilities in individual patients. The process involves a multi-step pathway leading to target identification and pre-clinical evaluation in relevant models. However, significant knowledge gaps exist, particularly in step processes toward patient outcome, preventing the rapid clinical deployment of target validation [44].

The imperfect nature of cancer provides chemical or genetic compounds that selectively restrict certain processes or cell sub-types. “Genome mapping” experiments interrogate the cancer gene hypotheses or offend cellular process, identifying vulnerabilities/interaction partners. Patient cases gleaned from the above research pathway have been subjected to multi-method, multi-dimensional, multi-time frame molecular profiling to map the network structure of candidate regulatory genes. The input to the “drug discovery” pathway is a candidate gene expansion with the power to broadly identify carcinogenic and therapeutic liabilities [45]. Regulatory approval of the targeted agent paving the way for implementation of clinical use.

21 Limitations of Current Research

The implications of harnessing CRISPR-Cas9 for functional genomic studies, specifically in the area of target validation in precision oncology are noteworthy. It allows an exploration of various promising areas of research while reiterating skepticism at the heart of its promise. As a guiding principle, the possibility of efficient target validation in precision oncology through the development of relevant mouse models would ensure a proliferation of the corresponding research. In a special issue devoted to ‘CRISPR-Cas9 in functional genomics’, article has focused on approaches that utilize CRISPR-Cas9 in an implementation phase while indicating relevant examples from both academic and industrial settings [24]. Although each of these representative examples demanded significant resources and commitment, they are feasible for health institutes dedicated to high-impact cancer research [46].

The well-characterized MOSE cells were utilized with a pooled CRISPR-Cas9 library in a tissue recombination model of ovarian cancer to identify genes that affect response to chemotherapy [47].

22 Funding and Resource Allocation in CRISPR Research

CRISPR continues to revolutionize biology and biomedicine through its diverse application in simplifying and accelerating gene editing, gene resequencing, transcriptome imaging, and transcription regulation. Over a few years, it has become an indispensable tool for empowering academia and industry research on genomics with wide-open application prospects in biomedicine, crop breeding, and microbiology. The open-access nature of CRISPR has led to growing hands-free applications ranging from self-editing bacterial genomes to ophthalmic treatments and agricultural engineering. However, such a rapid advancement of a new technology poses threats and risks that require implications on research funding and technical approaches [48].

As a fundamentally new technology, funding should focus on education, training, and recruiting not only fresh researchers but also senior researchers from non-CRISPR fields, especially in diagnostics, therapeutics, crops, and breeding, but also on R&D investments for tackling the new insight challenges presented by the CRISPR systemITDs. Finally, sponsors should accept honest projects and reports regardless of the outcomes to avoid a cycle of excessive optimism, research disinterest, or disaster denials [49].

23 Public Perception of CRISPR Technology

Public perception of genome editing is likely to influence its future use, just as public perception of biotechnology did a generation ago [50]. CRISPR technology is often characterized in the media as “cutting-edge” genome engineering, offering “precise” editing. Such descriptions

typically focus on anticipated benefits, with less attention to the limits of and risks associated with the technology, and hence the potential for unintended consequences [51]. Consequently, public perception of genome editing is fraught with emotion-laden comparisons to previous raises of biotechnological hope, and will likely shift as the benefits, limits, and risks of CRISPR technologies become clearer. Careful public engagement about CRISPRs is essential, but difficult. Decision makers may wonder if it is worth the time and effort to engage. Would any level of public information and engagement change the outcome of open versus restricted use [52]. Concerns about genomic inequality may worry some, while others may be inspired by the possibility of eradicating genetic disorders or creating designer babies [53]. Global biotechnology companies are already investing as much as $100 million in genome editing startups. At the same time, projects to create CRISPR-edited humans are underway across three continents [54]. Given earlier problems, it seems likely that some entrepreneurs will take regrettable actions that present the world's genome editors with stark choices. Containment efforts are already devising yet more approaches to editing, while knowledge-sharing initiatives aspire to present pressing bioethical, safety, and security concerns prior to public decisions on genome editing capabilities [55].

24 Training and Education in CRISPR Techniques

The rapid emergence of CRISPR technologies presents unique challenges for teaching and learning. In addition to genome engineering, CRISPR technologies can be used for various other applications such as multiplexing, epigenetic regulation, transcriptional regulation, protein localization and imaging, and the creation of biosensors [56]. Due to the fast-paced nature of CRISPR research, it is essential to teach students about the greater applicability of CRISPR technology beyond just genome engineering and to provide discussions on how new CRISPR-related technologies are developed. CRISPR-Cas9 genome engineering may not be easily applicable for all diverse organisms useful for class instruction and overall biology research. Therefore, other CRISPR technologies beyond Cas9-based genome editing should be incorporated into course materials to teach students about the expansiveness of potential tools that can generate, destroy, or regulate genetic information. The CRISPR Club is a collaborative group-based online resource forum designed to share practical methods for building a CRISPR community on campus, guiding course instruction on CRISPR-Cas9 technology, offering advanced multimedia materials, and posting new questions and projects as they arise [57]. It is open to anyone worldwide who is interested in developing their CRISPR-Cas9 instructional materials or wants to explore the increasing relevance of CRISPR-Cas9 in the classroom and outreach settings. The aim was to develop a CRISPR instructional webinar and work in groups to formally produce course materials, protocols, laboratory modules, and outreach resources [58].

