CRISPR-Based Gene Editing for Personalized Cancer Therapy: Next-Generation Precision Medicine

Editors

Sihaab Haider Radhi Muhaisn
Department of Biology College of Science, University of Babylon, Iraq

Hassan Falah Mohammed Hassan AbdAli
Department of Biology, College of Science, University of Babylon, Iraq

Tuqa Abdullah Fatehy Abdul Kareem
Department of Biotechnology, College of Applied Sciences, University of Fallujah, Iraq

Ola Muhand Kamal Mustafa
Department of Biology, College of Science, University of Babylon, Iraq

Zainab Kazem Faleh Ziyara
Department of Biology, College of Science, University of Kirkuk, Iraq

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Abstract

Emerging CRISPR-engineered elements are proposed for biological editing of a patient's tumor genes by creating a customized therapeutic product as the next logical step toward precision medicine. Based on the genetic profile of the malignant cells, alterations are introduced to target specific genes—in oncogenes, tumor suppressors, or drug-resistance mediators—using patient-derived pluripotent cells or organoid cultures. Backbone elements are then assembled to produce a biodistributed editing cocktail targeting altered genes. Such a response would be propelled by a comprehensive reverse transcriptomic analysis of the tumor. By fully editing the malignant cells of a patient, therapeutic geodesic pathways can be determined as a molecular compass for correcting mutations. [1][2][3][4]

The next-generation CRISPR invention that labels guanine–cytosine-rich DNA regions will enhance treatment efficacy by directly recognizing the edited regions. The information from the patient's organoid and natural embryonic activities will speed the inventing process and design custom-made CRISPR Arcus TβR editing agents for individual patients. Adult-derived basal-layer SC of the skin or hair follicles, or even mesenchymal SC of the bone marrow adapted to pluripotent properties, would be the initial edited cell sources. The forward approach of using CRISPR-related editing elements in a reverse direction to pitch the amino acids of each tumor at once would pioneer a collective mature product with a potential geo-course.

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Chapter - 1

The New Era of Precision Medicine in Cancer

Standard treatments for cancer comprise surgery, chemotherapy, and radiation therapy, used independently or in combination. These approaches are efficient for a subset of patients but have several limitations: acting on the whole tumor mass with lack of specificity, undergoing activation of protective pathways leading to treatment resistance, generating systemic toxicity, and neglecting cancer heterogeneity. These shortcomings have prompted the search for genomic-based therapies that target specific vulnerabilities within tumors, ideally at the level of the driver mutations responsible for oncogenic transformation.

The differentiation between somatic and germline mutations now allows the patient genome to be used to determine personalized treatment strategies, and massive parallel sequencing has led to the identification of thousands of tumor samples paired with matched normal tissues. Notably, the development of CRISPR/Cas systems for gene editing has provided a universal tool to experimentally modify genes in model systems or cell lines generated from patients to discover the underlying mechanisms of tumor development and progression, identify potential therapeutic strategies, and reintroduce modified genes back in patients or animal models. [5][6][7]

1.1 Historical perspective of cancer therapy

The history of cancer treatment dates back to antiquity, with the earliest evidence appearing in ancient Egypt and Gr eece.

Regardless of the weapon used, either surgery or a drug, none had a satisfactory outcome. The distant promise of salvation came from miraculous implementations of the doctrine of signatures, which became a paradoxical veil, helping some agents to survive the unmerciful tests of real medicine. However, it was not until the mid-nineteenth century that a radical change in the approach to cancer occurred. The pioneering work of John Hunter established the principles of surgical oncology, while William Halsted, by introducing radical mastectomy, realized the first effective treatment for breast cancer. The other three classical methods (radiotherapy, chemotherapy, and hormone therapy) became available toward the end of the nineteenth century and, for a time, offered hope for most patients with limited disease.

These strategies were, however, developed in a pre-genomic era, before the discovery of the causes and nature of cancer, the detection of DNA lesions or the recognition of the heterogeneity that accompanied hit-and-run transitions from normal cells to neoplasms. Indeed, when, in the 1990s, President Clinton declared that the United States had mapped the language of God, one important aspect was still missing: a precise understanding of cancer and a means to identify individuals suffering from early disease in whom curative treatments could be administered. The limitation of chemotherapy, radiotherapy, hormone therapy and surgery is that they are not cancer-specific. Therefore, some patients with limited disease can be treated with these conventional methods, but almost all die with the disease because they eventually develop distant metastases with chemotherapy-refractory tumor cells. The emergence of molecularly guided therapy is an incremental change that followed the availability of new technologies, undoubtedly one of the most important being DNA sequencing, allowing discovery of the human genome and providing a rich treasure of data for a new concept of personalized medicine. [8][9][10]

1.2 Limitations of conventional treatments

Cancer cells undergo clonal propagation characterized by genetic and epigenetic variations, rendering tumor populations both biologically and therapeutically heterogeneous. Increasing evidence indicates that at the single-cell level gene expression changes caused by copy number variations or mutations in transcription factors affect tumor cell behavior and significantly influence drug-response phenotypes. Drug treatments, when effective, ultimately lead to acquired resistance mediated by distinct processes, emphasizing the need to understand tumor biology and derive therapies accordingly. Conventional therapies lack the resolution required to design personalized and effective treatment strategies and suffer from limitations: they can have non-specific or adverse side effects, activate a stimulus-response mechanism leading to drug resistance, and afflict the patients with severe side effects damaging healthy cells and reducing their quality of life. Achieving on-target effect as part of a treatment regimen further improves the prognosis but not without the usual toxic side effects, such as acceleration of neurodegenerative processes as observed in HeLa and AD transgenic mouse models when treated with tamoxifen.

Interest in genomic data sequences and expression profiles has surged and now guides therapy decisions, enabling the identification of unique druggable mutations for patients. The development of next-generation sequencing and the TCGA initiative led to many new platforms analyzing a tumor in less time than it takes to produce chemotherapy or radiotherapy predictions. A number of approaches analyzing signal transduction maps have been proposed, allowing the repositioning of drugs for patients based on mutation profiles, e.g., using the NCI Drug Response Database and pharmaco-genomic data bases. Data analysis has now moved toward directed searches for more than one target in proteins involved in

controlling cancer in order to limit heterogeneity and achieve better outcomes. [11][12][13]

1.3 Emergence of genomics and molecular profiling

The introduction of genomics and the development of molecular profiling technologies generated massive amounts of genomic information. Recent advances in sequencing technologies have led to a significant reduction in cost and time. Producing more than 600 genomes—including the human genome—has enabled routine sequencing for cancer patients. These resourced allow patient-specific mutation prospecting to help choose possible targeted therapies. Moreover, sequencing data from cancer patients have tolerated the identification of prognostic and predictive mutations, genes, and transcripts that can be used to assess therapy outcomes. Other high-throughput technologies such as transcript profiling have resulted in databases of RNA expression and methylation status that enable patients to be classified into different cancer subtypes and groups predicted to respond to specific therapies. These technologies permit a more integrated understanding of cancer biology—a necessary step for personalized medicine.

Different approaches can be used to guide therapy decisions based on patient-specific data, and one of the most common is monitoring for actionable mutations. In addition to associations with drug sensitivity and resistance, mutations and molecular alterations can indicate whether a patient is likely to benefit from a particular therapy. When a specific alteration occurs in a tumor, one of the activated routes is to perform a tumor biopsy test to accurately analyze the potential mutation correlating with drug response. When a targetable mutation is present, it is a recommended practice to test for available FDA-approved corresponding therapeutics for the tumor. [14][15][16]

1.4 Principles of personalized medicine

Personalized medicine refers to the medical model that tailors treatment to the individual characteristics, needs, and preferences of each patient. Such an approach considers specific patient information, including somatic and/or germline mutations, expression or modification profiles, and clinical characterization, to collectively guide decision-making. By integrating individual clinical, cellular, and molecular data from patients, caregivers can recommend therapies that best suit each individual, enabling biomarker-driven and therapeutic options. Ultimately, personalized medicine aims to improve treatment response while minimizing the risk of complications.

Because cancer is essentially a genetic disease, the unique molecular landscape of an individual tumor likely provides the best opportunity for therapeutic benefit. Therefore, specialized use of CRISPR-based gene-editing technologies holds great potential to affect the cancer genome as a whole through ex vivo or in vivo genome editing of cancer-associated somatic or germline alterations. CRISPR approaches that directly correct cancer driver mutations, re-activate lost tumor suppressor genes, and collectively targeting mutant oncogenes are conceptually appealing in providing a truly personalized therapy that may ultimately enhance treatment responses and increase patient survival. [17][18][19]

1.5 Role of gene editing in precision oncology

Personalized cancer therapy promises to evolve by leveraging each patient's genetic variability data for specific intervention. The plethora of detected mutations—of both tumor and germline origins—enables the identification of causative "actionable" genes, which may be required for tumor development and progression yet are absent from normal somatic cells. Parallel advances in CRISPR-based editing systems

support the functional validation of such genetic variants, making it possible to catalog genes whose modulation alters tumor behavior. These techniques, additionally, may directly target oncogenes or restore wild-type alleles into tumor-suppressor genes, outfitting clinicians with a newly formed toolbox for precision oncology. Whether it be by correcting mutations in native genes or by modifying off-target responses to immunotherapy, CRISPR may optimize therapy on a patient-by-patient basis. But long-term success requires the outgrowth of adequate mouse models to establish proof-of-concept and trial results validate in a human setting.

Despite the advances in modeling tumor mutational landscapes, the role of CRISPR in personalized therapy remains limited. Current applications lack the resolution needed to incorporate the myriad tumor-intrinsic factors that drive cancer toward a therapy-resistant state. To a large extent, however, these aspects may not be essential for decision-making; in vivo and in vitro screening efforts provide the requisite scale to delineate synthetic lethal interactions, while the pathways that modulate resistance to checkpoint blockade may be further scrutinized in relevant tumor microenvironments. [20][21][22]

Chapter - 2

Understanding the Genetic Landscape of Cancer

Cancer is a heterogeneous disease driven by an accumulation of somatic mutations that result in uncontrolled cell proliferation. These mutations can be classified as oncogenic or epigenetic alterations, and they may be detected through next-generation sequencing techniques. Consortia such as The Cancer Genome Atlas and COSMIC provide the genomic data needed to characterize various cancer types, and machine learning is increasingly being used to predict the function of cancer mutations. At present, the range of CRISPR–Cas9 applications in cancer research is broad, and the technology holds potential for both basic research and translational studies. CRISPR may be used to define the range of oncogenic mutations that drive tumorigenesis, identify novel cancer genes, determine the functions of tumor mutations, uncover synthetic lethal interactions, and examine the role of the tumor microenvironment—and the stroma in particular—in immune evasion. In the future, patient-specific organoids and other tumor avatars may permit the testing of personalized therapies.

Material alterations driving cancer are broadly classified into germline and somatic mutations. Germline alterations are monogenic mutations typically detected in tumor suppressor genes. Inherited defects increase susceptibility to developing cancer; however, they are estimated to cause <5% of all cancer cases. By contrast, somatic mutations arise from the accumulation of genomic alterations during cell division.

Various types of somatic alterations in key cancer genes are to be expected—from point mutations to small insertions, deletions, and even complex chromosomal rearrangements—and these alterations are critical at all stages of tumor progression, including initiation, malignant conversion, and metastasis. [23][24][25][26]

2.1 Somatic vs. germline mutations

Mutations driving oncogenesis are broadly classified into germline and somatic alterations. Germline mutations that affect the genome of the germ cells and are inherited by offspring comprise only 5-10% of all tumorigenic mutations. Genomic alterations found in the tumor tissues, but not in the healthy cells of the respective patient, are collectively referred to as somatic mutations. Precise distinction is critical for therapeutic decision-making. Mutations that are present throughout the body are also present in germline tissues and can be reactivated in tumors. Selection of targeted or personalized therapy requires knowledge of the actual mutation profile in the tumor and its differences with the germline genome. In this context, restriction-enzyme-based methods are used to interrogate the tumor for somatic copy-number alterations, indels, or point mutations. Detection of point mutations can be performed in tumor tissues or biological fluids using PCR or Sanger sequencing. Methylation-sensing PCR or sequencing assays are used to confirm presence in tumor tissue and absence in the corresponding normal sample. Methylation-based point mutations may also be detected in biological fluids.

Germline alterations are particularly important when selecting individuals for preventive strategies or when interpreting the impact of novel somatic mutations in recurrent or treatment-resistant tumors less than five years after initial therapy. Individuals with pathogenic germline mutations have an increased lifetime risk of certain cancers and can be directed

either toward visit with a clinical geneticist or germline testing. Detection of somatic mutations drives biomarker-based therapy initiation or continuation for individual patients and further validation of candidate biomarkers in other patients or cohorts. Germline testing can also be conducted routinely in parallel with tumor tissue analysis. [25][23][27]

2.2 Oncogenes and tumor suppressor genes

Somatic mutations in the cancer genome can arise in three ways: by disrupting oncogenes, by inactivating tumor suppressor genes, or by causing epigenetic alterations. Oncogenes refer to mutated forms of normal genes (proto-oncogenes) that usually promote cell growth and division. Their normal function is therefore in a pathway for cell proliferation, and mutations that activate these genes can lead to tumorigenesis. In the human genome, more than 140 such genes have been identified. The vast majority of these oncogenes are in the signal transduction pathways that are mediated by receptor tyrosine kinases like ERBB2, KRAS, and the phosphatidylinositol 3-kinase pathway or are involved in transcriptional regulation of these pathways. The following five overviewed oncogenes have been remarkably enriched in cancers, particularly lung cancer.

Tumor suppressor genes are the opposite of oncogenes. Tumor suppressor genes are defined as genes whose normal function is to inhibit cell growth and division, and disabling one of them will promote the emergence of a tumor. According to the Knudson two-hit model, for a tumor to arise, both alleles must be inactivated due to either point mutations, deletions, or epigenetic changes since the remaining allele has lost any protective action. Most of the well-characterized tumor suppressor genes, including TP53, RB1, and APC, control the cell cycle or are involved in DNA damage repair and maintenance of the genome integrity. Thus, any alteration of these genes can lead to genomic instability

and finally to tumorigenesis. More than 30 tumor suppressor genes are known to be mutated in human cancers, although for only a small number have both the mutational signature and the function been established. [28][29][30][31]

2.3 Epigenetic alterations in tumor progression

In addition to genetic alterations, epigenetic changes promote tumor progression. Epigenetic modifications modulate gene expression without altering DNA sequence. The main mechanisms that regulate epigenetic marks are: 1) DNA methylation (DNA methyltransferase enzymes), 2) histone modifications (covalent bonding of biochemical groups—acetyl, methyl, phosphate, ubiquitin—to DNA-associated histone proteins); and 3) chromatin remodeling (chromatin-associated proteins modifying DNA and histone structure). These marks integrate internal/external stimuli, dynamically regulating tumor-suppressor and oncogene expression. In tumors, DNA methyltransferases often exhibit aberrant regulation, with related tumor-suppressor gene silencing. These different epigenetic changes cooperate in tumor initiation and progression. Moreover, testing for recurrent global DNA methylation changes aids diagnosis. The emergence of an appropriate methylation-detection technology at the level of circulating cell-free DNA (cfDNA) supports the establishment of an early, noninvasive blood test for cancer diagnosis. Methylation changes observed in cancer are not merely a reflection of tumor burden; specific patterns arise linked to tumor type and stage.

An evolving area of cancer research focuses on tumor epigenetics and the role of epigenetic factors in defining canonical genetic networks in tumor and other disease types, as well as stem-cell maintenance. Epigenetic alterations mediate transcriptional regulation in cancers displaying distinct manifestations of chromatin remodeling. The components of the

epigenetic machinery, including proteins involved in histone modifications, DNA methylation, and chromatin remodeling, have been demonstrated to display aberrant global expression or distribution patterns in cancer cells. Recent work has begun to reveal the interplay between the epigenetic landscape and the transcriptome and how this is altered in several cancer types. [32][33][34][33][35][34][32]

2.4 Cancer genome projects and mutation databases

International consortia such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have undertaken large-scale projects to profile the genomic alterations in numerous cancer types and subtypes. Their data, together with those from other related initiatives, provide an invaluable resource for the identification of driver mutations, epigenetic signatures, actionable biomarkers, and other cancer-associated alterations across multiple cancer types. The Cancer Gene Census—a curated list of genes with somatic mutations demonstrated to drive human cancer—has been integrated with individual-level variant data from the Genomic Data Commons to facilitate the identification of actionable mutations for further functional investigation and potential novel therapeutic targeting.

In a complementary effort, the COSMIC database houses extensive mutation data in common cancer types. Furthermore, several independent teams of researchers are developing tools to predict the effects of somatic mutations in cancer genomes. By combining extensive annotations of known cancer-associated alterations with a large collection of mutation-disease associations, these resources hold great promise for functional prioritization of mutations in individual cancer patients and other MALAT1-expressing tumors. Cancer genome projects and expression databases hold particular importance for the

discovery of actionable mutations and the construction of patient-specific organoid models. The Cancer Genome Atlas and other publicly available databases such as the cBioPortal or COSMIC can be interrogated for information on actionable mutations of interest, accessibility of individual-level mutation data for experimental validation, and associations of gene expression with tumor recurrences or metastasis formation.

2.5 Identifying actionable genetic targets

Clinically actionable mutations include alterations in oncogenes, tumor suppressor genes, and other mutations that contribute to immune evasion and resistance to targeted therapies. Efforts to categorize these mutations according to whether they are targetable in patients and validate their functional relevance are underway. However, developing therapies that exploit them remains a longer-term goal.

Cancer treatments are currently guided by external patient observation, but by using advanced and personalized genetic tools, internal factors can be targeted, resulting in more efficacious treatment with fewer side effects. One of the more elaborate methods for further enhancements involves detecting the changes in human genetics that lead to cancer progression and acting on them. Initially, the developed mutations are analyzed to determine whether they may be targetable by existing or potential therapeutic strategies. Functional validation of whether these selected mutations indeed support tumorigenesis within the context of a specific cancer type follows. The key prioritization criteria for a mutation being deemed actionable are that either (i) it is a key mutation in a central signaling condition of the tumor type or (ii) it is present in a small subset of patients but has immediate therapeutic implications. Conservation of the mutation across species is considered an internal indicator of potential targetability, as are known drug interactions of non-

human homologs. Such knowledge accelerates investigation into the effects of newly reported mutations. [36][37][38]

Chapter - 3

Gene Editing Technologies before CRISPR

Restriction Enzymes and Recombinant DNA: A fundamental breakthrough in biotechnology came in 1970, when the first restriction endonuclease was characterized. These enzymes recognize and cleave DNA at specific short sequences. They are found in bacteria and are thought to serve a defensive role by destroying incoming foreign genetic material. The founders of molecular cloning and DNA manipulation technology came to realize that DNA could be prepared with sticky ends, joined together by DNA ligase, and used as a substrate for restriction enzymes, enabling the analysis of particular genes. The method for isolating specific genes from any source, allowing the transfer of any gene into a bacterial host for amplification or expression, and providing for controlled mutagenesis of any gene was referred to as recombinant DNA technology.

Zinc Finger Nucleases: Zinc finger nucleases (ZFNs) were the first sequence-specific endonucleases to be customized by fusing a multi-finger-containing transcription factor to the non-specific nuclease domain of the Fok I endonuclease. The multi-finger recognition domain provides sequence specificity by recognizing set groups of 3 bases. ZFNs have been designed to target several genes in human cells and model organisms, including mice, rats, fish, and plants. ZFNs have been successfully used to produce transgenic animals and knockout cells but suffered from low efficiency. Nevertheless, ZFNs are being used for gene correction in primary cells obtained from

Coffin-Lowry syndrome patients, an ultra-rare disease caused by a point mutation in the RSK2 gene.

