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A Brief Glimpse on Advancement OFCRISPER/Cas in Research

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ABSTRACT-
CRISPR/Cas genome editing is a simple, cost effective, and highly specific technique for introducing genetic variations. In mammalian cells, CRISPR/Cas can facilitate non-homologous end joining, homology directed repair, and single-base exchanges. Cas9/Cas12a nuclease, dCas9 transcriptional regulators, base editors, PRIME editors and RNA editing tools are widely used in basic research. A variety of CRISPR/Cas based on the therapeutics are being investigated in clinical trials. Among many new findings that have advanced the field, I have highlighted a few recent advances that are relevant to action of CRISPR/Cas based gene therapies for monogenic human genetic diseases.
SpCas9 was applied in mammalian cells, other Cas9 proteins have been studied and developed as genome editing tools. For example, smaller Cas9 proteins derived from Staphylococcus aureus called SaCas9 and Neisseria meningitidis called Nme2Cas9 which exhibits more gene editing efficiency comparable to that of SpCas9. These smaller Cas9s are more amenable for in-vivo delivery than the large SpCas9.
CRISPR/Cas9 technological advances have also enabled various applications of nuclease deficient Cas9s, which can bind a specific region of the genome without creating DSBs. For example, catalytically inactive dead-Cas9(dCas9) can be fused with various transcription regulatory domains to create CRISPR-activators(CRISPRa) or inhibitors(CRISPRi) that activate or silence the expression of a target gene. dCas9 can also be used as a visualization tool. Chen and colleagues have used dCas9 fused to enhanced green fluorescent protein (EGFP) to visualize repetitive DNA sequences using one sgRNA, or nonrepetitive loci using multiple sgRNAs. David R. Liu's group has fused D10A Cas9 nickase with either cytidine or adenine deaminase to generate Cytidine Base Editors(CBEs) and Adenine Base Editors(ABEs), respectively. CBEs and ABEs generate transitions between A•T and C•G base pairs without causing maximum levels of double-stranded DNA cleavage in the target genomic region. Liu's group extended base editing to utilize H840A Cas9 nickase fused with reverse transcriptase to create prime editors (PEs), which can achieve all possible base pair conversions, as well as targeted insertions and deletions without DSBs or donor DNA templates.
In addition to DNA editing, Feng Zhang's lab has reported that an RNA-targeting CRISPR system based on Cas13 can target and cleave specific strands of RNA, and subsequently developed strategies called REPAIR(RNA Editing for Programmable A to I Replacement) and RESCUE(RNA Editing for Specific C to U Exchange) to edit RNA. Thus, RNA editing with CRISPR can efficiently modulate target genes at the transcript level in a transient and PAM independent manner.
CRISPER/Cas have a huge impact in the field of biosciences for curing several diseases.
Key words*-CRISPR/Cas, Gene editing, Gene therapy, Human disease, Genetic disease*

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I. APPLICATIONS OF CRISPER IN GENETIC DISEASES-

CRISPR/Cas systems have been used to investigate the target genes in genome modification, splicing, transcription and epigenetic regulation, it's used research setting to investigate and treat against genetic diseases, infectious diseases, cancers, and immunological diseases. Among the exciting advances, translational use of CRISPR/Cas in monogenic human genetic diseases has the potential to provide longterm therapy after a single treatment.

CRISPR/Cas9mediated gene knockout screens and target identification via wholegenome sequencing uncover host genes required for Picornavirus infection-

Several groups have used genome-wide libraries of lentiviruses encoding small guide RNAs (sgRNAs) for genetic screens. In most cases, sgRNA expression cassettes are integrated into cells by using lentiviruses, and target genes are statistically estimated by the readout of sgRNA sequences after targeted sequencing. We present a new virus-free method for human gene knockout screens using a genome-wide library of CRISPR/Cas9 sgRNAs based on plasmids and target gene identification via whole-genome sequencing (WGS) confirmation of authentic mutations rather than statistical estimation through targeted amplicon sequencing. We

used 30,840 pairs of individually synthesized oligonucleotides to construct the genome scale sgRNA library, collectively targeting 10,280 human genes (three sgRNAs per gene). These plasmid libraries were co-transfected with a Cas9-expression plasmid into human cells, which were then treated with cytotoxic drugs or viruses. Only cells lacking key factors essential for cytotoxic drug metabolism or viral infection were able to survive. Genomic DNA isolated from cells that survived these challenges was subjected to WGS to directly identify CRISPR/Cas9-mediated causal mutations essential for cell survival. Scientists were able to identify known and novel genes essential for viral infection in human cells. Scientists propose that genome-wide sgRNA screens based on plasmids coupled with WGS are powerful tools for forward genetics studies and drug target discovery.Heon Seok Kim et al. 2017