25 International Perspectives on CRISPR Regulation

The CRISPR (clustered regularly interspaced short palindromic repeat) is a bacterial immune system that protects the organism from exogenous genetic elements. Since the first realization in 2013 that this mechanism could be harnessed in a versatility manner for genome editing in nearly any organism, it has triggered an avalanche of research applications in both the biological sciences as well as in biotechnology, agriculture, and medicine [59]. Depending on the component proteins and crRNA (CRISPR RNA)-tracrRNA (trans-activating CRISPR RNA) that are used, CRISPR technology enables the editing or modulation of DNA, RNA, and epigenetic signatures [56].

CRISPR-Cas9 technology and derived CRISPR-based tools are a rapidly evolving field, whose continued development holds significant potential in research and clinical applications. However, relevant global standards are currently lacking, as are guidelines for the governance of CRISPR-Cas9 technology [23].

The importance of both its innovation output and number two position in terms of concern in CRISPR-Cas9 research are self-evident 8. Moreover, the robustness of its estimate makes it somewhat immune to potential confounds, such as differences in language. Nevertheless, further examination of that concern would certainly be warranted for more nuanced understandings of non-institution-based concerns [60] (Figure 1).

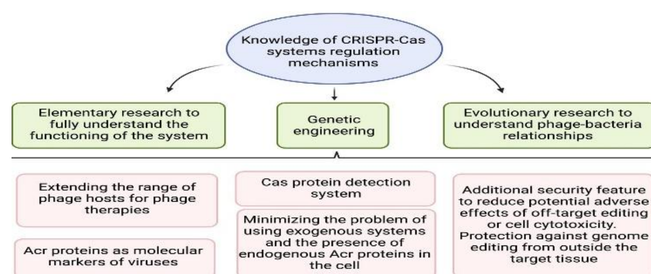

Fig. 1. International perspectives on CRISPR regulation (61).

26 Conclusion

Genome Editing Approaches with CRISPR/Cas9 for Cancer Treatment: Critical Appraisal of Preclinical and Clinical Utility, Challenges, and Future Research.

As for the appraised studies, AAV6-CRISPR/Cas9 delivery efficiently knocked out mutant HRAS in human tumors while reducing tumor growth in preclinical xenograft models. Targeting mutant K-Ras with LNP-encapsulated gRNA and Cas9 mRNA was proposed to increase preclinical anti-tumor activity by selecting the population of tumors shedding KRAS mutation copies in blood. Self-replicating RNA technologies assembling Cas9

and gRNA were studied for treating mutant HRAS and K-Ras, knocking down the target allele and decreasing growth in xenografts. Research assessing AAV8-delivered dual gRNA and spike protein modification noted the immunogenicity of Cas9 is a big challenge. Concomitant inhibition of histone deacetylases and diamond shows the capability to induce and enhance CRISPR cleavage rates. However, disease-triggered splicing could also present an elaborated way to target regions of the genome using an exogenous gRNA, Cas9 protein, or mRNA. Nevertheless, there are still some hurdles for CRISPR/Cas9 approaches to the clinic for cancer treatment and tackle malignancies such as solid tumors 16. Overall, wide demonstrations in vitro and in vivo indicate CRISPR/Cas9 approaches could ameliorate cancer therapeutic efficiency and safety. New approaches to nanosized bioconjugate delivery, PEM nanoparticle engines for a wide delivery concept, newly designed gRNA/Cas9 bioconjugates, advanced imaging, or sensing methods could improve current limitations.

Additionally, clinical translation studies on CRISPR/Cas9 cancer therapy are in progress, enabling CRISPR/Cas9-based approaches for personalized anti-tumor applications. In terms of safe designs or bio-successful delivery methods and comprehensive in vivo breeding, new-generation strategies and drugs might bring a New World into real-world cancer therapy. New CRISPR/Cas9 designs emerged due to off-target effects. The human genome is a wide genetic field for this engineering tool, and collateral damage can occur when applied for a specific goal. Researchers invested a great deal of effort in narrowing down these unwanted side effects. They partially ablated the nuclease activity to achieve higher specificity; however, this requires additional gRNA. Researchers envisioned a dead nuclease Cas9 that can prevent transcription of the target genes by blocking transcription factors. To augment the inhibition effect of dead nuclease Cas9, different peptides have been fused to block the binding sites of transcription factors. An elegant approach to accomplish tumor-specific gene inactivation explored unique genomic rearrangements in the tumor genome, resulting in distinct gene fusions. The breakpoint of such fusions can be used to design gRNA that targets the fusion point of the gene, proving efficient for specific gene knockout in tumor cells.

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