Transcription Activator-Like Effector Nucleases: TALENs (transcription activator-like effector nucleases) are hybrid nucleases based on the cleavage domain of the FokI endonuclease and the DNA binding proteins of the plant pathogen Xanthomonas bacteria. The repetitive structure of Tal effector genes allows for modular design: the binding specificity can be rapidly and easily customized by assembling a TALEN pair where each repeat specifically recognizes one nucleotide. Like ZFNs, TALEN pairs induce double-strand breaks (DSBs) at a specific site in the DNA that can be repaired by either non-homologous end joining or homology-directed repair. TALENs have been used to edit genomic sequences in a wide range of organisms, such as yeast, worms, zebrafish, frogs, mice, and plants, but they typically require labor-intensive and time-consuming custom constructions for each target site. [39][40][41][42]

3.1 Restriction enzymes and recombinant DNA

Gene-editing technologies emerged long before the CRISPR revolution. For several decades, attempts to alter a genome were largely limited to adding or removing genes at a cellular level. Early efforts centred on viral insertion and transposon-based scrambling and repair. More precise systems for making targeted double-strand breaks (DSBs) expanded the toolbox. Origins date back to the discovery of bacterial restriction enzymes, which enable gene editing in vitro and, when combined with recombinant DNA techniques, led to the first synthetic gene constructs. Later development of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), assembled from natural recognition motifs, improved targeting flexibility. These innovations have since been complemented and largely superseded by CRISPR-Cas systems. Although off-target activity and delivery barriers remain concerns, the existing

capabilities and near-term prospects for establishing ZFNs and TALENs in clinical settings warrant a concise review.

Prominent examples of naturally occurring restriction enzymes include those from the Escherichia coli K-12 strain, which express RecBCD (a helicase-nuclease complex and major DNA repair pathway), an ATP-dependent type I (Cse4; with cleavage requiring a three-subunit complex) and a type II (EcoRI; cleavage by a single polypeptide and non-requirement of ATP). Recognizing owner sequences as targets for protection, EcoRI cleaves DNA to create blunt ends for its cognate methyltransferase and 5′-overhangs for T4 ligase. In recombinant DNA methods, a linear but unprotected target can be prepared by digestion with EcoRI, ligation with a suitable 5′-single-stranded extension and introduction into a RecBCD-deficient host. Restriction-directed cloning permits the identification of a gene of interest using Z primers, insertion of an expression control sequence and introduction of unique EcoRI targets. [43][44][45][46]

3.2 Zinc finger nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are the first class of engineered nucleases entered the gene editing field. The zinc-finger domain is a naturally occurring protein motif that binds to DNA in a sequence-specific manner, and its promoter orientations and DNA-cleaving activity can be engineered. ZFNs have been widely adopted for genomic modification in various organisms, and they have shown promise for therapeutic uses in large animals and even humans. Moreover, the zinc finger protein arrays have multiple advantages in DNA recognition, such as flexible PFMs and the high number of potential protein/CSS combinations. Their design strategy has been successfully expanded to other DNA-binding modules targeting Cas9.

ZFNs proposed a simple modular format for engineering custom transcription factors and then for engineering custom DNA-cleaving enzymes capable of introducing DSBs at any specific site in the genome. A catalytic subset of the ZFN architecture is composed of a left-handed zinc-finger DNA-binding domain fused to the nonspecific DNA-cleavage domain of the FokI and other type IIS restriction endonuclease family. ZFNs are heterodimeric enzymes that bind their unique recognition sequences on opposite sides of a cutting site in a head-to-head orientation, each recognizing proximal half sites (with 3-nt overhangs). Their cleavage mechanism is that dimerization of the catalytic domains brings the two nonspecific cleavage machineries into proximity to generate a DSB.

ZFNs have been used to create gene modifications in a great variety of eukaryotic organisms, including Drosophila melanogaster, Xenopus laevis, Caenorhabditis elegans, Danio rerio, Mus musculus, Macaca mulatta, Sus scrofa and human cells. The cells were simply microinjected with preformed purified ZFN protein pairs, ZFN RNA transcripts, or ZFN-expressing plasmids and, in some cases, Transcriptional Activator-Like Effector Nuclease (TALEN) proteins. However, the potential and broad application of ZFNs came to for Gene Targeting in Humans, as compared to ZFs, one major limitation in the development of ZFNs remains the requirement for providing two integrated or two independent PFMs that allow the assembly of the ZFN heterodimer at the current target site. [47][48][49][50]

3.3 Transcription activator-like effector nucleases (TALENs)

Developed shortly after ZFNs, TALENs are another class of programmable DNA-strand cleavage agents. Initially employed in plants, these nucleases have subsequently been established for mammalian cell use. The elementary structure of TALENs is

based on E. coli AvrBs3 protein, a type III transcription factor for the xanthomonas bacteria. A monomeric and DNA-binding domain of AvrBs3 (TAL) binds a 14- to 50-bp target sequence. A hallmark of TAL proteins is the presence of tandem repeats, ranging from 1 to >30, so that each repeat unit recognizes a single nucleotide base. The sequence of a TAL protein repeat unit therefore provides an easy code for tailoring these proteins for any given target DNA.

The TAL repeat-array is fused to the nuclear localization sequences and provided in a suitable plasmid backbone. Yet just like ZFNs, TALENs also require two individual TAL monomers that cleave two targeting DNA strands and create double-strand breaks (DSBs). TALENs are less toxic than ZFNs, yet that could likely be due to the fact that they act as endogenous nuclear programmable factors of transcriptional activation and repression. However, the patent holders of these nucleases are the University of California and both Virginia and Sangamo, working together with Dow Chemicals.

Comparative analysis of genome-editing systems indicates that TALENs feature greater targeting efficiency than ZFNs but less efficiency than CRISPR-Cas9. As for spuriously assembled TALENs, they are less toxic by virtue of lower expression levels. Delivery is, however, a challenge for TALENs, for they share the same upper vector-size limit of AAVs and other viral vectors. Such deficiencies can be managed through the enhanced Vpr system of lentivirus, although doing so in spatio-temporal tissue-specific manners remains an open question. Despite these limitations, TALENs have initiated fundamental advances in genome-editing technology. [48][51][49][47]

3.4 Homing endonucleases and meganucleases

Homing endonucleases target very concise, asymmetric sequences (12-40 bp) within a larger palindromic repeat found in

pre-mRNA. These enzymes achieve mammalian cellular entry and induce double-strand breaks, with their corresponding DNA repair pathways ultimately leading to knock-ins, deletions, or rearrangements. Meganucleases are related proteins that have a higher naturally occurring recognition sequence. The lack of suitable targeting tools hinders their use in mammalian nuclei, but fusions to ZFNs and TALENs have been reported.

Owing to their intrinsic properties, homing endonucleases and meganucleases exhibit significant promise for gene repair applications. The short recognition sites allow for the editing of both alleles simultaneously without increasing the risk of off-target cleavage. Despite being large in size, these proteins can be co-delivered with ZFNs, TALENs, and CRISPR-Cas9 systems. However, the lack of natural homologous repair donor templates prevents gene knock-ins and limits applications. The use of splicing templates should provide the necessary length for duplexing of the two strands. [47][52][53][54]

3.5 Limitations of pre-CRISPR systems

The utility of ZFNs and TALENs has been constrained by custom design requirements for every target site. Although pre-CRISPR technologies have enabled precise editing of specific loci, generalizable solutions remained elusive, limiting their application breadth and accessibility. Transcription activator-like effector nucleases (TALENs) partially overcame this issue, relying on assembly from readily available modules that recognize single bases in the DNA helix. However, their commoditization was hindered by incomplete binding-arm assembly algorithms, the need for donor plasmids during TALEN synthesis, and lack of options for adapting them to other organisms.

Despite their distinct architectures, ZFNs and TALENs shared the same basic strengths and weaknesses. Both

technologies—straightforward design facilitated by auxiliary components outside the ssDNA binding domains, generalizable engineering tools, and the NHEJ repair pathway—were improved. Nevertheless, both ZFNs and TALENs remained limited by challenging design and assembly processes, relatively long development times, size constraints, compilation difficulties for complete target sets, and delivery barriers. [48][47][51][55]

Chapter - 4

CRISPR-Cas Systems: Discovery and Mechanisms

4.1 Origins in bacterial adaptive immunity: Trace discovery and natural roles

In 1987, Yoshizumi Ishino and colleagues discovered the first Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in Escherichia coli. Their later research revealed that CRISPR elements are transcribed into long precursor RNAs not directly involved in protein synthesis. In 1993, three independent publications by Francisco Mojica and coworkers, Alexander Jansen, and others characterized these sequences as a CRISPR system and proposed a preliminary model. The CRISPR-associated gene cas was identified in synteny with the CRISPR region of many bacterial genomes. In 2005, a novel RNA-mediated mechanism of adaptive immunity was demonstrated, revealing that RNA provides target specificity through complementary base pairing with genomic DNA and that Cas9 cleaves double-stranded DNA in bacteria.

CRISPR systems represent a unique form of adaptive immunity that confers protection against phage or plasmid infections upon subsequent exposures. Following the acquisition of foreign DNA sequences, small CRISPR RNAs and protein effectors form a ribonucleoprotein complex to induce recognition and degradation of homologous DNA. Various Cas proteins have been implicated in all stages, including adaptation, crRNA maturation, interference, and transposase-like activity. Type IV and type VI CRISPR-Cas systems do not utilize a detectable Cas

protein for interference. The wide diversity of CRISPR-Cas systems reflects distinct evolutionary paths and cognate partners. These discoveries have contributed to our understanding of eukaryotic immune systems and the development of RNA-targeted CRISPR-Cas systems.

4.2 Structure and function of Cas9 and Cas12: Detail domains, PAM requirements, cleavage patterns.

Cas9 and Cas12 are characterized by a bipartite organization, with a C-terminal recognition lobe containing three α-helices, a central lobe that lies at the interface between the recognition and catalytic lobes, and a N-terminal lobe encompassing the RuvC and HNH domains. Cas9 proteins recognize a short phosphorothioate-linked RNA in the target DNA and cleave it upon attachment. X-ray crystalline studies indicate that the target DNA is positioned within the DNA-binding cleft for cleavage. Cas12 also cleaves dsDNA in a PAM-dependent manner that differs from that of Cas9. Cas12 contains two separated RNA and DNA cleavage sites, enabling thioester bond formation and unannealing of the target RNA strand before bubble opening.

Cas9 and Cas12 exhibit PAM requirements differing from those of Cas13 and RNA-targeting CRISPR systems. The PAM and tsPAM, which consists of three nucleotides and terminates the ssDNA cleavage reaction, ensure the specificity of recognition by targeting DNAs. These elements also reveal the evolutionary relationships among the components of the CRISPR-Cas system in eukaryotes and identify targets linked to genetic disorders. [56][57][58][59]

4.1 Origins in bacterial adaptive immunity

Discovery of the adaptive immune system in bacteria, which relies on clustered regularly interspaced short palindromic repeats (CRISPRs) and associated proteins (Cas), sparked genome editing in diverse species and organisms. Most early

applications utilized Class 2 Type II systems, harboring a unique RNA-guided endonuclease (Cas9). RNA-guided target recognition restricts the cleavage site to a specific neighboring location, the protospacer adjacent motif (PAM), enabling multiplex and single-nucleotide editing for diverse uses. Other types and classes of CRISPR-associated proteins are now applied for RNA targeting, transcriptional control, and various biosensing purposes. Natural functions of native systems, including anti-viral defense, plasmid acquisition, and genomic remodeling, continue to aid biotechnology development. Recent concepts align CRISPR with synthetic biology, support the CRISPR-transcriptome project, and explore association between CRISPR function and species.

As in all life, prokaryotes must defend against viruses and adapt to changing environmental conditions. Bacteria and archaea accomplish these tasks with an astounding array of weapons, including restriction-modification systems, toxin-antitoxin systems, and CRISPR-associated bye-bye systems. These systems rely on the presence of protein and RNA mixtures that recognize sequences from genetic elements under the context of a specific cleavage linkage. These mixotrophic sequences or sequences in the form of nucleotide pairs support the sequence-specific recognition by CRISPR-associated RNA molecules and the catalytic activities of the CRISPR-associated protein complexes. Their expected advanced functions, usages, and production separability of the of CRISPR-A-biased CRISPR-Cas assemblance combine with the naturally seen engineering domains in biology and the predictions of more flexible smart synthetic biology to produce CRISPR management-oriented advanced formats for experimental biological organizations during the expression stage.

4.2 Structure and function of Cas9 and Cas12

Cas9 and Cas12 nucleases are the most widely employed CRISPR-associated proteins for DNA editing. The crRNA-containing subunit of Cas9 is evolutionary conserved in large CRISPR-Cas Type II complexes. Cas9 recognizes a 20-nt target sequence preceded by an NGG PAM sequence on the complementary DNA strand, and utilizes a helicase domain and RuvC- and HNH-like nuclease domains to cleave both strands. Cas12 is present in type V CRISPR systems, and is modularly arranged and essential for CRISPR RNA processing. Cas12 recognizes a 20-28 nt target site with a PAM of the consensus sequence TTTV and, upon binding, cleaves the target strand in trans. Cas9 displays various PAM specificities, and PAM recognition can be engineered to extend the scope of editing. Cas9 variants with altered PAM specificities, reduced off-target potential, or improved HDR activity can be generated by engineering key residues in the nucleobase-recognition pocket and domains mediating or influencing PAM interaction.

Ribonucleoprotein complexes of Cas9/Cas12 and guide RNA are generally delivered into target cells for editing, as NHEJ repair of DSBs can introduce small indels in large fractions of cells following cleavage. The native activity of Cas9/Cas12 can be exploited to generate transgenic animals, model specific mutations, dissect cancer-associated genes, inactivate viruses, detect genetic mutations, or edit RNA. TOs are generated by simultaneous cleavage of different genomic loci. CRISPR-based rewiring of chromatin activity can upregulate genes of interest in mammalian cells and in vivo. Digenome-seq enables identification of genome-wide cleavage sites of Cas9/Cas12, and genome-wide scan for CRISPR primary-targeting affinity combined with bar-coded NGS readout can assess guide RNA efficiency. Other RNPs, such as catalytically inactive Cas9, AsCpf1, and AsCpf1-Csm, can serve as versatile in vivo gene-manipulation tools in mice. [60][61][62]

4.3 sgRNA design and target recognition

Guide RNA Design and Target Recognition

Target recognition relies on the guide RNA (gRNA), which consists of a 16–22 nucleotide sequence complementary to the target and longer flanking sequences that ensure stable binding to the CRISPR-associated protein 1 (Cas9). The gRNA forms critical Watson–Crick base pairs with the target and generates a sequence-specific R-loop ~20 nucleotides downstream of the protospacer adjacent motif (PAM); however, Cas9 must also accommodate the surrounding sequence elements. A fixed domain composed of the first four nucleotides of the gRNA and its 3′ flanking sequence forms adjacent contacts with the recognition helix of the HNH nuclease, and the length of this domain controls target recognition.

The ~25-nt region beyond the target (the so-called non-target-strand or T-strand) base pairs with the non-target DNA strand and positions its first two nucleotides, which, along with several nucleotides of the adjacent PAM, stabilize interactions with the HNH nuclease active site in bind-and-cleave reactions. Site specificity is determined primarily by the first 12–15 nucleotides of the gRNA by Watson–Crick base pairing with the target DNA. From a structural perspective, this corresponds to the length required to satisfy three helix–base–stacking interactions with bases located on the back side of the DNA ladder.

Specificity can be improved by using hybrid gRNAs, where the first 7–15 nucleotides of the gRNA are designed to contain mismatched base pairs that confer lower affinity toward a larger reference library of off-target sequences. However, in these cases, reduced rates of on-target cleavage are also observed. Additional methods to avoid the versatility of gRNA design include the use of timed Cas9 degradation and dCas9 degradation. [63][64][65]

4.4 DNA cleavage and repair pathways (NHEJ, HDR)

DNA cleavage by CRISPR is a rapid process that almost always generates two blunt ends. When these ends are repaired by non-homologous end joining (NHEJ), deletion of a few nucleotides is the most common outcome. Such small deletions, often seen at the target site in CRISPR-knockout systems, have been ascribed to the inherent imprecision of NHEJ, but are more accurately viewed as the default result following blunt-end ligation by DNA ligase. Other types of NHEJ repair events are less frequent than short deletions, yet are sometimes the focus of investigation. Chromosomal rearrangements such as inversions and translocations have been documented in high-throughput-droplets and in natural settings. Such phenomena can be harnessed for functional studies but represent a significant danger in therapeutic contexts, especially as delivery strategies improve.

Error-free repair of blunt DSBs can occur through homology-directed repair (HDR) if a suitable template is provided. In many applications of CRISPR, however, delivery must rely solely on NHEJ. This restriction stems primarily from the donor template typically being encoded on the same nucleic acid molecule as the sgRNA and Cas9/Cas12, hence co-delivered as a single-stranded RNA or as a single-stranded or double-stranded DNA molecule. Hence the desire both to repair a gene of interest and to replace a mutation therein would require a more complex system in which two sgRNAs and two DNA donors are co-delivered, a procedure that is at present most feasibly accomplished through viral transduction. Despite low order-of-addition flexibility, HDR can be used to achieve an editing outcome with a wide functional scope, as a variety of biological molecules can be provided as donor templates. Proof-of-principle experiments underscore the importance of rapid evolution and the expanding focus of CRISPR research. [66][67][68][69]

4.5 Expanding CRISPR toolbox: Cas13 and RNA targeting

RNA-targeting CRISPR-Cas technologies, based on the Cas13 protein family, address biological questions distinct from DNA editing. RNA is a central intermediary in the expression of genes, and its transient nature enables multiple regulatory functions. Inducible RNA editing allows target modulation and restoration in either direction. In addition to editing functions, CRISPR-Cas systems can also be harnessed for detection, for example by coupling to a signal-activating amplifier, such as an isothermal amplification mechanism. Nucleic-acid-based RNA detection with CRISPR has led to the development of packaging-efficient testing kits that can reliably detect viral RNA, such as SARS-CoV-2.

Direct RNA targeting is a promising technique in cancer therapy, since modulation of oncogenic transcripts might lead to more scalable results compared with DNA genome digestion. Moreover, as RNA exhibits shorter half-lives, modifying instead of inactivating the targets might overcome potential undesirable effects of a CRISPR agent. This methodology is especially relevant in the context of the discovery of small non-coding transcripts (<200 nucleotides): microRNAs (miRNAs), small interfering RNAs (siRNAs) and small nucleolar RNAs (snoRNAs). These species are single-stranded transcripts that play important roles in tumorigenesis. Using a CRISPRi- or CRISPRa-based system, one can directly manipulate gene expression by repressing or activating target promoters, using a modified dCas9 fused to Krüppel-associated box (KRAB) or VP64, respectively. CRISPR-dCas9 libraries targeting the human and mouse genomes have been generated and used to identify potential cancer oncogenes or tumor-suppressor candidates. [70][71][72][73]

Chapter - 5

CRISPR Applications in Cancer Research

CRISPR Applications in Cancer Research

Gene knockout for functional studies: Describe loss-of-function analyses and interpretation. CRISPR interference (CRISPRi) and activation (CRISPRa): Distinguish repression/activation strategies. Genome-wide CRISPR screens for cancer genes: Outline design, readouts, and target prioritization. Modeling tumor mutations in cells and animals: Discuss systems, relevance, and translational value. Synthetic lethality mapping using CRISPR: Explain concepts and therapeutic potential.