APPLICATIONS OF CRISPER AGAINST INHRITED EYE DISEASE-

Leber Congenital Amaurosis (LCA) is a rare genetic eye disease manifesting severe vision loss at birth or infancy. LCA10 caused by mutations in the CEP290 gene which is a severe retinal dystrophy. CEP290 gene is too large to be packaged into a single AAV. To overcome this limitation, Editas Medicine developed EDIT-101, a candidate genome editing therapeutic, to correct the CEP290 splicing defect in human cells and in humanized CEP290 mice by sub-retinal delivery. SaCas9 is used to remove the aberrant splice donor generated by the IVS26 mutation. In the human CEP290 IVS26 knock-in mouse model, over 94% of the treated eyes achieved therapeutic target editing level when the dose of AAV was not less than 1×10^{12} vg/ml. Allergan and Editas Medicine have initiated a clinical trial of EDIT-101 for the treatment of LCA10.

Autosomal dominant Cone Rod Dystrophy 6(CORD6) is induced by gain of function of GUCY2D mutation. CRISPR/Cas components delivered by AAV specifically disrupt the early coding sequence of GUCY2D in the photo-receptors of mice and macaques by NHEJ. This study was the first to successfully perform somatic gene editing in primates using AAV delivered CRISPR/Cas to cure inherited retinal diseases.

APPLICATIONS OF CRISPER AGAINST β-THALESSEMIA DURABLE FETAL HEMOGLOBIN INDUCTION-

Inherited blood disorders are good candidates for gene therapies because gene therapy can modify the causative gene in autologous hematopoietic stem cells (HSCs) and correct the hematopoietic system β-thalassemia and sickle cell disease are two genetic blood diseases β-thalassemia is due to various mutations including small insertions, single point mutations or deletions in β-globin gene, resulting in loss or reduced β-globin synthesis. Sickle cell disease is caused by a Glu->Val mutation in β-globin subunit of hemoglobin, leading to abnormal hemoglobin-S. Re-expressing the paralogous γ-globin genes is a universal strategy to ameliorate both β-globin disorders. The Bauer group applied CRISPR/Cas-based cleavage of the GATA1 binding site of the Erythroid Enhancer(EE). This approachmakes the erythroid expression decreased of the γ-globin repressor BCL11A and in turn it increases γ-globin expression.

This strategy is therapeutically practicable to produce durable fetal hemoglobin induction.Three clinical trials aiming to treat patients with β-thalassemia and severe sickle cell disease by transfusion of CRIPSR/Cas9 edited CD34+ human HSCs (CTX001) have been initiated by CRISPR Therapeutics in 2018 and Allife Medical Science and Technology Co., Ltd in 2019.

APPLICATIONS OF CRISPER AGAINST GENETIC LIVER DISEASE-

Hereditary Tyrosinemia TypeI(HTI) patients with loss of function FAH mutations accumulate toxic metabolites that cause liver damage. CRISPR/Cas mediated HDR has been used to correct $FAH^{mut/mut}$ in the HTI mouse model by hydrodynamic injection of plasmids encoding CRISPR/Cas components or by combined delivery of AAV carrying HDR template and sgRNA and of nanoparticles with Cas9 mRNA. VanLith transplanted edited hepatocytes with corrected FAH into recipient FAH knockout mice and cured HTI mice. Scientists have used ABE in an adult mouse model of HTI to correct a FAH point mutation. In addition to correcting FAH, several groups have knocked out Hydroxyl-Phenyl Pyruvate Dioxygenase(HPD), which acts in the second step for tyrosine catabolism and is an upstream enzyme of FAH, to prevent toxic metabolite accumulation and treat HTI metabolic disease.

Patients with α-1 Antitrypsin Deficiency(AATD) develop liver disease due to a toxic gain of function mutant allele, as well as progressive lung disease due to the loss of AAT antiprotease function. CRISPR/Cas mediated NHEJ has been used to disrupt mutant AAT to reduce the pathologic liver phenotype, while HDR has been used to correct an AAT point mutation.

APPLICATIONS OF CRISPER IN CANCER RESEARCH-

(1)CRISPR-mediated TCR replacement generates superior anticancer transgenic T cells:-

Adoptive transfer of T cells genetically modified to express a cancer-specific T-cell receptor (TCR) has shown significant therapeutic potential for both hematological and solid tumors. A major issue of transducing T