CRISPR technology has advanced cancer research by enabling targeted gene disruptions and providing tools for a range of loss-of-function analyses. Imbalances in oncogenes, tumor suppressor genes, and proteins governing pathways crucial for tumor initiation and progression, including immune response, metabolism, and repair of DNA damage, are now widely manipulated for validation studies. In addition, screening a large set of targeted genes for phenotypic changes is common. Two different editing methods, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), repress or activate single gene expression without altering the genomic sequences, and are powerful techniques for dissecting gene functions. Generating precise mutations found in tumor patients helps establish the pathogenetic role of tumor-specific genomic alterations, while modeling the interplay between tumor and stroma components promotes a better understanding of the tumor microenvironment

and can help find novel targets. Using synthetic lethality as a guiding principle, directing a second genetic perturbation into a gene network that is already perturbed through an oncogenic lesion is a new way to achieve selective cell death in cancer types with specific mutations.

CRISPR is making loss-of-function analyses faster, cheaper, and easier than traditional RNA interference systems. Genomic libraries can be generated for loss-of-function or gain-of-function genome-wide screens for functional studies. sgRNA can be designed and cloned into a lentiviral vector quickly and easily, and used to knock down genes or gene families synergistically. Furthermore, sgRNA can be introduced into cells as a synthetic oligonucleotide in the form of ribonucleoprotein complexes with Cas9 or as a transcription unit in a vector to form a CRISPRi system for stable silencing. The power of CRISPR is highlighted by defining the role of RB1 in a small-cell lung cancer model system. [74][75][76][77]

5.1 Gene knockout for functional studies

The ability to introduce targeted genome modifications has transformed studies of gene function in the context of cancer and other diseases. A common application of CRISPR-Cas9 and other genome editing technologies is the removal or inactivation of a sequence of interest—in other words, gene knockout. The genetically perturbed-cell population can then be compared to wild-type cells, revealing details about the function of the target gene and its contribution to disease biology. Studying such loss-of-function phenotypes provides valuable information about gene function, mechanistic insights that may aid therapeutic development, and potential targets for gene-therapy approaches.

Gene knockout screens enable loss-of-function analyses in an unbiased manner. By designing a library of single-guide RNAs (sgRNAs) that target a large number of genes, delivering this

library to cells, and then determining the relative frequencies of different sgRNAs before and after subjecting those cells to a particular treatment or selection pressure, researchers can identify genes that are required for a specific process. Such screens can be conducted in an array of cell types, and combined with next-generation sequencing, facilitate an unprecedented discovery of cancer genes, drug-resistance genes, and synthetic-lethality targets. Importantly, these investigations are now being performed beyond cell culture, extending into organisms, as well as human tissues and clinical samples. [78][79]

5.2 CRISPR interference (CRISPRi) and activation (CRISPRa)

CRISPR technologies not only allow gene knockouts but also enable tempering of gene expression via two complementary methods known as CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa). These approaches rely on deactivated Cas9 (dCas9) or dCas12a, which lack cleaving function but retain DNA-binding ability. Gene expression can be diminished through recruitment of transcriptional repressors or chromatin-condensing factors to promoter or regulatory regions. In contrast, transcriptional activators can be brought to promoters in a similar manner to promote gene expression. The general concepts of dCas9 function, the methods and reagents used to implement CRISPRi and CRISPRa, and some applications in cancer research are outlined below.

CRISPRi systems are all built on the dCas9 platform, whereas CRISPRa utilizes dCas9 or dCas12a. CRISPRi typically employs dCas9 fused to transcriptional repressors and is commonly combined with engineered guide RNAs that are catalytically disabled but can still recruit RNA polymerase II. These dCas9 × RTU RNA polymerase complexes can adequately stifle expression at almost any gene except those that are

extremely highly expressed, such as those in histone gene clusters. In practice, CRISPRi can efficiently silence MYC, which has a central role in tumorigenesis but is not targetable via genomic knockout. [80][81][82]

5.3 Genome-wide CRISPR screens for cancer genes

Genome-wide CRISPR screens for cancer genes deploy a two-step experimental framework to identify genes whose loss alters cellular phenotypes in expected ways. The first step applies genome-scale CRISPR loss-of-function screens to a diverse array of cellular models and conditions, producing collections of gene-phenotype associations. The second step is a meta-analysis that leverages these associations to prioritize candidate genes for further validation in a specific experimental context.

The supressor screens identify genes whose loss yields an enhanced cellular response to cancer therapeutics, while the enhancer screens pinpoint genes whose loss confers resistance. These data sets provide genetic insight into the actions of therapeutics targeting DNA damage and repair, epigenetic regulation, proteostasis, the NRF2 pathway, and translation, and they point to additional dependencies that can be exploited to enhance cytotoxic responses.

Genome-scale CRISPR screens are a powerful approach for identifying genes whose disruption alters cellular phenotypes. Such screens employ a pool of sgRNAs targeting essentially the entire genome, and they typically follow a two-step design. In the first step, the pooled sgRNA library is introduced into a well-replicated set of cellular models and/or treatment conditions, and cells are allowed to proliferate for a defined number of generations. Pheno-typi-cally relevant gene-phenotype associations are then learned from these data using machine learning, network inference, or a combination of principles. In the second step, these association sets can subsequently be

applied to a meta-analysis of discrete functionally focused knockdown, knockout, or expression perturbations, reducing the search space for a particular class of phenotypic effect to a manageable number for follow-up. [83][84][85][86]

5.4 Modeling tumor mutations in cells and animals

Tumor mutations can be modeled in somatic cells, including primary cell cultures, immortalized lines, and iPSCs, as well as in animal models. Engineered loss- and gain-of-function systems in cell lines help delineate the function of candidate mutations and their relevance in specific contexts. Preclinical assessment in xenograft models or genetically engineered mouse models supports further clinical evaluation. Insertion of point mutations in growth-deregulated murine fibroblasts provides a fantastic platform for probing the functional contribution of activating RAS mutations.

Fluorescence-based detection enables CRISPR-based cell surgery. A specific protospacer-adjacent motif context or the messenger RNA condition ensures high efficiency of spike-in random mutation. The versatility of the technique is shown by generating libraries that mimic the RAS oncogenic spectrum in the RAS-activated model. p53, the most frequently mutated tumor suppressor, can be assayed through transcriptional inactivity in a pool of HEK293 cells. Mutations of the TP53 gene found in human cancers can be functionally and phenotypically characterized in vivo by gene editing integrated with signal-sensing technology. Such approaches deconvolute the deleterious effects in images. The tumor angiogenic model is applied to investigate the pro-angiogenic effects of vascular endothelial growth factor-A. [87][88][89][90]

5.5 Synthetic lethality mapping using CRISPR

CRISPR-based synthetic lethality mapping reveals underlying cancer vulnerabilities, such as DNA damage repair

deficiencies, activation of alternative pathways, or sensitivity to metabolite depletion. Using this framework, candidate vulnerabilities can be validated and subsequently targeted for therapeutic benefit. As an example, primary screen selection for synthetic lethality with loss of FBXW7 uncovered requirement for the G2/M checkpoint kinase CHK1 in FBXW7-deficient cells, to which greater sensitivity was confirmed subsequently. This hazard matrix approach combines CRISPR screens with an annotated cancer mutation dataset for prioritized screens and validation of dysregulation of the tumor-suppressive gene (TSG) NKG2A in head and neck cancer, especially in HPV-negative disease.

The mapping of cancer dependencies has been transformed by the application of genome-wide CRISPR-Cas9 lethality screens. Which genes, particularly TSGs, are mutated or transcriptionally repressed. They engage with a wide range of cellular processes, include chromatin remodelling, direct the expression of endogenous retrovirus loci, and govern DNA damage repair pathways. These latter roles mean that when TSGs are inoperative, tumours develop with marked deficiencies in HR and FA repair pathways, which can be exploited by inducing synthetic lethality with mutations or drug-mediated inhibition of BRCA1/2, PALB2, RAD51C or RAD51D. In parallel, Coley et al. show that mutation of one arm of the FA-BRCA pathway generates hypersensitivity to substrate depletions that compromise fundamental cellular processes, such as nucleotide and amino-acid metabolism, and alter signalling through the mTOR pathway.

Together with many previous studies, the findings indicate the power of genome-wide CRISPR-Cas9 screens to expose dependencies in specific cellular contexts, including that defined by the inactivation of individual TSGs. Indeed, by interrogating data that define the properties of TSGs and other mutation events

across a wide range of cancer types, an integrated ‘hazard matrix’ emerges that dramatically streamlines the selection of candidates for genome-wide synthetic-lethality screens followed by focused validations. Exploratory screens targeting candidate dependencies also reveal that CRISPR-Cas9-induced deletion of NKG2A in HNSCC cells potentiates tumour-associated immunosuppression and may represent a novel therapeutic avenue for the disease. [91][92][93][94]

Chapter - 6

Targeting Oncogenes and Tumor Suppressors

Cellular oncogenes are usually mutated or overexpressed in tumors, while tumor suppressor genes are frequently silenced or mutated. These alterations drive tumor initiation and progression, establishing oncogenes and tumor suppressors as prime targets of molecularly guided therapeutic interventions. In principle, the underlying genetic alterations can be directly corrected using CRISPR gene editing. Indeed, recent groundbreaking studies have demonstrated the feasibility of such editing in cancer, showing that CRISPR-mediated correction of driver mutations, re-activation of silenced tumor suppressor genes or reversal of immunoediting can be achieved, with notable therapeutic benefit in preclinical models. Despite the much-publicized challenges faced by CRISPR in the clinic, these proof-of-concept demonstrations have laid a solid foundation for the therapeutic application of tumor-targeted genome editing in patients.

In cancers driven by well-defined mutations, the most straightforward editing approach is to repair the alteration. Such repairs can be delivered using CRISPR-Cas9 or alternative editing tools, and have been validated in primary human cells from patients with hematological malignancies. Several studies have shown that re-activating silenced tumor suppressor genes (TSGs), especially through the resolution of pathological DNA methylation, has therapeutic potential in cancer by restoring the corresponding TSG functions. Tumor-specific redundancy can also be exploited: for example, restoration of a silenced TSG may not reduce tumor growth if a second copy of the gene is

expressed, but should nevertheless still impede tumor regrowth after therapy. [95][96][97][98]

6.1 CRISPR correction of driver mutations

Correcting pathogenic mutations in cancer driver genes is a logical application of CRISPR gene editing. Patient sequencing data provide information about the specific mutations in a patient's tumor. In principle, precise gene correction, when performed in the tumor itself, could restore the wild-type sequence, potentially normalizing gene expression and function. Attempts to repair point mutations in cancer-related genes were made almost as soon as CRISPR editing first became available; however, many early studies recapitulated driver mutations in model organisms rather than correcting them in relevant biological systems. Various strategies for repairing driver mutations within appropriate cellular and tissue contexts have since been reported and are now summarized.

The most straightforward method for repairing a pathogenic DNA point mutation is to provide a donor DNA template with overlapping sequences flanking the target mutation—often simply the wild-type sequence cloned into a plasmid vector. Such a template would normally be sufficient to drive homologous recombination (HR) repair in a dividing cell, and even non-dividing cells are sometimes amenable to this repair pathway. In practice, however, recruitment of a donor template for repair at a specific target site occurs at a very low frequency, commonly rendering HR replacement impractical. To remedy this problem in other contexts, methods have been developed to artificially promote HDR at a desired chromosomal site. Some of these approaches have been adapted for use with cancer driver repair. Given the potential for correcting pathogenic mutations in individual patients, such efforts merit close attention. [99][100][101]

6.2 Re-activating silenced tumor suppressor genes

Tumor suppressor genes can become inactive or downregulated due to mutation, promoter methylation, or histone modification. Genetic editing (and replacement via transgenic mouse models) can restore function, but demethylation or histone deacetylase inhibitors can also induce expression, with relatively little risk. For recently identified putative tumor suppressor genes, these latter approaches should be preferred.

DNA hypermethylation co-occurs with gene inactivation in many cancers. However, direct editors that can confer such DNA marks on tumor suppressors may be riskier than indirect demethylation strategies. CRISPR-dCas9 fusions can induce DNA demethylation by recruiting demethylases or blocking inhibitory complexes, enabling expression of genes such as RASSF1A or APC in neoplasms where these genes are normally methylated. Knockout of specific DNMT3A or DNMT3B alleles, or such downregulation achieved with shRNA, can relieve silencing of other RASSF genes. Inducible histone deacetylase inhibitors can also alleviate RASSF1A downregulation in neoplasms lacking DNA methylation.

Direct DNA-demethylation tools (such as dCas9-fusion DNMT3As along with TET1 or TET2) enable restoration of expression, allowing further probing of yet-uncharacterized putative tumor suppressor genes where DNA hypermethylation contributes to loss of expression via induction of DNA marks. Restoration of decitabine-inducible expression adds evidence for true tumor-formation-suppressor function. In addition, screening for small molecules to replicate the phenotype in a more rapid and druggable manner in vivo would open new avenues for approaching aberrantly regulated genes. [102][103][104]

6.3 Editing of RAS, TP53, and EGFR pathways

Key developments in the field, using the RAS, TP53, and EGFR pathways as central examples, highlight the delivery of therapeutics or the rescue of cellular responses through targeted CRISPR-Cas9 editing, including the important concept of restoring drug sensitivity. Both the somatic mutations of RAS and TP53 (the two genes most frequently mutated in cancer) and the recurrent mutations of EGFR provide major targets, given their established associations with oncogenesis, therapeutic response, and poor prognosis. Preclinical proof-of-principle studies have demonstrated that targeted correction of these mutations can restore the functional integrity of the pathways involved, whereas reactivation of TP53 may provide therapeutic benefit when tumor cells are edited to acquire sensitivity to RAS- or EGFR-targeting drugs.

The RAS pathway, TP53 pathway, and EGFR pathway are key players in malignant transformation and drug response. The RAS pathway is frequently activated through mutation in many cancers; TP53 is the most frequently mutated gene in human cancers; and recurrent mutations within the kinase domain of EGFR represent a well-known mechanism of drug sensitivity in certain lung adenocarcinomas, alongside an acquired resistance mechanism in other tumor types. More generally, CRISPR-Cas9-based editing has been proposed as a means to restore normal function to mutated genes in order to enable a return to "normalcy." These targets are therefore exemplars of the potential therapeutic benefit of precisely correcting oncogenic mutation drivers within cancer genomes. [105][106][107]

6.4 Gene editing for drug resistance reversal

Resistant tumors hinder the effectiveness of various cancer therapies, necessitating innovative strategies. Drug resistance emerges from genetic mutations, aberrant expression of specific

genes or proteins, and epigenetic factors. CRISPR technologies have been harnessed to reverse resistance to targeted therapies, immunotherapies, and chemotherapies in multiple cancer types.

Conversely, targeted rewiring of the tumor genome using gene editing has shown the potential to restore therapeutic sensitivity. CRISPR approaches for reversing resistance to targeted therapies against the EGFR, BRAF, and ALK loci, as well as to immune checkpoint inhibitors and chemotherapeutic agents, have been documented. Two notable examples illustrate the possibilities in this area.

Acquiring functional resistance to targeted therapies is notably associated with mutations in the EGFR kinase domain of non-small cell lung cancer (NSCLC) cells. Park et al. employed a CRISPR-Cas9-based "trojan horse" strategy to deliver sgRNAs targeting the EGFR mutation site along with Cas9 into NSCLC cell lines & mice bearing tumors driven by resistant EGFR mutants. In vivo, Cas9-associated tumoral NF-κB activation provoked immunogenic death, further promoting the clearance of local-treated EGFRmutant tumors, together with off-target effects on distanced resistant cells bearing identical mutations. These mutations in EGFR also confer resistance to anti-PD-1 therapy. Hence, achieving specific excisions of the mutation as a strategy to circumvent PD-1 blockade clears the resistant tumors. Similar approaches should be applicable for gene-editing of other tumorigenic drivers and to reverse drug resistance. [108][109][110]

6.5 Case studies in targeted gene correction

Case studies illustrating the utility of CRISPR for oncogene and tumor suppressor gene modification in clinical or advanced preclinical settings are summarized. These projects demonstrate the application of CRISPR for precisely targeting cancer-causing genetic alterations in patient-derived tissues. Such studies serve as proof-of-concept for functional and therapeutic approaches

involving targeted gene correction in advanced diseases.

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease associated with a multitude of chromatin-regulating mutations. Quant et al. have explored a CHD4 knockout, which is recurrent in carcinoma-ascitic fluids, employing lifecycle RNA-seq, metabolic flux analysis, and other techniques in pancreatic cancer models compiled with high-dimensional imaging and network inference. They identify CHD4 loss promotes fatty-acid synthesis and certain lipogenesis-derived oncometabolites, supporting a metabolic vulnerability that may be clinically deployable through CHD4 loss.

Aerospace valuable physiological tissue, but the technologies for the manipulation of islets and induction of multiple islets into combined pancreas-like structures, suitable for transplantation, need improvement. The CRISPR-Cas9 system for the intermittent removal of growth-arrested or dysfunctional cells reprogrammed from human somatic cells into islet-like spheroids is explored. An advanced tissue engineering strategy that coupled CRISPR-Cas9-mediated excision of cell aggregates combined with multiple PDAC exosomes, uniformly stimulating the growth of islet-like spheroids, provides a powerful tool for the development of islet- like spheroids and the efficient production of multiple islets. [111][112][113]

Chapter - 7

Advanced Editing Techniques — Base and Prime Editing

Base editing mechanisms and types: Explain cytosine/adenine base editors and outcomes.

Prime editing innovations and precision control: Describe mechanism and tunability.

Comparison with traditional CRISPR-Cas9: Side-by-side efficiency, specificity, and scope.

Applications in correcting point mutations in cancer: Examples and limitations.

Technical challenges and future directions: Delivery, off-targets, and clinical translation.

7.1 Base editing mechanisms and types

Base editing, a precise genome-editing technique, empowers cytosine-to-thymine and adenine-to-guanine conversion without causal DNA double-strand breaks. Cytosine base editors (CBEs) consist of an error-prone cytosine deaminase fused to a Cas protein that forms a complex with an sgRNA and a DNA repair protein or complex, which deaminates the target cytosine into uracil, inducing conversion to thymine during repair. Adenine base editors (ABEs) combine an adenosine-deaminase domain with deaminase cofactors, an engineered Cas protein, and an sgRNA to recognize the target DNA and finally catalyse adenine to guanine conversion during DNA repair. By achieving point mutation replacement within the endogenous genomic context of

target cells, base editing has gained traction in cancer and disease research as well as precision medicine development.

Despite the technological potential shown by base editing systems designed, tested, and applied in diverse model backgrounds, extensive analysis of system strengths and weaknesses is needed, as well as strategies to overcome limitations. Base editing in cancer research has made early-stage but pioneering contributions by addressing the spectrum of diseases, chemoresistance, and long-term expression and viability of gene-based systems targeting concern genes and pathways. Integration of base editing with conventional CRISPR-Cas9 systems and other genome-editing tools will ensure rapid evolution of technology applications towards a broad range of future biotechnological and biomedical needs. [114][115][116][117]

7.2 Prime editing innovations and precision control

Prime editing represents a leap forward in precise genome editing technologies. Its distinct mechanism uses a specialized fusion protein composed of a Cas-derived endonuclease and reverse transcriptase in concert with a prime editing guide RNA (pegRNA) that encodes the target edit and the corresponding template sequence. During the editing process, the first portion of the pegRNA guides the Cas domain to make a double-strand break at a defined target site, followed by introduction of the reversion mutation using the template domain. The lack of DNA cleavage at the second target site minimizes indel formation and reduces the need for donor template delivery. This concept of delivering a "genotype-reverter" pegRNA that encodes a mutation of interest, coupled with the ease of designing a DNA gRNA, opens a novel and powerful approach to genome editing in human cells.

Prime editing stands out for its unprecedented precision. An extensive comparison of prime editing with traditional CRISPR–Cas9 and base editing systems using well-characterized sgRNAs with known off-target and bystander activity confirms both the excessive bystander and off-target activity observed with the CRISPR delivery system. Prime editing, which does not cause double-strand breaks, has a similar specific activity to base editing yet opens a much broader spectrum of application. Prime-editing windows can be constructed to amend, insert, delete, and even expand long homopolymeric repeats, all using identical mechanisms. Applications for cancer include converting polymorphisms in fundamental genes such as TP53 and KRAS involved in tumorigenesis, enabling precise repair of pathogenic mutations linked to familial cancer predisposition syndromes, altering predicted tumor response to targeted therapies, and investigating side effects associated with therapeutic or nudging mutations. [118][119][120]

7.3 Comparison with traditional CRISPR-Cas9

Both side-by-side comparisons highlight the superior efficiency and specificity of base and prime editing over traditional CRISPR-Cas9 editing, especially for gene therapy applications targeting point mutations. The limitations of standard CRISPR-Cas9 editing are reflected in the fact that approximately 60% of proofreading-deficient Cas9s still produce undesired by-products, such as large insertions or deletions, when injected into zebrafish. Notably, 2,198 out of the 2,259 modifications in the Digenome-Seq dataset are associated with undesired by-products, leading to the concept of either omitting the donor template or adopting a multi-modular and multi-pronged strategy to enhance precision. However, although Cas9- and Cas12-based editors are limited to single-nucleotide changes, synthetic reactants can even achieve the insertion of seven consecutive adenines.

In addition to base editing, prime editing represents a new CRISPR innovation. The reaction is not based on double-strand breaks but rather follows a different mechanism that exploits the reverse-transcriptase activity of prime-editing Cas9. Indeed, the prime-editing system has been likened to a form of in-cell polymerase, enabling longer and more precise insertions while allowing the knocking-out of genes that play a role in tumorigenesis. Moreover, prime-editing efficiency can be fine-tuned by modulating RT and Cas9 concentrations, ensuring that optimal conditions do not promote unwanted by-products. In addition to deletions and point mutations, long insertions have been documented in various studies, expanding the repertoire of applications. [121][122][123][124]

7.4 Applications in correcting point mutations in cancer

Base editing and prime editing achieve single base changes with reduced risk of long-range editing. For point mutations, CRISPR-Cas9 efficiency drops to immunogenic PAM sequences or when NHEJ repair introduces deletions/insertions. Base and prime editing address these problems, enabling mutation repair by installing or substituting a single base pair without DSBs. Base editing possesses clear advantages over the traditional CRISPR-Cas9 approach and has been employed to rectify known pathogenic mutations in diseases such as hypertrophic cardiomyopathy, Usher syndrome, and A1AT deficiency. Nevertheless, it remains unclear whether the breadth of point mutations that can be addressed by base editing extends to all types of cancer. Certain hotspot mutations appear well suited, while others combine low targetable frequency with high editing failure. Prime editing enables CT-to-CA, TA-to-CG, and CTG-to-CCG repairs but lacks a comprehensive efficiency assessment at cancer-relevant loci.

Base and prime editing, by correcting point mutations without introducing DSBs, are anticipated to diminish the risk of undesirable editing events at distant genomic locations. Nonetheless, unintended long-range effects may still arise. All such new tools must therefore remain subject to the same rigorous safety, functional, and preclinical effectiveness testing that preceded the initial application of traditional CRISPR-Cas9 gene editing. [125][121][123]

7.5 Technical challenges and future directions

Base and prime editing introduce new types of DNA alterations for CRISPR-Cas9. In particular, base editing provides a targeted means of converting a C•G base pair to a T•A pair (cytosine base editing; cytosine deaminase fused to a nicking Cas9) or an A•T base pair to a G•C pair (adenine base editing; adenine deaminase fused to a nicking Cas9). Prime editing allows any canonical base pair to be changed to any other canonical combination (e.g., C•G to A•T) while avoiding double-strand breaks, with the result that unwanted alterations (head-to-tail duplications, large indels) are suppressed. Although base and prime editing deliver gains in efficiency and specificity when correcting single-nucleotide cancer mutations, they are complemented by side-by-side comparisons confirming the superior performance of traditional CRISPR-Cas9 systems in many cases.

Successful application of base and prime editing to the correction of recurrent cancer mutations is limited by three factors. First is the need for efficient and non-toxic delivery of the complex editing systems to tumor tissue. Second is the inevitable off-target activity associated with any guide RNA search strategy, which must be sufficiently low to avoid complications in clinical use. Finally, the limited set of alterations that can be made by these new types of editing must

be considered. In particular, single-nucleotide conversion is likely to remain the preferred strategy for “fine-tuning” a hybrid therapeutic index, such as re-sensitizing tumors to a previously used drug. [121][122][125]

Chapter - 8

CRISPR Delivery Systems for Cancer Therapy

CRISPR delivery systems determine the feasibility and safety of therapeutic applications. Different approaches excel in distinct aspects.

Viral delivery methods utilize modified viruses to transfer the CRISPR components into target cells. Adeno-associated virus (AAV) is preferred for its broad tissue tropism, low immunogenicity, and ability to persist in non-dividing cells. However, its small payload limits applications to linear constructs with low activity. Lentivirus and adenoviral vectors can deliver larger RNAs, but their risk of insertional mutagenesis and stronger immunogenicity complicate use in tumors. Safety is critical when delivering nucleases to healthy tissues. As AAV is considered safe for human use, danger mainly stems from off-target cleavage. Hence, efforts to minimize off-target activity are especially important when using AAV-mediated delivery. Importantly, disorders of the CNS or the retina may be cured through direct administration of the AAV-CRISPR or AAV-anti-CRISPR complexes, thereby bypassing safety concerns associated with viral vectors.

Non-viral approaches overcome the limitations of viral delivery systems. Nanoparticles can load different RNA components and launch controlled release to minimize toxicity. Liposomes are another common non-viral delivery method, although they require careful optimization for in vivo applications. Physical delivery methods, such as electroporation

and microinjection, are highly efficient but limited to tissues that can be accessed during surgery. For tumors, electroporation is routinely adopted in the clinic and can therefore be complemented with CRISPR for a more effective local treatment. Nevertheless, using CRISPR simultaneously with an established therapeutic strategy remains challenging, as it requires very specific targeting of the tumor bed to minimize unwanted side effects in surrounding tissues.

Additional advantages were recently proposed for CRISPR delivery. Targeting ligands can be covalently conjugated on the exterior of either viral or non-viral vectors to improve the targeting capabilities of the system. Tumor-promoter-driven expression cassettes can also provide tumor specificity during the delivery of CRISPR in a viral system. Despite the exciting opportunities opened up by these elegant ideas, the success rates remain relatively low. It therefore remains challenging to target CRISPR to specific tumors or even deliver CRISPR only to the cancer tissue during gene editing. Exploiting the normal homology-dependent DNA repair pathway or RNA splicing instead of relying on tumor-suppressor genes for tissue-specific RNA expression might improve the safety profile of CRISPR. Finally, CRISPR delivery systems must also overcome the innate immune response against the Cas9 protein. [126][127][128]

8.1 Viral delivery: AAV, lentivirus, adenovirus

Various viral vectors have been applied for gene delivery in mammalian cells, with an obvious emphasis on safety and payload capacity. Adeno-associated virus (AAV), lentivirus, and adenovirus (AdV) vectors have distinct advantages and challenges.

Adeno-associated vectors (AAVs) are small, non-pathogenic viruses of the dependence virus family, originally categorized as replication-defective adenoviral subrepositories. The AAV viral

genome is too small to accommodate genes encoding all major structural proteins and hence replicates effectively only in cells infected by wild-type adenovirus. AAV-based vectors can be produced to express at least one of the AAV capsid proteins; these encapsidate the AAV packaging signal and any insert sequence. The isolated capsids can tolerate 2.5-fold larger inserts than in native virions. AAVs achieve broad tissue tropism by natural infection of many cell types. Adeno-associated vectors have several advantages for CRISPR delivery compared to lentiviral vectors or other alternatives. They persist in non-dividing cells, express low immunogenicity, do not recombine, and do not induce substantial cytotoxic T-cell responses. However, their applications continue to be constrained by relatively low genome transfer efficiencies, an inability to replicate in non-dividing cells, and small cargo capacity. Moreover, the exceedingly low prevalence of natural AAV infection in humans might not justify vaccine development.

Lentiviral vectors, derived from human immunodeficiency virus type 1, can transduce both dividing and non-dividing cells, are non-cytotoxic, and induce long-lasting transgene expression. Their major limitations lie in immunogenicity and safety: lentiviral genomes integrate into host cell chromosomes, randomly, leading to potential insertional mutagenesis.

Adenoviral (AdV) vectors are also applied in mammalian cells. Derived from a large family of non-enveloped double-stranded DNA viruses, they do much of their intracellular damage to infected cells by stimulating strong innate and adaptive immune responses; thus, they have not been widely adopted as expression vectors in routine studies. AdVs can mediate CRISPR delivery to the liver, lung, nervous system, and possibly other tissues. Because they rapidly spread throughout the body following systemic delivery, they have been proposed as delivery vectors for the CRISPR-Cas system for use against viral infections such as HIV. [129][130][131]

8.2 Non-viral methods: nanoparticles, liposomes

Mammalian cells evolved within interstitial fluid containing high concentrations of free nucleotides and nucleosides, allowing them to acquire exogenous nucleotide-free complex macromolecules naturally without the use of membrane transporters. The ability to give rise to cells through nonviral delivery methods also offers other unique advantages. Compared with viral vectors, the volume and mass of nucleic acids introduced into cells can be orders of magnitude greater using nonviral methods. Nonviral transfection methods do not impose the processing requirements on their payloads that must be satisfied for mediating an efficient transduction of viral vectors. Electroporation can be an effective strategy for transporting molecules of diverse sizes, including large DNA plasmid molecules, RNA and RNA enzyme inhibitors, the CRISPR/Cas9 ribonucleoprotein complex, and large protein and protein-enzyme complexes. When establishing expression systems of low-risk viruses, these nonviral delivery systems have the distinct advantage of bypassing the need for establishing infectious disease biosecurity protocols while achieving transfections in defined cell types that could otherwise be achieved only with virally mediated transduction.

Poor transport efficiency through the cell membrane provides the most serious limitation for all nonviral transfection systems. The overall transport efficiency of negatively charged molecules, required for CRISPR applications, can thus be improved by electrostatically condensing them into positively charged nanoparticles that harness the endocytic pathways for cellular entry. Relatively small lipid nanoparticles through which RNA silencing and RNA enzyme inhibition were first demonstrated provide one example of this approach. Nucleic acid nanoparticles consisting of small synthetic oligonucleotides in addition to lipid nanoparticles constitute another class of positively charged

particles that has been shown to efficiently transport larger nucleic acids such as siRNA and RNA enzymes into mammalian cells. There is an extensive literature comparing the cell transport efficiencies of these different classes of synthetic nonviral nucleic acid transfection agents. [132][133][134]

8.3 Physical delivery: electroporation, microinjection

Cellular delivery is often most difficult to achieve efficiently and safely, especially in vivo. Electroporation enhances the uptake of nucleic acids by exposing cells to electric fields that induce transient permeabilization. Electroporation of plasmid DNA encoding various Cas proteins and sgRNAs has been shown to enable the robust genome editing of CRISPR-Cas9 in numerous cell types; yet, off-targets are still observed. Detection of Beclin 2-Cas9 complexes using in situ proximity ligation assays in mouse pancreas clearly depicted the complexes in pancreas cells, hitherto reported only in cultured systems. The simplicity and low cost of electroporation make it attractive for small-sized tissues and cells. Moreover, it has been elegantly integrated into three-dimensional organotypic PanIN models.

Microinjection enables direct cytoplasmic delivery of nucleic acids into fertilized eggs, single cells, or even subcellular territories of living embryos. Although highly efficient, the complexity, cost, and putative damage prevent wide application. In mice, Cas9 protein coupled with reporter sgRNA was used to create mutagenesis in all three Pten alleles in admixture with fluorescent DNA. In embryos, constructs for tagging cyan-expressing protein for endogenous expression control were equally injected into a subgroup; unlike other animals of the same litter, the tagged embryonic alleles were active, serving as reporters for embryonic stages of development.

The label and knock-in also functioned in several embryonic tissues of tag-reporter crossing progeny mice. In zebrafish,

microinjection at the one-cell stage of Cas9 with target-specific sgRNA induced efficient, multiplex genome engineering—deleting different loci in the same genome in 70%–80% molecular-sanger-validated embryos. By coupling the ATP-inducible dimerization strategy with the ubiquity of RNA-processing and translation machinery, spatiotemporal regulation of CRISPR-Cas9 was elegantly achieved within the embryos through nuclear-cytosolic proteolysis. [135][136][137]

8.4 Tissue-specific and tumor-targeted delivery

Tissue expression patterns of plasmid, or viral, vector components can enhance delivery efficiency to selected organs. ACR type (Adeno-Associated Virus) vectors utilize a rep family protein from AAV2 and rationally engineered capsid proteins derived from AAV2/8 that enable high infectivity and hepatic tropism. Tumor-cell-specific promoters facilitate transgene expression in particular tumor types, such as the prostate-specific promoter pPSP, and in prostate carcinoma but not in other malignancies. Where appropriate, the tissue-selective promoter controls expression of both components in CAR T cells. Nevertheless, vectors that are normally not tissue selective often carry homologs of A-, B-, C-, D- and E-type adenoviral fiber proteins. These proteins localize viral expression to the liver, spleen, and skin after intravenous injection by interacting with host-cell receptors.

Targeted delivery to malignant tissues increases therapeutic efficacy and may reduce toxic side effects. Tumor-targeted and systemic liposomes that render tumor cells sensitive to chemotherapy are substantial delivery vehicles. These liposomes encapsulate a mixture of the anticancer drug and a prodrug with a chemical composition that prevents cellular penetration. Following uptake of the prodrug-liposome complex via the overexpressed folate receptor, enzymatic cleavage of the prodrug

within the cytoplasm produces the active drug. Targeting ligands conjugated to gold or silica nanoparticles increase CRISPR delivery efficiency. These ligand-CRISPR complexes then enter cells via receptor endocytosis and release their cargo via lysosomal escape, enabling ribonucleoprotein translation and subsequent activity of the Cas9 protein. [138][139][140]

8.5 Overcoming delivery barriers and immune responses

Strategies to enhance uptake and persistence.

A wide range of therapeutic candidates adapted for CRISPR-based approaches are currently under clinical development. To achieve optimal therapeutic efficacy, it is critical to enhance the delivery of the active CRISPR-Cas ingredient to the target site, and to improve the stability of these delivery systems. For example, using different CL4-lipid-modified D-ssRNA, the shown efficacy of DIP microinjection in activating the immune pathway, however, this liposome-based DP encapsulating and delivering the whole CRISPR system still needs to be evaluated. In addition, combining the solid magnetic heterostructured micro-particles (SMH) and EUS, Chitosan-pDNA/VaxG still required further optimization for low numbers and short time for TCR-T engineering; thus examining alternative immune reactions should be considered.

Engineered CAR T cells and TCR T cells are capable of evading immune rejection. Multiple clinical trials are in progress that aim to engineer immunoregulatory circuits directly into TCR T cells that prevent the T cells from exhaustion and promote T-cell adaptive-crisis dynamic. However, engineered NK cells still suffer from rapid and significant decline after transfer. Consequently, in vivo imaging systems that allow real-time tracking of adoptive T-cell fate are actively being developed, with the goal of better understanding T-cell immune response and persistence in cancer regulation. [141][142][143]

Chapter - 9

CRISPR in Cancer Immunotherapy

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) has helped realize the long-held ambition of engineering the human immune system to eliminate tumors. By reprogramming cytotoxic T cells to specifically target tumor antigens through chimeric antigen receptors (CAR-T) or T cell receptors (TCR-T) and by enhancing Masquerade and immune checkpoint inactivation for other functional immune cells such as natural killer (NK) cells, efficient induction of an anticancer response is ultimately feasible. In addition, CRISPR technologies can also aid vaccine development by inducing a cellular immune response against cancer cells. Several studies have now translated such concepts into in vivo or trial setting, but clinical results thus far provide a mixed picture.

CAR-T therapy can be thought of as one of the most promising cancer treatment strategies. It consists of extracting patient T cells, engineering them to express CARs targeting tumor-specific antigens, and finally reinfusing them into the same patient using lentiviral or retroviral vectors. Engineering the target cells before reintegration into the patient solves many issues associated with direct administration of TNF-α or CD40L cytokines. However, not all cancers are sensitive to CAR-T therapy. For some cancers, TNF-α, IL-12, or CD40L are not expressed by commonly validated CAR steps. Targeting the replacement or reinforcement of these signals using CRISPR is thus attractive. Positive results have been reported when

engrafting such issues in non-cancerous cells and treating them with the first principal CAR-T cells gene. To enhance the overall ratio of tumor-infiltrated T cells, reinforcing the capacity within NK and NKT cells by using the perforin pathway appears a logical way to assist in extinguishing the tumor as a whole. Targeting these cell types using CRISPR at different stages seems a good manner to increase the global CAR-T therapy effect for more malignancies. [144][145][146][2]

9.1 Engineering CAR-T and TCR-T cells

In the CAR T-cell platform, transgenic T cells express a specific chimeric antigen receptor (CAR) that targets an antigen present on malignant cells, enabling antigen-specific activation of T cells and destruction of tumor cells upon reinfusion. CD19-directed CAR T cells have already shown remarkable clinical efficacy in patients with relapsed/refractory B-cell malignancies. However, several issues hamper the broad applications of CAR T-cell therapy, including: (1) limited availability of T cells, (2) unpredictable expression level, (3) immunogenicity, (4) cytotoxicity in the body, (5) an immunosuppressive tumor microenvironment, and (6) the limited capacity for multispecific targeting.

CRISPR-based gene editing has emerged as a powerful tool to overcome these functional limitations by engineered CAR T cells with various attempts. To enhance therapeutic efficacy, these studies provided a technical landscape for developing more optimized and next-generation CAR-T cells with CRISPR/Cas9 technology by simultaneously knocking in multiple genes involved in improving the efficacy and persistence of CAR-T cells. Specific gene mutations, such as PD-1 in TCR-T cells, may enhance the functions of these T lymphocytes and provide resistance to exhaustion, further increasing their safety profiles against solid tumors. Moreover, the capacity to knock out PD-1,

and CTLA4 makes CRISPR a powerful tool for multiplexed immune checkpoint knockout targeting at once. [147][17][148]

9.2 Enhancing natural killer (NK) cell activity

Natural killer (NK) cells are vital innate immune effectors directly involved in eliminating malignant and virus-infected cells, and they play fundamental roles in both tumor establishment and rejection. The development of therapeutic strategies to augment the cytotoxic action of NK cells has enormous clinical potential. The hyper-activation of NK cells in cancer, however, is tempered by several immunosuppressive mechanisms in the tumor microenvironment, and therefore the reprograming of these immune cells has been a major focus of immunotherapy. The power of CRISPR technology has also been harnessed to provide new avenues to improve NK-cell-mediated anti-tumor activity.

NK cells are the major source of interferon gamma (IFN-γ) crucial for the activation of adaptive immunity, and high levels of IFN-γ are associated with good prognosis in patients with cancer. As CRISPR-Cas9 technology allows genetic alterations to be easily achieved, much effort has gone into maximizing NK-cell activation against tumors through gene editing. IL-15 has emerged as an important cytokine for NK-cell homeostasis and function, and its delivery to the tumor microenvironment or NK cells by various methods has been investigated. Efforts to express IL-15 in NK cells by the deletion of PD-1 have also been efficient in enhancing their antitumor activity. Human NK-92, a cell line expressing CD16, is an ideal platform to explore further to enhance their anti-tumor effect via armoring techniques in combination with checkpoint inhibition. [149][150][151]

9.3 Editing immune checkpoints (PD-1, CTLA-4)

Gene editing for immune evasion checkpoint Committee on the academic protocol immune checkpoints (PD-1, CTLA-4);

CRISPR is under consideration because of its capacity to insert gene sequences of significant length, particularly. The rationale behind these uses stem from how these proteins play vital roles in modulating the function of immune cells such as T or B-cells and can lead to immune evasion in the tumor microenvironment resulting in progression or metastasis. Various combinations of editing these checkpoints are being investigated and have been engineered for CAR-T cells for enhanced therapeutic efficacy.

Successful tumor immunotherapy requires that CAR-T relieves immune checkpoint-mediated T-cell inhibition, thereby facilitating T-cell activity in the tumor microenvironment. PD-1 and CTLA-4, a negative and positive immune checkpoint molecule for T-cells, respectively, are frequently co-expressed in exhausted CAR-T cells. PD-1 blockade augments the expansion and potency of CAR-T cells, while clearance of CTLA-4 supports T cell survival and proliferation in the tumor environment. Therefore, the simultaneous ablation of PD-1 and CTLA-4 in CAR-T cells might reinforce their function in immunotherapy against cancer. With CRISPR technology demonstrated efficient inactivation of both genes in primary human CD8+ T cells, and ablated protein expression with no apparent effects on T-cell growth and phenotype.

Although recent preliminary reports involving CTLA-4 knockout CAR-T cells showed superior anti-tumor effects, they also revealed impaired T-cell activation with excessive CTLA-4 levels. Therefore, CRISPR/Cas9-mediated double-arm editing of immune checkpoints PD-1 and CTLA-4 by exploiting the advantages of Nanog- and PD-1 promoter-targeting gRNAs is very promising and warrants further investigation in tumor-bearing mice or clinical evaluation. [152][153][154]

9.4 CRISPR for vaccine development

Preclinical trials have shown that mRNA vaccines can elicit

a strong immune response against tumor-associated antigens (TAAs), but solid tumors can evade immune detection by downregulating these antigens. Current cancer vaccines targeting neoantigens, which are produced by protein-coding somatic mutations, have delivered better results. Vaccine development is a complex and lengthy procedure since the mutational landscape of many cancers remains yet uncharacterized. The rapid development of the COVID-19 mRNA vaccine has inspired the pursuit of new mRNA vaccines targeting different diseases. CRISPR technologies could facilitate this effort by generating a library of mRNA vaccines in an engineered cell system tailored for patient-specific treatment.

The rescue or generation of HLA-fusion mRNA libraries using a CRISPR/Cas9-based gene knock-in system in antigen-presenting cells can speed the development of vaccines for various infectious and other diseases. Moreover, previously reported CRISPR-Tag vaccines provide a novel strategy for generating custom multiplex vaccines at high speed and low cost. Such platforms would help exploit the noise in the tumor microenvironment, produce immune checkpoint inhibitors, or reactivate immune, metabolic, and glycolytic pathways during vaccination. Such combined or induced vaccine-drug pairs would allow the eradication of combinatorial cancer in various patients. A CRISPR-supported discovery and PreMAP strategy would speed new HLA-associated neoepitope mRNA-vaccine development and patient-specific sequencing-guidance. [155][156]

9.5 Case studies of immuno-oncology trials

The first clinical CRISPR trial using T cells engineered to target PD-1, a checkpoint receptor responsible for dampening cytotoxic T cell activity, demonstrated safety and feasibility while providing insight into the persistence and effects of Cas9 editing in vivo. Strong anti–PD-1 T cell responses were detected

in circulating and tumor-infiltrating T cells following treatment, yet no tumor regression was observed, potentially linked to the low dosage used. TCR-engineered T cells were also generated for targeting NY-ESO-1 in patients with resectable melanoma. An additional trial aimed to delete CEACAM1 in anti-CD19 CAR-T to enhance efficacy against CEACAM1-expressing tumors. Another trial utilized CRISPR-edited PD-1–deficient TCR-T targeting hTERT in solid tumors, and a further study explored lentivirus-mediated delivery of Cas9 and sgRNA targeting PD-1 in TCR-T. TCR-T targeting glypican-3, using T cells derived from patients with resectable hepatocellular carcinomas, also incorporated the knockout strategy.

A phase I trial assessing TALEN-engineered allogeneic NK cells for treatment of recurrent ovarian cancer demonstrated safety and a favorable immunogenicity profile, with four out of ten patients achieving clinical benefit (three stable diseases and one partial response) and resolution of ascites. Another trial utilized TALENs to delete the inhibitory receptor NKG2A in CIK cells targeting head and neck squamous cell carcinoma. CRISPR technology was applied to genetically modify NK cells by knocking out the immune checkpoint molecule PD-1 to potentiate antitumor immunity in patients with colorectal cancer. A first-trimester miscarriage vaccine was also developed in mice through a CRISPR/Cas9-mediated approach for immunological endometrial enhancement in maternal-fetal tolerance and promoting fertility. [157][158][159]

Chapter - 10

Tumor Microenvironment and CRISPR Modulation

Cancer represents a systemic disease orchestrated by a network of altered cellular and subcellular players. Nonetheless, the advances in exploiting CRISPR for targeting the cancer cell compartment should not prevent also studying and manipulating the supporting tissues and cells that provide important support. Progress has been made in exploring and modifying the tumor microenvironment using CRISPR. One main area of investigation has regarded the stroma and endothelial cells of the tumor. Clinical cancer therapy has often focused on targeting the TME, in particular the vasculature, to normalize blood flow and pressure, enhance perfusion, and thus improve the therapy efficacy. Delivery of cytotoxic agents to the tumor tissues might also benefit from improved vascular function.

The excision of endothelial cells can provide insight into the tumor support they provide and is necessary for studying tumor angiogenesis and the effects of angiogenesis-inhibiting therapies. CRISPR has been useful to genetically manipulate tumor endothelial cells to assess the consequences of deletion of functionally important genes or to produce knockout organoids that allow characterization of tumor-promoting mechanisms. Combinatorial approaches involving the tumor stroma have also been explored. CRISPR has been applied to genes encoding cytokines and chemokines secreted by proximal or distal tumor cells and regulating recruitment and activation of immune cells

that can infiltrate tumor tissues and promote the cancer cell immune evasion. Moreover, modified cancer cells have been tested in advanced preclinical models, revealing unexpected effects and enabling the design of more efficient treatments. Finally, the effects of genetic editing in the stroma on cancer cell behaviors other than tumor growth, such as tumor cell metabolism and metastasis, have also been explored using CRISPR engineering. [160][161][162]

10.1 Genetic manipulation of stromal and endothelial cells

Cancer cells survive and thrive within a host environment often named the tumor stroma, which includes all non-tumor components found within the tumor. The tumor stroma consists of a supporting cellular scaffolding containing the ECM, blood vessels, lymphatic vessels, and infiltrating immune cells such as myeloid cells, neutrophils, and lymphocytes. In addition to cancer cells, stroma cell types (endothelial cells, pericytes, and fibroblasts) are also subjected to genetic alteration (mutations, epigenetic changes, or viral integration) that support tumor progression. The main genetic pathways altered in non-tumor cells have not yet been extensively investigated, but research has begun to uncover the potential contribution of alterations in stromal and endothelial cells.

CRISPR-Cas systems have been used to genetically manipulate the expression of several genes in these cells to modify their behavior in tumors. Inhibition of these cells with CRISPR tools has allowed researchers to dissect and validate their effects on cancer cell proliferation, invasion, therapy resistance, and metastatic spread. CRISPR-edited tumor-associated endothelial cells have been utilized to evaluate their role in supporting tumor angiogenesis and progression. These approaches permit more careful dissection of the role of these cells and represent an important advance in evaluating their

contribution to tumor development and therapy resistance. The ability to edit the gene expression of tumor-supporting cells enables questions regarding their role in tumor biology to be examined in a more definitive manner.

Changes in the tumor microenvironment (the composition and activity of surrounding non-cancer cells) represent the culmination of several processes, including selection and adaptation to promote immune evasion. Functions essential for tumor take and growth may be shared among many or all tumors, while changes enhancing malignant cell dissemination should be epistatic and thus not necessarily required while the primary tumor is still present. Given that they will often be critical for cancer spread, changes in cancer-stroma interactions leading to altered angiogenesis should be a major area of focus. Since endothelial cells form a relatively well-defined, specialized population with relatively few other functions beyond but supporting growth and dissemination, criteria for targeting should therefore be less stringent than the usual considerations applied for CRISPR to be clinically applicable. [163][164][165]

10.2 CRISPR in studying immune evasion

Structural and functional alterations of CAFs and endothelial cells enable cancer cells to evade the immune system. CRISPR activation of chemokines like CCL4 improves CD8 T cell recruitment and anti-tumor activity in animal models. Induction of nonsynonymous mutations enhances CD8 T cell infiltration but without achieving durable protection. Deletion of the α5 subunit of fibronectin or of lysyl hydroxylase-2 in the extracellular matrix of tumors reprograms immune evasion and increases anti-tumor immunotherapy efficacy. Targeting the Kynurenine pathway, which impairs T cell function and favors tumor immune evasion, by editing Kynurenine 3-monooxygenase shows promise.

Various approaches have been developed to engineer stem and progenitor cells for tumor immunotherapy. CRISPR-Cas9 systems are proving useful for reprogramming different tissues and specialized cell types, including T cells, NK cells, iPS cells, DCs, and macrophages, for either allogenic or autologous engineering. Moreover, the genetic reprogramming of CFs, SCs, and TE can impair tumor growth.

It is increasingly evident that tumor microenvironment plays an essential role in cancer progression. The complexity of the tumor microenvironment poses challenges in identifying the key populations or signals responsible for therapeutic resistance. In the context of multi-faceted stimulation, CRISPR systems provide a powerful solution for both spatial-temporal locus design and multiplex disruption or activation. Using these tools, specific populations or signals related to immune evasion or chemoresistance can be characterized and, subsequently, targeted by employing a combination of CRISPR and existing therapeutic strategies. [166][167][168][169]

10.3 Remodeling tumor metabolism

Disruption of metabolic pathways alters tumor growth and drug resistance. Cancer cells deve-lop dependencies on specific nutrients, including amino acids, lipids, and carbohydrates that favor tumorigenesis. To exploit these vulnerabilities, experimental approaches modulate the availability of respective metabolites using small-molecule inhibitors. Combining such inhibitors with genetic manipulation highlights the importance of metabolic remodeling.

CRISPR-based techniques aid the dissection of metabolic pathways by targeting enzymes involved in metabolic turnover. Metabolism-modulating oncometabolites such as lactate, itaconate, and fumarate are associated with immune evasion and treatment resistance. CRISPR activators targeting glycolytic

enzymes, including hexokinase 2, forward metabolic flux toward lactate production and promote tumorigenicity in TLR3-knockout mice. These studies establish metabolic rewiring as a viable therapeutic avenue for cancer treatment.

Modification of tumor metabolism has therapeutic potential by impacting tumor-associated processes such as growth, drug resistance, and immune evasion. Targeting glycolysis or lipid metabolism in tumors alleviates the negative effects of immune checkpoint blockade in preclinical models. Genetic approaches elucidate candidate metabolic regulatory genes, particularly those involved in lactic acid production, in the context of metabolic remodeling. CRISPR-mediated overexpression or deletion of metabolic enzymes such as hexokinase 2 directs metabolic remodeling, offering experimental proof of principle. However, further investigations are needed for translation into a therapeutic strategy. [170][171][172]

10.4 Editing cytokine and chemokine pathways

The immune microenvironment profoundly impacts cancer development, progression, and therapy efficacy and is characterized by presence, abundance, and localization of specific infiltrates rather than immune cell composition per se. Cytokines and chemokines act as messengers exchanged by tumor and immune cells, coordinately orchestrating the tumor–microenvironment dialogue through differential and dynamic expression. Tumors can hijack this communication by overexpressing soluble factors that interact with their own or neighboring cells to support tumor growth, immune evasion, metastasis, and therapy resistance. Cancer cells and stromal cells (mostly fibroblasts and immune cells) may also release tumor-promoting cytokines, such as IL-6 and IL-1, that recruit other protumor cells to form a pro-tumorigenic network.

Evidence indicates, for instance, a role for IL-31 produced by tumor-associated macrophages in recruiting IL-31 receptor-expressing myeloid-derived suppressor cells that promote resistance to immune checkpoint blockade in melanoma. Expression of the chemokine CCL22 by tumor-associated macrophages in different types of tumors, and its recruitment of Treg cells, also represents a well-studied example of immune evasion through buildup of immunosuppressive components. Such findings point toward opportunities for therapeutic strategies that aim at knocking down supportive cytokines (e.g., CCL22) or promoting protumor-suppressive responses (e.g., IL-31 receptor knockdown).

Studies on the role of specific cytokines and chemokines on tumor progression and therapy response are often facilitated by gain or loss of function in the respective receptors. Such approaches naturally rely on gene editing technologies. Successful applications include CRISPR-mediated knockout of the CCL2 receptors CCR2 and CCR4, which enhanced the antitumor efficacy of anti-PD-1 therapies through a reduction of tumor-associated macrophages in the microenvironment and a concomitant increase of cytotoxic T lymphocytes, and disruption of the IL-35 receptor in T cells, which improved their antitumor effects in prostate cancer. Further investigation and manipulation of the cytokine and chemokine pathways hold great promise for new avenues in cancer immunotherapy. [158][173][174]

10.5 Combining CRISPR with microenvironment therapies

Strategies combining CRISPR with other modalities targeting the tumor microenvironment hold great promise for therapeutic advancement. Tumor-associated stromal and vascular cells provide both structural support and regulatory signals during cancer progression. Eliminating such supportive features is a potential therapeutic avenue. Conversely, restoring

normal stroma development can also suppress malignant growth through indirect mechanisms. Notably, CRISPR-engineered endothelial cells producing pro-inflammatory chemokines markedly inhibited tumor progression in preclinical models. Interfering with tumor-induced immune suppression is another rational approach. Reversion of defective chemokine expression in tumor cells or genetic engineering of nearby myeloid cells for enhanced inflammatory stress are strategies that may promote cytotoxic T-cell activity against tumors. Finally, the tumor's altered metabolic landscape creates a therapeutic window that can be explored using CRISPR approaches. Attenuation of oncometabolite production, modulation of lipid metabolism, or elimination of distinctive metabolic traits represent strategies under investigation.

In summary, CRISPR technology is advancing our understanding and manipulation of the tumor microenvironment. Modifying non-malignant cells can help elucidate tumor-supporting roles throughout different cancer stages. Further, increased mechanistic insight may open new therapeutic avenues. [175][176][160]

Chapter - 11

CRISPR-Based Diagnostics and Biomarkers

CRISPR biosensors: SHERLOCK, DETECTR: Mechanisms and detection capabilities.

Detecting oncogenic mutations and fusions: Specificity and clinical relevance.

Early detection and minimal residual disease: Sensitivity requirements and implications.

Liquid biopsy integration with CRISPR tools: Workflow and interpretative framework.

Portable and point-of-care cancer diagnostics: Feasibility and limitations.

11.1 CRISPR biosensors: Sherlock, Detectr

Recent advances in CRISPR technology have enabled the development of CRISPR-based biosensors for the detection of RNA and DNA sequences. One of these sensing systems is termed SHERLOCK (Specific High Sensitivity Enzymatic Reporter unlocking), and it uses an RNA-cleaving Cas13 protein that is programmed to target a specific RNA sequence and generates a fluorescent reporter signal. A second system, DETECTR (DNA-Encoded Testing for the RNA of Targeting), employs the RNA-guided DNA-cleaving activity of an engineered Cas12 protein to detect DNA sequences. The potential of SHERLOCK to detect target RNA sequences in biological samples has been demonstrated, as has DETECTR's ability to detect DNA, including from pathogenic bacteria.

Detection of target sequences mediated by SHERLOCK or DETECTR can simultaneously occur in one reaction, enabling the distinguishing of nucleic acid sequence polymorphisms in both DNA and RNA without the need for labeled primers per target, thus greatly simplifying nucleic acid testing for both quantitative and qualitative purposes. The catalytic detection formats permit the use of very low concentrations of the biosensor, and both catalytically active Cas proteins can be expressed in vivo together with the target sequence-recognizing guide RNA. The data demonstrate the potential for CRISPR/Cas-based catalytic detection systems to make both potent and sensitive biosensors for the in situ analysis of pathogen infections and abundance. [177][178][179]

11.2 Detecting oncogenic mutations and fusions

CRISPR-based cancer immunotherapy aims to harness the immune system's capacity to eradicate cancer by genetically engineering immune cells (CAR-T and TCR-T), boosting the activity of immune effector cells (NK cells), modifying immune checkpoints (PD-1 and CTLA-4), or constructing vaccines against cancer-specific antigens. Successful human trials have showcased the potential of immunotherapy-driven CRISPR/Cas9 technology to combat cancer. In addition, CRISPR/Bioinformatics cancers offered a plethora of successful approaches in the detection of oncogenic mutations that may assist in achieving genotype-phenotype correlations.

The powerful multiplexing capability of CRISPR/Cas9 technology has enabled the detection of clinically relevant oncogenic mutations in specific cases. However, such mutations usually require tumor DNA, which is unequally accessible in clinical practice. In those cases, fusion transcripts, among others, could be utilized. SCOT is being heralded as an innovative approach capable of detecting RNA synergistically amplified by

CRISPR-based transcript and cDNA amplification, and offers sensitivity and specificity. Utilizing a similar dually amplifier-shrinker scheme may also allow for the highly sensitive detection of HDR, transkenomic, TAR, stTAQ, or short- and long-crRNA transcripts in engineered organisms and cell lines.

Creating sensitive and reproducible detection tools and reagents for generating functional data are essential for reliable clinically pertinent genotype-phenotype conclusions. Moreover, CRISPR/Cas9-mediated detection tools based on simply nucleic acid amplifiers in combination with an inappropriate DNA polymerase and SCOT should be highly sensitive. The integration of liquid biopsy principles with CRISPR/Cas9 technology may provide an accurate point-of-care diagnostic device. [180][181][182]

11.3 Early detection and minimal residual disease

Sensitive detection of cancer-associated mutations could enable diagnosis long before clinical symptoms emerge, facilitating and improving the chances of treatment success. In a complementary approach, CRISPR-based technologies could reveal the presence of minimal residual disease following treatment or active and early progression of recurrent malignancy. Such early diagnostic testing may facilitate the initiation of timely treatment, sustaining a good prognosis and improving patient outcomes.

Cancer early detection relies on the identification of established molecular alterations commonly observed at tumor initiation. The expression of oncogenes at the target site frequently occurs at low levels and may be captured with very sensitive DNA or RNA detection technologies, such as next-generation sequencing (NGS). Because of its high sensitivity for the detection of specific nucleic acid signatures, SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) enables

extremely low numbers of targets to be identified accurately. The SHERLOCK platform relies on Cas13 based detection of specific RNA sequences. Cas13 is an RNA-targeting RNA-guided RNA endonuclease that cleaves non-target RNA in the presence of a DNA–RNA duplex, and its activity also activates trans-cleavage activity, resulting in the degradation of any nearby RNA transcript. In a similar manner, DGCR8, dCas9–RNaseC and Cas9 from Streptococcus thermophilus provide a DNA detection platform termed "detection of transcribed RNA and other nucleic acids" (DETECTR). [183][184][57]

11.4 Liquid biopsy integration with CRISPR tools

CRISPR tools for cancer detection through blood sampling, or liquid biopsy, are of considerable interest because they can close the gap between table- and bed-side approaches. While response prediction and therapeutic decisions are generally aided by large-scale genomic efforts such as The Cancer Genome Atlas and the Genotype-Tissue Expression project, actionable mutations in formalin-fixed and paraffin-embedded tumor samples are often detected too late to benefit the patient. Liquid biopsy can fill these gaps; however, the ability of superbly sensitive CRISPR-based detection systems to detect residual neoplasia in plasma of patients with complete response to therapy has not yet been tested. Despite the versatility of CRISPR detection systems, they are better suited for detecting a known target than for screening a sample for the presence of a previously unidentified target. Integrating CRISPR-based detection with liquid biopsy therefore requires defining a range of potential targets. This can be achieved by screening solid tumor samples using the CRISPR-associated RNA-guided-engineered endonuclease system and focusing on oncogenic mutations, fusion genes, and insertions/deletions present only in the tumor. Hematopoietic tumors can be further reduced in scope by focusing on cloned T or B cell receptors, while loss-of-function

targets can be considered separately. Pooling. Information about context is critical for determining design. The presence of the target can subsequently be detected in a plasma sample using a CRISPR detection system with appropriate spatiotemporal oversight.

The speed of response is dependent not only on analyte detection but also on the time taken to design a detection system and on the presence of a suitable plasma sample. Long-standing structural knowledge facilitates easy and rapid detection of many oncogenic mutations. Detecting point mutations in circulating cell-free DNA usually requires two primers and two probes, but adding an internal control to the process simplifies development of the detection system. Pan-cancer panels designed with an appropriate probe facilitate the all-in-one detection system. Point-of-care diagnostic tools require CRISPR detection systems that operate the same way as glucose meters. Addressing these considerations has already enabled the rapid development of CRISPR detection systems for other disease areas. [185][186][187]

11.5 Portable and point-of-care cancer diagnostics

Portable and point-of-care devices based on CRISPR sensing have been established and can be combined with liquid biopsy technologies for noninvasive disease detection. Cancer diagnostics are often too complex and time-consuming to be deployed in the field. A new class of CRISPR-based pathogen-detecting biosensors has been introduced that relies on Cas13-based trans-cleavage of a fluorophore-quencher-reporting RNA probe. These highly specific, sensitive CRISPR-based tests have a short turnaround time and employ specialized detection portable devices for point-of-care application. They are also able to detect extremely low quantities of target nucleic acids to provide early-stage disease diagnosis. Combining these advantages with CRISPR technologies has produced

SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) and DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), which can detect DNA and RNA from viruses, bacteria, and fungal pathogen targets in environmental samples well below clinical levels.

A prospective point-of-care SHERLOCKNA detection test using Cas13-as-ribonucleoparticle technology has been developed to enable the noninvasive early detection of genoperipheral nervous system tumors through genomic analyses of saliva. Liquid biopsies with CRISPR-based analyses are expected to become important diagnostic tools. CRISPR technology involving the EPCRAS cluster and Cas9 has expanded the potential scope of application and detection ability. Detection of small amounts of disease-related informative molecules is critical; few additional molecules in a complex matrix (for example, urine) can mask or bypass the informative ones. Non-invasive detection tests can provide accurate early-stage results in real-time monitoring, and portable desktop units can assist routine point-of-care testing. [181][188][189][190]

Chapter - 12

Personalized Cancer Models Using CRISPR

The greatest promise of CRISPR in cancer research lies within personalized therapy: patient-specific genomic data can be used to select pertinent genetic alterations in tumors, which are subsequently engineered into relevant models. CRISPR is being employed for the generation of organoids and directly edited patient-derived cells, allowing for the construction of organoid avatars that closely mimic the original disease and can guide treatment decisions. Together with patient-specific, high-throughput, drug-screening platforms and scaffolded assemblies that faithfully recapitulate the genetic architecture of cancer metastasis, these personalized models allow tumors to be dissected accurately in landmark studies validating the CRISPR-enabled paradigm shift toward precision oncology.

Proof-of-concept studies covering various aspects of personalized treatment are emerging across multiple cancer types. Addressing some of the main caveats of current tumor avatars, CRISPR-generated models containing distinct and clinically relevant mutations are being developed on a large scale, paving the way toward advanced selection systems capable of guiding therapy design and alleviating tumor-associated mortality and suffering. [191][192][193][194]

12.1 CRISPR in organoids and patient-derived cells

Patient-derived organoids and cells recapitulate the cancer landscape of individual patients and thus represent useful platforms for personalized medicine. CRISPR technology makes

it possible to develop organoids and patient-derived cells that carry complementary genetic aberrations. For organoid generation, limited modifications are typically incorporated within donor genome to address several limitations posed by existing organoid culture systems. Patient-derived cells can also be generated from cultures of direct reprogramming, integration-free expression of pluripotency-associated factors in the cells. In this context, CRISPR-based gene editing can drive the conversion of patients' somatic cell into induced pluripotent stem cell (iPSC) for drug screening in combination with pluripotent stem and TG techniques.

Patient-derived organoids may also be used for personalized drug screening. CRISPR technology allows introduction of cancer-associated mutations in organoids establishing a high-throughput screening platform. Lipid-based delivery system improves the transfection efficiency of NHEJ, HDR donors and cytotoxic markers. CRISPR focuses on elucidating the origin of cancer and its malignant properties describe organoids take a model for direct comparison with the initial tumours of the patients. Patient-derived organoids are next generated and used for high-throughput drug testing. Organoids contribute to the rapid expansion of patient-derived organoid banks providing a valuable resource for precision cancer medicine. [195][196][197]

12.2 Generating patient-specific tumor avatars

Patient-derived tumor avatars are enabling platforms for personalized therapy design. First, CRISPR has been applied to generate organoids or patient-derived tumor cells with intact patient mutations, which constitute the foundation for patient-specific avatars. These avatars can be fabricated in large sets and used to explore potential therapeutic strategies at scale. Finally, some patient avatars are deeply characterized and tested to probe translational aspects of patient-specific therapy design.

Cancer drivers define the cancerous status of a cell, thus any model recapitulating these mutations represents a patient-specific avatar. Based on this premise, CRISPR has been employed to generate organoids or patient-derived cells containing patient mutations. However, the field is still in its infancy, as optimal conditions for establishing patient-derived organoids are not yet available for all cancer types. Nonetheless, when conditions are fulfilled, the number of generated organoids can be amplified at a high scale within a short period. Such an extensive supply allows for the rapid identification of possible therapeutic combination strategies, and these organoids represent the first steps toward using patient-derived organoids as tumor avatars. [197][198][199]

12.3 High-throughput drug screening platforms

Cancer therapy decision-making benefits from individualized consideration of patient omics. Personalized cancer models provide unique opportunities for patient-tailored therapy design and assessment of therapeutic responses prior to actual treatment. CRISPR-based gene editing enables the construction of patient-specific cancer models by editing patient-derived primary cells, organoids derived from tumor biopsies or resections, and patient-derived xenograft (PDX) mouse models. Combination with high-throughput drug screening capabilities can support the rapid selection of optimal therapies for each patient.

Patient Avatar Co. (PAC) developed a platform to create personalized cancer avatars using patient-derived organoids for subsequent drug testing through multiplexed screening. PAC relies on primary cell-derived organoids developed in Geltrex (Thermo Fisher) to support high-throughput drug screening. Drug combination screening tests over 20 anticancer drugs in parallel to identify therapeutic responses. An initial technology-in-service study engaging 54 organoid models from patients with

various advanced cancers, including early-stage disease, set out to benchmark clinical correlations for predicted responses, summarize the proportions of actual clinical benefits, and assess the specificity of the system. A comprehensive systematic study for selection of the organoid medium is required to provide a robust and reliable organoid-based screening platform. [200][200][201][202]

12.4 Modeling metastasis and heterogeneity

CRISPR technology enables the modeling of complex biological processes, including metastasis and heterogeneity. Though the established models—mouse, zebrafish, and fruit fly—are useful, they are limited by ethical concerns and resource requirements. Organoids and patient-derived xenografts present more reliable options but cannot easily be modified or maintained to support metastasis. CRISPR gene editing addresses these limitations by generating models of metastasis and heterogeneity in organoids, embedded in chicken embryos. Such systems serve as accurate tumor avatars for therapeutic response assessment.

Metastatic progression of cancer involves the loss of epithelial features and acquisition of a mesenchymal and invasive phenotype. Cancer stem cells possess a hierarchical structure and play a critical role in the metastatic spread of tumors. Genetically manipulating these events allows the formation of an organoid-based chicken embryo model of metabolism. These "living metastasis chips" serve as a platform for the analysis of drug efficacy against invasive cancer. In combination with specific conditions, CRISPR-based gene editing systems modeling metabolic dysfunction and cancer stem cell formation in organoids can also verify chemotherapeutic responses. The generated organoids become a living chip that diagnoses and tests therapeutic responses to any drug candidate.

Diverse cell types and components interact to drive tumor growth and dissemination. Accurate, efficient, and specific modeling of heterogeneous and multifunctional tumors remains challenging. Given the ability of CRISPR-mediated genome editing systems to create complex genetically modified tumor models, using CRISPR to establish models that precisely recapitulate the development of tumor heterogeneity and metastasis is warranted. Although conventional multimodal tumor models depend on approaches yet to be standardized in the field, establishing mammalian tumor avatar models that integrate organoids and chicken embryos would facilitate a variety of applications.

12.5 Case studies in personalized therapy design

Select examples illustrate the CRISPR-assisted delineation of individualized treatment regimens, highlighting the potential of precision therapy design.

A personalized strategy for responsive therapeutic selection was devised for a patient with early-stage lung adenocarcinoma and multiorgan metastases who had refused surgery or drug treatment. Organoids were derived from both the primary tumor and liver metastasis, and a pattern of actionable mutations in the corresponding tumor tissue was replicated in vitro. Subsequently, drug screening with 165 compounds across the two organoid models uncovered differential sensitivities; the matched organoids responded to a novel combination treatment targeting lipogenesis. An alternative approach merged genome editing with patient-derived tumor avatars to guide a 44-year-old woman's biopsied rectal cancer therapy. CRISPR-enabled introduction of bona fide cancer mutations into 32992116-SNP-discordant normal colon organoids enabled synthetic lethality prediction and therapy choice.

In summary, functional CRISPR-based modelling of patient tumors combined with high-throughput drug or radiation scheduling opens a new avenue toward personalized anticancer therapy.

Chapter - 13

Artificial Intelligence and Bioinformatics in CRISPR Design

Machine learning is being deployed to refine the essential guide RNA design process by predicting on-target efficacy ranks governed by thermodynamic principles, and anticorrelating off-target potential. Aided by these advancements in guide RNA generation, early-stage clinical proposals seeking to target the proto-oncogenic human phosphatase dual specificity phosphatase 6 (DUSP6) have emerged. Efforts are also underway to expand existing CRISPR databases, predicting the likelihood of detecting any particular off-target site within the global set. These resources are invaluable, especially when the CRISPR experiments or screens probed remain unconnected to the whole-genome sequencing of the cellular substrate.

Integrating CRISPR data with cancer multi-omics is being pursued to train classification models for predicting cancer origin from sequenced, cellular CRISPR datasets, and to furnish resources predicting sample mutation burden. The application of AI technologies to facilitate patient-specific and personalized CRISPR-cas9-tabbed either preclinical or clinical trials and interventions is gaining momentum. Specifically, building a CRISPR database relevant to cancer and its associated features may empower decision-making for therapy, immunotherapy and CRISPR-based employing the analysis of artificial intelligence methods. [203][204][205][206]

13.1 Machine learning for guide RNA optimization

Machine learning algorithms can help optimize the design of guide RNA (gRNA) sequences for CRISPR systems. Predicting gRNA efficiency is difficult because numerous sequence and structural features contribute to activity. Prior predictive models focused on gRNA sequence, ignoring other characteristics that may influence Cas9 loading or function. Recent models leverage sequence, structural, and genomic feature sets. One integrates 61 gRNA features from seven types spanning codon, condition, genomic, mRNA secondary structure, position, secondary structure and nucleotide position of the CRISPR complex, and target-SNP sets. The method uses a multi-class support vector machine, trained on 777,370 previously published gRNAs with blacklisted off-target cleavage. It outperforms or is competitive in accuracy with the best prior codon-specific models for predicting targeted gRNA editing activity.

Another approach optimizes gRNA design for genome-scale clustering using ensemble learning techniques, incorporating sources of signal and noise inherent in CRISPR screens, data augmentation principles from natural language processing, and five different machine learning backbones. Performance is evaluated using binary classification (Hamming loss) and multi-class rank-based objectives. The model is used to evaluate the design of gRNA pools targeting the λ virus, and guide design recommendations support gene recovery in targeting screens. Separate modeling of CRISPR interference libraries further enhances library design and performance prediction. [207][208][209][207][208][209][210]

13.2 Predicting off-target activity

Models predicting potential cleavage by CRISPR-Cas9 and other systems can be built using machine learning and high-throughput experimental information. The accessible datasets

have grown tremendously, though the parameters vary widely; for example, several only consider the protospacer adjacent motif sequence. Consequently, the various models cover disparate activity ranges. A recent, extensive study benchmarked six leading prediction tools—CCTop, CFDB, GPP, CRISP, GuideScan, and C-RNA19—and four guide RNA design platforms—CRISPRdirect, CrisprZ, CRISPR-ERA, and CHOPCHOP—across five datasets. Although designed primarily for predicting Cas9 target sites, the GPP web application offers alternative scoring functions for SpCas9, SaCas9, and FnCas9 activity prediction, and there are parameters for Cpf1. Likewise, the Cas12a RNA-targeting system has several dedicated resources: DARTS, CLOWN, CRISPRoff, CRISPR-RNA, CRISPRain, and CRISPRisA.

Predicting off-target cleavage locations for CRISPR-Cas9 remains challenging. Guides with one mismatch may be cleaved, especially in high-abundance genomic regions, and even greater changes may still retain activity. Many early analyses detected unanticipated cleavage events, confirming that off-target activity must be considered and carefully monitored. Validation especially matters for therapeutic CRISPR-Cas9 applications. Gaining a more comprehensive understanding of Cas9 fidelity has inspired many strategies to predict off-target activity, including two high-throughput techniques: GUIDE-seq and CIRCLE-seq. The data generated by both methods are valuable for training machine-learning models. Though promising, the performance of any machine-learning algorithm depends strongly on the quantity and quality of the training data. Expanding the databases storing this and other relevant information will further advance the machine-learning approach.

13.3 Integrating CRISPR data with cancer omics

Two complementary strategies can be pursued to enhance the

precision of editing approaches: the integration of CRISPR systems with machine learning and the incorporation of expression proteomics and metabolomics data into CRISPR design and functional annotation. By associating FUSIONS with tumor expression profiles, it becomes possible to identify targetable mutations in putatively druggable cancer genes and assess the corresponding therapies in specific patients. Such efforts are expected to enrich the library of actionable cancer mutations identified by the COSMIC database, expanding the spectrum of lesions that can be targeted by precision CRISPR editing.

Another application of the integration of multi-omics data with CRISPR platforms lies in the prediction of cancer-relevant editing effects of guide RNA insertions. From a predictive modeling perspective, supporting an artificial intelligence-trained genetic-fitness-reward model based on RNA-Seq, DNA-Seq, and evolved tumorsome alterations of deep functional loss-of-function CRISPR screens can assist CRISPRa editing design to identify specific gene activation that can yield distinct plasticity fitness rewards, offering potential new hypotheses of gain-of-function editing effects. Together, these ideas demonstrate that multi-omics data supporting FUSIONS and AIFRES-6 can boost and standardize the utility of integrated cancer genomic architecture of CRISPR in precision oncology. [211][212][213][214]

13.4 AI-assisted personalized therapy planning

AI-assisted prognosis and therapy planning integrating multi-omics informatics with CRISPR gene editing hold great potential for precision medicine. Artificial intelligence, or more specifically machine learning, interprets complex data signatures and helps to make sophisticated inferences apparently beyond human reach. In CRISPR research, machine learning has been

mainly employed to distinguish true-target from off-target activity of guide sequences. Since high-throughput sequencing gives rise to an unprecedented wealth of experimental data, the fusion of large-scale CRISPR activity databases with other cancer omics presents new opportunities.

Targeting retained genomic alterations represents a promising strategy to improve clinical outcomes. A versatile data fusion framework is outlined that combines candidate mutations from sequencing; transcription; methylation; copy number; proteome; CRISPR sgRNA and biosensor detection; patient-derived organoids; and drug response data for individualized target selection and therapy planning. All data types can be integrated and mined by machine-learning methods, and the approach has implications for designing personalized CRISPR gene therapies and supporting clinical decision-making. This considers decision-support systems for clinical practice—in the form of an interactive website able to identify candidate mutations, gene targets, approved/pending drugs, and recommended therapies. [215][216][217]

13.5 Future trends in computational CRISPR biology

Next-generation precision oncology will benefit from a diversity of emerging computational tools, including RNA design algorithms, predictive models for off-target activity, integrated multi-omics approaches, and AI-assisted therapy planning.

The optimization of guide RNA sequences for CRISPR-Cas9 applications is currently one of the most active areas of convolutional neural networks (CNNs) applications in novel drug design. Several key determinants – such as the GC content and positional features of gRNA, the sequence identity with the target genomic region or gene, the secondary structure, and possible motifs for non-specific interactions with the Cas9

protein – can now be used as features for training CNNs that predict editing efficiency. Some of these predictors have been tested in massive-scale HTS in animal models.

The decrease in off-target effects is the second most prominent area of machine-learning project development in CRISPR biology. Most existing models provide site-specific raw scores that indicate a predicted tendency for off-target cleavage according to features of gRNA and gRNA-targeted locus sequence. Recently, these scores have been integrated into a prediction system that detects potential off-target cleavage.

Combining data from CRISPR perturbation studies with multiomics information from projects such as the Cancer Genome Atlas may also facilitate the discovery of epistasis among tumorigenesis regulators. Structures of clinical bioinformatics analysis pipelines, including a machine-learning component tailored for the identification of patient-sample-specific gene expression patterns from transcriptional profile data, may allow decision-support systems for precision cancer therapy to be trained. Other early approaches combining transcriptomic, genomic, and clinical information are already suggesting target profiles linked to specific therapies and outcomes. Integrating these converging fields would strengthen their predictive and prescriptive capacities over the long term.

Chapter - 14

Clinical Translation of CRISPR Therapies

Effective application of CRISPR technologies in cancer research intends to translate therapeutic approaches to the clinic. A systematic pathway is required to ensure that first-in-human gene modifications conform to all quality and safety requirements. Preclinical validation in appropriate cancer models supports true patient benefit, while regulatory oversight guarantees patient welfare and risk mitigation. The pioneering phase of human trials provides proof-of-concept for in vivo CRISPR-based gene-editing applications in humans.

Specificity and safety concerns mean that gene-editing procedures ordinarily require thorough in vivo validation prior to patient-experimental application. Conventional animal models can assess the therapeutic potential in an initial proof-of-principle exercise; subsequent development in tumorgraft systems generated from the patients' own cancers enables demonstration of true personalised medicine under stringent experimental conditions. Modified T cells have passed through those tests and are now being applied in first-in-human trials. Since a range of other methods are being explored, those goals alone are insufficient to establish the CRISPR-Cas technology for broad clinical use in oncology. Regulatory approval, quality-control procedures, and patient-selection considerations all require attention, not only for the pioneering trials but also for future approvals within that therapeutic space.

Early-phase clinical trials aim to establish safety and biological activity in fewer than 20 patient volunteers; importantly, a formal measure of clinical response is not obligatory. These fundamental safety datasets can then support the initiation of larger Phase 2 or Phase 3 studies designed to definitively assess therapeutic efficacy, often against an active comparator or historical data instead of a classic placebo control. Despite the pioneering nature of the approach, there is considerable public and regulatory oversight governing path-to-patient applications. Donor-derived T cells are genetically modified ex vivo and infused back to the patient, rather than directly into the patient, reducing the risk of in vivo infection or expression of the modifying agent within the recipient and thus limiting off-target activity. [218][219][220]

14.1 Preclinical validation and model selection

Thorough preclinical testing is essential for demonstrating safety and efficacy before independent investigators can proceed to human trials. CRISPR offers a variety of models for such evaluations, allowing the views and expertise of individual labs to help guide resilience and vulnerability questions.

Selecting an appropriate model is vital for generating meaningful preclinical data that will justify commencing patient trials. As with any experimental design, the simplest, most efficient choice that still delivers the necessary information is preferred. Mouse models tailored for evaluating efficacy in specific interventions may require only a small group of naive animals to assess the treatment paradigm. For the other end of the spectrum, many tumor types are susceptible to direct transplantation into immunocompromised mice, in which a recipient strain is chosen to provide the most appropriate support for local growth and organ dissemination. In such cases, the principal consideration is the size of the experimental cohort

needed to overcome the genetic heterogeneity of the tumor cells, with sufficient replicates employed to align data reliably with clinical observations. A different hurdle arises when developing a drug for more personalized — and potentially predictive — applications, where the aim is to collect extensive data across many candidate compounds.

14.2 Early-phase CRISPR clinical trials in oncology

Ongoing and planned clinical trials involving CRISPR-based therapies are currently registered in clinicaltrials.gov, covering a range of malignant pathologies. The primary objectives focus on determining the safety and feasibility of the intervention rather than evaluating therapeutic efficacy. The enrolled patient population is heterogeneous, with low patient numbers—no more than a handful for each CRISPR-based strategy—limiting the ability to draw generalizable conclusions. Nevertheless, these studies provide a firsthand glimpse into the clinical application of CRISPR technologies.

Covalent bond-forming and breaking reactions are central to molecular biology. The efficient and specific cleavage of target DNA through a double-stranded break constructs a favorable framework for harnessing the cell's own repair mechanisms for a variety of purposes. Most clinical trials are centered on the genome-editing of immune effector cells. Designing and producing programmed immune cells for repeated infusion into tumor-bearing patients has become the first opportunity for clinical application, due in part to the fact that the eventual outcome relies entirely on the immune system of the patient, rather than being a direct effect of the edited cells or a cell therapy. The immune-editing process actively and dynamically removes neoantigens under selective pressure during tumor evolution, even in the absence of an engineered TCR against individual mutations. A growing number of T cell-focused

clinical trials involve introducing CRISPR-induced changes to enhance function or prevent immune evasion. In addition to T cells, natural killer cells are also being engineered to achieve similar effects, as well as to target tumor cells more potently. Selectively dampening the innate checkpoint inhibitory mechanisms also reduces the risk of inducing autoimmunity in the case of ex vivo edited T cells. [221][222][223]

14.3 Manufacturing and quality control

Manufacturing of CRISPR agents for human studies requires compliance with good manufacturing practices (GMP) to ensure product quality and safety. CRISPR products have large payloads, often exceeding 20 kb, and are produced in adenoviral, lentiviral, or adeno-associated viral vectors, as plasmids, or as protein–RNA complexes. Any of these preparations are subjected to stringent material release criteria based on identity, purity, safety, and potency.

Identity control encompasses a molecular confirmation of the product when compared to the designed sequence, whereas contaminants testing includes either absence of replication-competent adenovirus in adenoviral preparations or quantification of helper viral particles in AAV production. A number of methods are applied to ensure safety standards, including labelling of lentivirus preparations to exclude replication-competent lentivirus, quantification of lipopolysaccharides, and testing of viral preparations for toxicity in mammalian cells. Potency assessment must determine both the in vitro effects in cell models and the in vivo functions in animal models before CRISPR agents enter human clinical trials.

14.4 Challenges in patient selection and dosing

Selecting the appropriate patient group is essential for minimizing variability in clinical trials. For CRISPR products that alter gene expression or modulate immune TCR or CAR

specificity, tumor and germline genotyping offer guiding parameters. Patients with saliva or blood malignancies are especially suited for early-phase trials because sample origin, mutation triggering, and tissue dissemination are evident, and minimal residual disease relates to treatment failure. Also beneficial are patients with cytogenetic abnormalities or relevant mutations for CRISPR targeting and with limited treatment options despite conventional therapies.

By analogy with CAR-T cell therapy, CRISPR products should be dosed at cytotoxic thresholds. Therapeutic window determination can draw on the broad expression profiles of Cas9 and Cas12, as well as genotoxicity assessments in tumor cells, natural killer cells, and stem cells. Population pharmacokinetics models can be developed for candidates such as AAV. However, drugs are often prematurely classified as tumor- or pathogen-targeted, and clinical translation relies on thorough testing, validation, and documentation. Testing combinations of two- or three-target appropriate CRISPR or delivery systems has merit. [224][225][226][227]

14.5 Lessons learned from ongoing human studies

Lessons learned from ongoing human studies provide insights into the challenges and considerations when applying CRISPR-based therapies for patients with cancer. Among the first clinical applications, data from eight ongoing trials testing allogeneic CRISPR-engineered αβTCR-negative T cells expressing the TCR specific for tumor-associated MAGEA4 epitopes have shown promise. This is associated with increased safety, including improved, more accessible and affordable supply chain due to closed non-viral universal T cell system, reduced risk of tumor morphological or functional reversion, rapid response to checkpoint therapy and enhanced anti-MAGEA4 and anti-other-tumor-antigens responses.

In the trial conducted by The Chinese Center for Disease Control and Prevention, HDAd5/35+-based E6 and E7 therapeutic vaccines targeting HPV associated malignancies were constructed and demonstrated safety and efficacy. Better prognosis in the therapeutic group was attributed to Ab production against the HPV viral oncoproteins. HPVs cause various malignant tumors, including cervical squamous cell carcinoma, precursor lesions and cervical adenocarcinoma, and contribute to about 500 000 resurgent cases of cervical cancer globally; the vaccines can be readily extended to treat precancerous lesions and other HPV related malignancies. As it is on-demand, portable and low-cost, CRISPR technology will facilitate vaccine development against emerging infectious diseases. [228][229][230]

Chapter - 15

Off-Target Effects and Genome Integrity

Off-target cleavage often arises from the imperfect recognition of the protospacer adjacent motif (PAM) and the target DNA sequences by the sgRNAs within heterogeneous local microenvironments created by the high concentration of Cas9 or Cas12 around the target sites. Design-dependent factors, such as the distinct nucleotide preference of the Cas/Cas9 sgRNA complex for the last base in the 3′ PAM-distal region, may also contribute to the off-target cleavage.

Several experimental methods have been developed to unveil potential off-target cleavage by endonucleases such as CRISPR-Cas9 and -12. Genome-wide analysis of double-stranded breaks detected by sequencing (Digenome-seq) enables identification of off-target sites by examining all cleavage products at single-base resolution. Genome-wide CRISPR knockout and knockin detection sequencing (GUIDE-seq) relies on the detection of tagged double-strand breaks to map all in vivo Cas9 and sgRNA-induced breaks, revealing potential sgRNA off-targeted sites both in vitro and in vivo. Circle-seq offers a high-resolution, designed-independent method to identify genome-wide off-target sites by proximity ligation and high-throughput sequencing.

Unintended editing events not only occur at these off-target sites but can also create chromosomal rearrangements such as translocations, deletions and inversions, which may severely affect cell function and genomic stability. Genotoxicity-related

side effects also arise from the collapse of replication forks at the introduced DSBs.

15.1 Mechanisms of off-target cleavage

Off-target cleavage has long been recognized as a potential drawback of CRISPR-based gene editing. Because the CRISPR-Cas system relies upon complementary base pairing between the guide RNA and the target genomic sequence for target identification, it is theoretically possible for a gRNA directed at one specific site within the genome to also exhibit varying degrees of activity at other genomic sites with sequences of partial homology. Indeed, it has now been conclusively demonstrated that the CRISPR-Cas system is capable of producing unintended gene edits at thousands of locations throughout the genome. Such unintentional cleavage is thought to primarily occur as a result of imperfect base-pairing between the gRNA and off-target sequences.

Factors that can influence the likelihood of off-target cleavage are plasmid gRNA design and delivery system components. Distinctions in plasmid design, including the presence of a tracrRNA or modification of the scaffold sequence, can not only affect on-target activity but also lead to differences in off-targeting behavior. In general, delivery of Cas9 and the gRNA in separate plasmids can be expected to yield substantially lower off-targeting levels than delivery by means of a single plasmid. Concentration of the gRNA may also play a role, with a lower concentration tending to favor on-target activity. Delivery of Cas9 and the gRNA through AAV vectors appears to effectively eliminate off-target cleavage and associated DNA mutagenesis.

15.2 Detection methods (GUIDE-seq, CIRCLE-seq, Digenome-seq)

Detection methods for unwanted CRISPR-Cas9 mutations

include GUIDE-seq, CIRCLE-seq, and Digenome-seq. These methods feature different experimental designs, but their primary goal is to identify off-target cuts with a firm understanding of how these cuts interact with the genome and what mutagenesis and repair patterns occur.

GUIDE-seq identifies potential off-targets via 5′-end-biotinylated double-stranded oligodeoxynucleotide that bind to the DNA blunt end in a sequence-complementary manner after cleavage by Cas9. The captured biotin-labeled cell lysis products are enriched by matrix-streptavidin beads, and their identity profiled by high-throughput sequencing. GUIDE-seq is not limited to cell lines but also suitable for tissue samples or tissues with detectable Cas9 activity.

CIRCLE-seq detects double-stranded breaks created by Cas9 together with NHEJ repair without relying on a known off-target site. The DNA adjacent to the off-target sites, which is cleaved by Cas9, is circularized by T4 DNA ligase. The products are then purified using the ExoI enzyme and identified by high-throughput sequencing. The methods contribute a further dimension by directly revealing the mutational changes introduced at off-target sites. [231][232]

15.3 Minimizing unintended edits

Minimizing unintended edits

Although Cas9-induced cutting can be harnessed for therapeutic purposes, it often causes off-target cleavages. Continuous DNA unwinding during the stably formed DNA–RNA hybrid, but only limited DNA unwinding in the complementary strand, could lead to single-stranded cavities. Randomly occurring cytosine deamination by AID/cytidine deaminase family enzymes is another potential source for unintentional alterations, resulting in an A3A–A3B-induced premature stop codon in the HIV-1 gag gene. Epigenetic

mechanisms, long-term insertions of exogenous donor template DNA, and Coxsackie virus A6 infection followed by genomic analysis of labelled A6-positive cells have also been detected under Cas9 infectious conditions. CD recombinase-based analysis revealed persistent cleavage in G1. Pre-emptive strategies include avoidance of TFs or chromatin regulators present at the target sites defined by Chromatin immunoprecipitation-sequencing. Translocating a minigene into the appropriate chromosomal context with Cas9 could further correct splicing defects.

Editing the β-globin gene is includes long-range deletions and chromosomal duplications involving the hunched-back locus, typically the HBB locus itself, but can also affect neighbouring genes (e.g. TLL1 on chromosome 11), although deletion of both alleles is always detrimental for viability. A refined design incorporating the latest advance in RNA-sequencing analysis at base resolution disclosed further unexpected consequences after gene president-gene editing. Owing to the presence of replication origins close to the target region, duplications or potentially even conversions are frequent in at a hotspot adjacent to the target site. Such precautions would thus seem a prerequisite for clinical gene corrective editing. [233][234]

15.4 Chromosomal rearrangements and genotoxicity

Genotoxicity refers to the ability of a chemical or other agent to damage the genetic information within a cell, leading to mutations that may contribute to cancer. Several non-canonical DNA repair events can arise after Cas9-mediated cleavage, including chromothripsis, which is proposed to cause extensive genomic instability in some tumors. Chromosomal rearrangements have been documented in Cas9-expressing mouse models and cells treated with Cas9 mRNA and gRNAs.

As is the case for off-target activity, translocations are more probable when Cas9 expression is sustained. Genotoxicity has been observed for high doses of Cas9 and, similarly, in human cells with active TP53 and P53.

However, other studies have shown that short Cas9 and gRNA expression does not lead to chromosomal rearrangements or alteration of global chromatin structures. More refined analyses may result in less controversial conclusions about potential risks. Moreover, using dual-gRNA systems and non-integrating viral vectors, which together induce lower levels of Cas9 than other approaches, appears to further reduce the risks. Finally, high-throughput analysis of off-targets and chromosomal alterations may confirm acceptable safety for preclinical trials and clinical applications. [235][236][237]

15.5 Ensuring long-term genomic stability

Because of their induction of double-strand breaks (DSBs), gene editing technologies can also cause chromosomal rearrangements, large deletions, or other genotoxicity. Such undesirable genomic instability can drive tumorigenesis and must therefore be monitored when applying CRISPR-Cas9 in somatic cells. Current methods for assessing treatment-induced changes in genomic integrity primarily rely on whole-genome sequencing (WGS) or multiplex PCR at candidate loci, both of which are costly and time-consuming, especially for large-scale studies.

Therefore, more cost- and labor-efficient methods would be valuable to enable high-throughput testing of clinical CRISPR-Cas9 platforms as well as numerous CRISPR applications that combine gene editing with other strategies (e.g., activation of endogenous retroviral elements) to probe the effects of genomic instability on cell physiology and ontogeny. Such a platform has been developed by applying CRISPRinglass in human-induced

pluripotent stem cells (iPSCs) to specifically monitor the response of model cells to Long-Read RNA Sequencing (LR-RNA).

Long-term stability and integrity of the genetic code is required for all organisms to ensure the species' survival. Therefore, the defect on DSB repair pathways would affect all model organisms, including yeast, mouse, and human cells. Experiments using a CRISPR-Cas9 approach to study the long-term effects of choosing different DSB repair mechanisms in Transcriptome were performed in mouse iPSCs, embryo fibroblasts, and NK cells. Depending on the presence of intact transcripts in these molecules, the best way to avoid the induction of stable chromosomal defects and eventual development of cancer in mice was tested. The findings demonstrate how the DNA damage repair pathway choice can impact the cellular transcriptome over time and the optimal way of modeling and following those changes.

Chapter - 16

Ethical, Legal, and Social Dimensions

Arguments concerning the prudence or feasibility of germline editing diverge sharply. Proponents cite extensive research, reformulation of the false dichotomy of public apprehension versus technological maturation, and the remarkable reduction of unintended DNA changes accompanying side-by-side editing applications. Opponents emphasize the dangers of transgenerational genome editing experimentation on humans, particularly without expansive indications and complete understanding of relevant potential long-term risks. Central to what constitutes prudent progress in germline editing are realistic appraisals of the likelihood of benefit exceeding risk as a function of specific circumstances. Distinct concerns shape the somatic gene-editing discussion. With somatic editing, the stakes are less about transgenerational consequences or experimentation on patients in the absence of sufficient consensus to warrant widespread clinical application. Rather, germline editing is primarily about the risk borne by others—their children and grandchildren.

Informed consent for somatic gene editing is not simply a matter of providing clear information about the technology and its implications for patients. The nature of the alteration—its permanent and unchangable character—demands that researchers and practitioners account also for its ramifications for the recipient's unborn and, potentially, all future offspring. Just as informing patients receiving chemotherapy of the associated risk of congenital disabilities or other serious conditions in their

offspring is an ethical requirement, so too is attention to the possibility that somatic gene editing may likewise have far-reaching implications. Present in both somatic and germline editing is the critical issue that the consent process be appropriately tailored to the intended beneficiaries of the intervention, which remains the patient alone in the case of somatic editing. [238][239][240]

16.1 Germline vs. somatic editing debates

Ongoing discussions on engineered germline editing remain polarized across scientific, ethical, and social domains. Supporters emphasize the potential for eradicating heritable conditions, while critics warn of unknown risks, unforeseen consequences during early development, lasting effects on entire lineages, adverse impacts on genetic variation, and the risk of exploitation for social enhancement. Current regulations across the globe restrict germline editing. Although germline therapies hold the promise of curing heritable disorders through somatic intervention, uncertainty exists regarding the introduction of edits into the germline, and public anticipation of germline therapies appears far ahead of scientific readiness.

On the other side of the debate, support for germline therapies is growing. Public health experts point to the possibility of eradicating sickle cell disease; geneticists argue that harmful mutations can be corrected before they accumulate; and discussions of future therapies for mitochondrial DNA disease are elegant and straightforward. The most famous germline intervention, moreover, altered CCR5 to protect against HIV. In the face of applicant requests, the deliberative bodies of the United Kingdom and Canada have acknowledged these potential benefits while recommending cautious engagement and consideration of the precautionary principle. Proposed regulatory guidance in the US and Europe extends similar reasoning to

confirming somatic gene therapy's safety before broadening to germline applications. [241][242][243]

16.2 Patient consent and risk communication

Patient consent and risk communication represent some of the fundamental processes in translational research that are crucial to enable responsible progress of the biotechnology enterprise. Although the ethics of patient consent are often discussed at the level of germline editing, every CRISPR trial in humans uses somatic editing in sick patients who are being treated, sometimes as victims of autoimmunity or cancer, and any biosafety issue is generally looked at primarily from the risk-benefit ratio of these patients. A two-part strategy has emerged from risk management and clinical bioethics: standard informed consent procedures integrated with risk communication to research participants. Informed consent is based on three principles: that patients affected by a serious condition with few or no effective treatment alternatives can be treated with a novel therapy if awareness of the inhuman risk is ensured; all patients involved can choose whether or not to participate; and the procedure is conducted by experienced medical practitioners in a secure clinical environment. Risk management involves labeling of serious, wounded or dying disease patients so that surgeons can inform and explain without creating panic in others.

This issue is especially pressing for gene editing, as these techniques carry unforeseen risks of altering the regulation or integrity of the entire genome, including gene duplication, deletion, translocation, chromosomal fusion and other forms of genomic instability. For somatic editing—new genetic cures for severe, rare diseases—sidebar ethical difficulties are ameliorated by the fact that patients are fully informed of the massive level of intrinsic risk—massive in relation to the risk of dying of the disease. The principle of precaution also applies: the risk of gene

therapy using nucleases and donor DNA for correcting mutations is far and away outweighed by the intrinsic risk carried by the serious condition; the problem of risk is confined mainly to the off-target effects and their long-term consequences.

16.3 Public perception and media influence

Public perception is a decisive factor in the success or failure of any scientific advancement, and media coverage shapes public opinion—especially on issues that involve science or technology with ethical dilemmas. CRISPR technology is no exception. Criticism of gene editing was primarily triggered by its application on human germline editing, which subsequently forced a pause and reassessment worldwide. Despite the scientific rationale securing broad public acceptance, prudence is warranted whenever new biotechnologies hold promise for curing devastating diseases but carry the potential to unwittingly compromise biodiversity. Furthermore, studies of CRISPR-Cas9-ShERLOCK as a technique capable of detecting DNA or RNA of viruses from infected plants demonstrated broad acceptance among participants who correctly perceived the technique as a success of science that, in addition to enabling genetic editing, will reduce food security loss and possibly even prevent war. However, there remains considerable public disquiet regarding applications in multifunctional animals or crops, alongside fears of spreading modified genes to wild relatives, a potential reduction in biodiversity, and the commercial control of the technology. Moreover, simulated online news coverage reporting a gene-editing technology capable of preventing/utilising genetic diseases clearly influenced public perception, raised awareness of a sensitive/controversial issue, and positively affected germline editing-related attitudes. Nevertheless, news media had a limited impact on individuals' behavioural intentions towards germline editing. [244][245][246][244][245][246][247]

16.4 Global governance and policy frameworks

Effective research and clinical use of CRISPR systems—particularly in cancer treatments—demands collectively established governance principles and policy frameworks. Existing international legal frameworks regulate biomedicine and biotechnology. However, if society—both at an operational level and through media, politic, and public opinion—continues favoring biomedical research with the argument of humanitarian reasons, the looming consequentialist-terrorist techniques debate unavoidably emerges. Global institutions face serious challenges to promote equitable and optimal use of all these powerful techniques, both in-territory and cross-territory, applying flexible and adaptive procedures through a lifelong reconsideration process. Thus, the strong pressure applied for the establishment of international strands—having an impact on national and transnational levels—provides the social safeguard for systemic technologies.

In particular, problems arise from strict legislative frameworks and their interpretation regarding CRISPR genetic modifications, especially when considering their possible applications regarding people's embryos and/or germinal lineage. In that case, there are very strong arguments against and in favor of germ line genetic modifications. The initial generation of genetically edited people at the blastocyst level, CRISPR population screening, and treatment pose new ethical, bioethical, and legal issues. Yet rapid technological advances may override the issues before a stable conclusion reshapes public opinion. The need for responsibility is by now the only recommended guideline by scientists and ethics/blastocyst boards. Any violation of responsible behavior in the use of these techniques must indeed entail the strongest consequences.

16.5 Responsible innovation in gene therapy

Partnering the promise of CRISPR technology with a robust reminder that health care innovation should always proceed with caution is one way to mitigate the justifiable fear that this transformative technology will come to be viewed as the next "recreational biological science kit." For Professor Stuart L. Hazen, the message is both simple and one applied repeatedly with success: "It's a great tool, but you have to know how to use it."

Preparation and control exercised by institutional bioethics committees, education and awareness-building within the population, a culture of reflection and debate on technological and medical questions, fair use of research findings and technologies, and respect for the principle of precaution, combined with a broad social debate on acceptable boundaries for the applications of biotechnology, can help the democratization of science and provide a sound ethical basis for the development of experimental genetic retouching technology. Society has the right to demand answers to its doubts and questions about gene editing, but it must do so free of prejudice and fanaticism. [248][249][250][251]

Chapter - 17

Regulatory Pathways and Commercialization

17.1 FDA and EMA guidelines for gene editing: Regulatory criteria and processes

CADTH's approach to the review of CRISPR-Cas medicine is multidisciplinary, integrating inputs from medical and economic experts, along with information about the relevant considerations from industry. Due to its early development stage, there is limited evidence to validate its potential clinical or medical–economic impact. Therefore, CADTH has examined the scientific principles and technical requirements needed to progress toward formal clinical investigation. The primary focus is on clinical and technical requirements for products that use CRISPR technology to induce localized, targeted gene editing in patients and that aim to modify specific somatic cells in cancer or other disease treatments without germline effects. Commercialization pathways for these products must also be viable.

Currently, two-based gene editing platforms (TALENs and ZFNs) have been approved for clinical use; however, these technologies utilize the same principles and require highly skilled personnel to create them, whereas CRISPR-Cas9 can be engineered quickly and inexpensively. Nonetheless, the CRISPR-Cas systems differ in precision, delivery, robustness, and cost. Both on-target and off-target mutagenesis must be evaluated because the former leads to intended genetic alterations, while the latter can have safety and efficacy

consequences. इन क्षणों में, डिज़ाइन किए गए श्रेणियों के वर्गीकरण का उपयोग

Regulatory agencies across the global health networks, such as the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA), and the World Health Organization (WHO), have published documents outlining the main criteria for approving. These include: the indication for use, risk: benefit ratio, product quality, and compliance with good manufacturing practices (GMPs). In summary, the FDA considers cellular gene transfer and manipulation for somatic therapy using CRISPR technology to be within its regulatory scope. [252][253][225]

17.1 FDA and EMA guidelines for gene editing

Recent advances in gene editing techniques have led to the implementation of knowledge gained from basic and translational research in clinical settings. The Food and Drug Administration (FDA) and European Medicines Agency (EMA) are currently developing guidelines and recommendations for the use of gene editing in somatic cell products. Groups of basic and clinical researchers have outlined several considerations that may serve as a foundation for protocol development and review. The analysis of security and productive ingestion into the natural environment is of special interest considering the revolutionary nature of gene editing. The results of the application of these approaches are expected to permit rapid but careful steps in responsible gene editing clinical applications for patients.

CRISPR/Cas-mediated processes may have broad therapeutic value in biomedical areas. These methods have potential applications for the prevention, treatment, or cure of diseases, conditions, or defects through genome editing in somatic tissues, including those of the hematopoietic system, delivery of CRISPR/Cas components in permanent or transient expression systems, and the training of immune systems against specific agents. With the ease of using natural systems from

bacteriophage and bacteria, CRISPR has become a standard editing tool in growing lists of mammalian and non-mammalian systems, with many components available for potential clinical applications.

The use of these technologies has led to the development of many infectious disease indications, including oral vaccines to prevent gut invasion by cholera pathotypes or intestinal tropic Norwalk and other viruses, active immunization against lentiviruses, such as HIV-1, Zika, Chikungunya, and influenza virus, delivery systems targeting viruses causing upper respiratory tract infections, and the engineering of cholera toxin--based enterotoxin vaccines with safety and efficacy potential. [254][255][256]

17.2 Intellectual property and patent issues

Intellectual property and patent issues: Ownership of CRISPR inventions remains debated, with the University of California and the Broad Institute pursing separate claims. Fundamental patents grant freedom to operate with early-phase technologies and have been assigned to Emmanuelle Charpentier's group. Budding academic spin-offs, particularly in diagnostics, benefit from established implementation of key technologies. Commercialization of CRISPR-based medicine is limited to gene therapies targeting single diseases. Growing interest in more complex uses introduces additional uncertainties and potential hindrances related to ownership of preclinical alterations in CRISPR technologies or their applications. Addressing these concerns is crucial for rapid translation of basic research discoveries into impactful biomedical innovations. Finally, appropriate pricing policies need to ensure broad access to affordable CRISPR applications. [257][258][259][260]

17.3 Industry-academia collaborations

Building CRISPR-based therapies is expensive, technically challenging, and increasingly regulated. Because of these factors, many biotech companies have opted to partner with established pharmaceutical and biotechnology companies, which may help engender buy-in from regulatory agencies. Research universities and related institutions have also seen the benefits of infancy-stage partnerships with companies. By licensing technology or enabling a dedicated research program on site, companies can retain a stream of scientific validation and innovation while, in turn, providing the institution with funds and state-of-the-art equipment that might otherwise not be available. The final elements of the cycle are product research and development, which are the main focus of commercial biotechnology and pharmaceutical companies. The technical and financial burden tends to be high in these phases.

Under the binary classification of CRISPR treatments, somatic genome editing is the field actively pursued. Academic researchers are conducting many of the leading human clinical trials. As the pipeline develops further and with a growing understanding of patients' genetic profiles, subtle issues of risk assessment will come into sharper focus. For example, the Cytogen group in New York has used CRISPR to modify immune T cells from a human patient diagnosed with severe Epstein-Barr virus (EBV) positive, lymphoproliferative disease and persistent EBV infection. Major clinical actors have entered the field as a result of these pioneering efforts, constituting the initial phases in the medicine cycle. [261][262][263][264]

17.4 Economic and access considerations

Gene therapy is undeniably a costly process, encompassing both manufacturing and treatment expenses. For patients not covered by health insurance or who lack the financial means to

afford these therapies, manufacturers may need to make special arrangements, which can span across countries. Optimizing manufacturing processes is an important step towards making these therapies more affordably without sacrificing patient safety. In the short term, decisions on pricing and reimbursements for CRISPR-based therapies will heavily rely on the link between treatment response and data from the patient cohorts. However, obtaining long-term follow-up data on the treatment response remains a challenge, especially for heritable conditions, as most patients die before they become parents, thus making a potential benefit from received germ-line therapy unattainable.

Patient costs should be based on treatment value rather than manufacturing costs or revenue maximization. The initiation of CD-19 CAR-T cell therapy is seen as the beginning of a revolution in cancer therapy, and "addressing the challenges of current chimeric antigen receptor T-cell therapy is key for its broader application". To tackle this, delivery of CD-19 CAR-T cells at an affordable cost is essential. In the case of heritable conditions, "a planting-thought approach can further aid patient stratification and reduce costs" so that germ-line editing may be delivered at a loss to stimulate take-up. For preclinical sterile-insect-controlled gene-driving bugs, the deployment price focuses on economic equilibrium rather than research costs. For herpes simplex virus antibody against the HSV-2 glycoprotein D (gD2) in vaginal delivery, the median avoidance probability and non-responders were crucial for pricing. "Pricing gene therapy products in resource-limited settings requires particular consideration" and demand estimates are of great importance for determining whether a sustainable market exists for gene therapy products.

17.5 Future of CRISPR-based biotech startups

The CRISPR toolbox has opened exciting opportunities in the OTT, enabling innovative solutions for cancer diagnoses, therapies, and monitoring. Drug development takes a long time and is expensive, requiring up to 15 years and $50 billion to develop a single drug. For drugs targeting oncogenes or relying on the tumor microenvironment, the actual development and preclinical research phase lasts less than two years. Targeting well-known cancer mutations and malignancies while paving the way for novel therapies is therefore alluring to researchers and investors alike. This growing interest is reflected in the increasing number of CRISPR-based biotech companies with clinically validated candidates. However, the limited commercial opportunity, high regulatory barriers, extended timelines for some players, and early-stage nature of others pose significant challenges. Research can de-risk a start-up, but preserving its identity and value as a nimble innovator is essential for success.

The therapeutic CRISPR-Cas9 toolbox represents a highly engaged trend in biotech, encompassing cancer detection, diagnosis, therapy, prognostication, and even prevention. Start-ups have innovated in three areas: optimizing the therapeutic CRISPR-Cas9 system, developing clinically validated solutions (diagnostics, drugs, etc.) for a specialized niche, or applying synthetic biology to develop CRISPR-analyzed detection methods. To transform such a promising yet diffuse field into an efficient therapeutic ecosystem, any one start-up must remain focused and provide a solution of sufficient breadth, depth, commercial appeal, and timely delivery. [265][266][267][268]

Chapter – 18

The Future of CRISPR in Cancer Medicine

Graduate Module Title: CRISPR-Based Gene Editing for Personalized Cancer Therapy: Next-Generation Precision Medicine

Integrating multi-omics for precision editing: Multi-omics approaches, leveraging genetic, epigenomic, transcriptomic, proteomic, and metabolomic profiles to characterize cancer, improve biomarker discovery, and enhance CRISPR functionality, notwithstanding technical hurdles. Investigating CRISPR systems as components of synthetic biological circuits, with promising applications and inevitable challenges. Current efforts in biology and medicine gravitating towards preventive genome editing. Aspirations of total patient cures, borne from success for rare indications, beckoning impassioned yet cautious support. Toward superior design of precision cancer therapy and amplification of patient survival.

Sophisticated multi-omics strategies unveiled the genetic, epigenetic, transcriptional, proteomic, and metabolic landscapes of diverse cancers. CRISPR rendered possible drug responses and resistance models, drug and disease signatures, and detection sensors for key oncogenic events. The animal rationale adopted by organoid CRC models adapted to patient-derived xenograft avatars, signified the cost-effective next-generation strategy for functional ex vivo patient exploration. Physiological and animal access unlocked synergies with immune checkpoint inhibitors and other immune therapies. Synthetic biology combined with

CRISPR improved animal pathology modeling. The clinical community contributed successfully curated clinical solution responses to infrequently used cancer drugs induced in organoids and organs, be it successful CRISPR- or RNA-based vaccine preparations derived from intelligent virus circuits, or dedicated models.

What originates from science and technology striving for a cure in one rare case resonates across patient groups and appeals for support, be it stress disorder correction, prevention of AIDS or cancer, and common viral infectious diseases. In that sense, total cure, be it virological, bacterial, genetic, or tumor cancer, is the goal people wish to achieve. It is also a sign of colorful development. But visions of "preventive gene editing" are now at a turning point. Recent warnings from functional insights into mice remain sane rather than scaring; stepping back however is neither possible nor in line with the noticed progress toward many simpler and safe methods, including organoid CRISPR- or RNA-based vaccine preparations, intelligent virus circuits for vaccines, cancer vaccines, and synthesis-resistance preparations. [269][270][271][272]

18.1 Integrating multi-omics for precision editing

The full spectrum of mutations across multiple cancer types is now well-characterized, following the establishment of large-scale genome sequencing initiatives. Comprehensive multi-omics studies further emphasize that beyond simple base changes and copy-number variations, other genomic modalities, including epigenetic alterations, chromosomal instability, and expression variation, contribute to tumorigenesis (e.g., Beck et al. 2019). However, clinical implementation pathways remain largely siloed, with separate efforts underway to develop CRISPR-based therapies for individual mutation classes. The clinical engineering of CRISPR pathways for genomic mutations driven

primarily by chromatin accessibility offers compelling promise. While current progress remains exploratory, developing systems with sufficient resolution for broad-spectrum drug discovery is a feasible next step.

An added layer of complexity will arise when synthetic endothelial-templated designs seek to recapitulate the higher-order mixing of both transcriptional and epigenetic systems present in native tumors. Beyond discrete drug products, emerging interest in therapeutic vaccines raises the prospect of homing in on T cell responses against a patient's own tumor. Collectively, the above advances converge to provide a blueprint for developing next-generation onco-therapies in which precision CRISPR editing is combined with systems that directly or indirectly address the variable epigenetic states of specific tumors. These initial fusions should offer attractive beta-testing grounds on an inevitable path toward the integration of CRISPR editing into a full multi-omics framework with potential for precision editing of any tumor type.

18.2 CRISPR and synthetic biology fusion

Two emergent domains of biology, CRISPR gene-editing technologies and synthetic biology, have recently converged, with promising implications for several sectors, particularly cancer medicine and biotechnology start-up industries. Such dual applications would provide double-driven advances for incubated start-ups by significantly reducing the intellectual cost of establishing demonstration usable prototypes, and thus accelerating getting commercially launched syntheses into the market.

The fusion of the two domains is well illustrated by the CRISPR-Dx platform, integrating the best-sensitive enzyme-based detection of specific DNA/RNA fragments with integrated logic gates directing cleavage of probes, both of which are

controllable by detection-specific synthetic CRISPR-transcriptional sequences. Also worthy of note is the construction of CRISPR-based biosynthesis systems for chitobiose and cyclodextrin, microbiome leader chemotaxi circuits, and reconfigurable dual representation, using CRISPR technology to direct established synthetic pathways. The prospect of combining CRISPR-mediated gene-editing capabilities with biosystems that generate new chemosynthetic taxa is also a topic of increasing interest. [273][274][275]

18.3 Toward curative cancer therapies

The concept of CRISPR-based technologies for treatment resistance and personalized therapy is an enticing notion, an optimism strongly reflected in society and expressed in art and scientific publications. Today's generation conceives the prospect of being able to exclude the imminent risk of cancer development among the health priorities of the future and of treating any cancer with possibility of cure, thus moving from precision medicine to filling the gaps of today's cancer treatments. Nevertheless, although completion of clinical trials is important for human health, coalescence of collective efforts and competence on CRISPR and cancer research and the scientific answer to the question of whether the application of CRISPR can indeed prevent cancer and cure any cancer are indispensable for a promising future of the next cancer generation.

Innovations, ideas, discoveries, and projects have taken shape during the past 10 years, and numerous CRISPR clinical studies in humans are underway. It is therefore realistic to foretell that treatment of the first patients will soon be completed or started. At the same time, important steps in therapy planning, safety, and follow-up are necessary to pave the way and offer an example for fulfilling the wishes of society and innovation beyond this first round of trials.

18.4 Preventive genome editing: promise and peril

The potential of gene editing therapies to eliminate disease-causing mutations prompts speculation about using the approach preventively. Repetitive NHEJ repair associated with HDR pathway suppression may favor chromosomal translocations and DNA damage, while inefficiencies increase the number of cells required to express an edited allele for phenotypic correction. Together, these features could enhance the formation rate of deleterious genotypes after preventive editing. Moreover, the expanding world population and mounting public concern could intensify the balance-wagering pressure on genome-editing technologies. Whether the prospect of using gene editing medicine in large populations convinces society to abandon such applications remains open to debate.

CRISPR-based approaches hold great promise as a means of preventing cancer before either its initiation or the formation of additional mutations that would offer a survival advantage. Complex considerations involve both scientific challenges, such as the extensive heterogeneity of primary tumors and the experimental evidences that CRISPR systems only temporarily modify the DNA sequence in edited cells, and ethical discussions. Additional questions remain regarding public perception and the implementation of somatic cell editing through genetic therapy.

18.5 Vision for the next generation of precision oncology

The rationale and planning for next-generation precision medicine, particularly for cancer, will follow a similar trajectory to initiatives in genomics, transcriptomics, proteomics, and metabolomics: first, generate comprehensive big datasets using multi-omics technologies from many specimens, then develop a research model based on patient-derived specimens, and finally apply the multilevel data and real-world modeling to assist in

decision making. Patient- and disease-specific data, preferably from the same background, will thus ideally be synthesized and integrated for artificial intelligence-supported decision making in precision oncology. CRISPR is both a technology and an idea generator, inspiring extensive research in cancer management and precision medicine despite still requiring a breadth of validation before real-world application. Advanced and perhaps next-generation cancer treatments will partially resemble curative treatments for genetic diseases, targeting the same cellular mechanism. The excitement triggered by efficient, precise, multimodal, and universal gene-editing tools is already directing interest toward therapeutic correcting surgery for aging and cancer as previously achieved for monogenic diseases.

Finally, some researchers advocate preventive genome editing in early embryos or germ cells, with the goal of removing alleles predisposing to diseases from future generations. Opponents of human germline editing counter that prudent evolutionary conservation should avoid altering human evolution in the species' entire history. Overall, the diverse stories behind the manifold inputs and outputs from omics studies depict an exhilarating journey across the ocean of biological and medical research toward an unfamiliar land with untold secrets. The experience offers insights supporting the quest for the next generation of precision oncology. [276][277][278]

Conclusion

CRISPR-Cas technology has revolutionized gene editing by lowering the technical barrier for generation of precision cuts in DNA at virtually any location and has rapidly gained attention in directing therapy against a range of human diseases, including cancer. CRISPR-based interventions for personalized cancer therapy have been positioned in the wider context of precision medicine, which aims to integrate individual patient characteristics and data with the goal of improving treatment outcomes. Artificial intelligence and bioinformatics are expected to play a key role in realizing that vision within the near future, in synergy with CRISPR and multi-omics information.

Considerable challenges remain before CRISPR's potential for curing cancer can be realized. Its current applications are only beginning to extend beyond simple repair of known mutant driver genes toward the more ambitious goal of complete genetic correction of a tumor. Moreover, CRISPR remains one of many promising gene therapy methods poised to help patients—together with gene therapies that restore lost function, overcome addiction, or induce synthetic lethality. Nonetheless, as multiple experimental approaches combine with the latest developments in CRISPR technology, the DNA-targeted therapy industry is already placing the first precursors of new curative strategies within reach.

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