cells with a transgenic TCR is the preexisting expression of TCRs in the recipient cells. These endogenous TCRs compete with the transgenic TCR for surface expression and allow mixed dimer formation. Mixed dimers, formed by mispairing between the endogenous and transgenic TCRs, may harbor autoreactive specificities. To circumvent these problems,scientists have designed a system where the endogenous TCR-β is knocked out from the recipient cells using clustered regularly interspaced short palindromic repeats CRISPR-associated protein-9 (Cas9) technology, simultaneously with transduction with a cancer-reactive receptor of choice. This TCR replacement strategy resulted in markedly increased surface expression of transgenic αβ and γδ TCRs, which in turn translated to a stronger, and more polyfunctional, response of engineered T cells to their target cancer cell lines. Moreover the TCR plus CRISPRmodified T cells were up to a thousandfold more sensitive to antigen than standard TCR transduced T cells or conventional model proxy systems used for studying TCR activity. Finally, transduction with a pan-cancer–reactive γδ TCR used in conjunction with CRISPR/Cas9 knockout of the endogenous αβ TCR resulted in more efficient redirection of $CD4^+$ and $CD8^+$ T cells against a panel of established blood cancers and primary, patient-derived B-cell acute lymphoblastic leukemia blasts compared with standard TCR transfer. Our results suggest that TCR transfer combined with genome editing could lead to new, improved generations of cancer immunotherapies. (Andrew K. Sewell et al. 2018)

(2)Targeted Delivery of CRISPR/Cas9Mediated Cancer Gene Therapy via Liposome-Templated Hydrogel Nanoparticles:-

Due to high efficiency, the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology has emerged as one of the most promising approaches for treatment of a variety of genetic diseases, including human cancers. However, further translation of CRISPR/Cas9 for cancer gene therapy requires development of safe approaches for efficient, highly specific delivery of both Cas9 and single guide RNA to tumors. Here, novel core shell nanostructure, liposome-templated hydrogel nanoparticles (LHNPs) that are optimized for efficient co-delivery of Cas9 protein and nucleic acids is reported. It is demonstrated that, when coupled with the minicircle DNA technology, LHNPs deliver CRISPR/Cas9 with efficiency greater than commercial agent Lipofectamine 2000 in cell culture and can be engineered for targeted inhibition of genes in tumors, including tumors the brain. When CRISPR/Cas9 targeting a model therapeutic gene, polo-like kinase 1 (PLK1), is delivered, LHNPs effectively inhibit tumor growth and improve tumor-bearing mouse survival. The results suggest LHNPs as versatile CRISPR/Cas9-delivery tool that can be adapted for experimentally studying the biology of cancer as well as for clinically translating cancer gene therapy.Xiaoying Wang et al. 2017

(3)CRISPR-mediated ablation of overexpressed EGFR in combination with sunitinib significantly suppresses renal cell carcinoma proliferation:-

Receptor tyrosine kinases(RTK), such as VEGFR, PDGFR and EGFR, play important roles in renal cancer. *EGFR* knockout as a therapeutic approach in renal cell carcinoma (RCC). We showed that a renal cell carcinoma cell line (RC21) has higher expression of EGFR as compared to other frequently used cell lines such as HEK293, A549, Hela and DLD1. Ablation of *EGFR* by CRISPR/Cas9 significantly restrained tumor cell growth and activated the MAPK (pERK1/2) pathway. The VEGFR and PDGFR inhibitor, sunitinib, attenuated the expression of MAPK (pERK1/2) and pAKT induced by EGFR loss and further inhibited $EGFR^{-/-}$ cell proliferation. We showed that loss of EGFR eventually leads to resistance to SAHA and cisplatin. Furthermore, EGFR loss induced G2/M phase arrest and resulted in an increased resistance to TNF Related Apoptosis Inducing Ligand(TRAIL) in renal cell carcinoma. Thus, ablation of overexpressed EGFR by CRISPR/Cas9 alone or in combination with sunitinib may be a new treatment option for renal cell carcinoma. D Chen et al. 2020

(4)CRISPR Mediated Reactivation of DKK3 Expression Attenuates TGF-β Signaling in Prostate Cancer:-

DKK3 gene encodes a secreted protein, Dkk-3, that inhibits prostate tumor growth and metastasis. By promoter methylation*DKK3* is downregulated in many types of cancer, including prostate cancer. Gene silencing studies have shown that Dkk-3 maintains normal prostate epithelial cell homeostasis by limiting TGF-β/Smad signaling. While ectopic expression of Dkk-3 leads to prostate cancer cell apoptosis, it is unclear if Dkk-3 has a physiological role in cancer cells. Treatment of PC3 prostate cancer cells with the DNA methyltransferase (DNMT) inhibitor decitabine demethylates the *DKK3* promoter, induces DKK3 expression, and inhibits TGF-β/Smad-dependent transcriptional activity. Direct induction of DKK3 expression using CRISPR-dCas9-VPR also inhibited TGF-β/Smad-dependent transcription and attenuated PC3 cell migration and proliferation. These effects were not observed in C4-2B cells, which do not respond to TGF-β. TGF-β signals can regulate gene expression directly via SMAD proteins and indirectly by increasing DNMT expression, leading to promoter methylation. Analysis of genes downregulated by promoter methylation and predicted to be regulated by TGF-β found that DKK3 induction increased expression of PTGS2, which encodes cyclooxygenase-2. Together, these observations provide support for using CRISPR-mediated induction of

DKK3 as a potential therapeutic approach for prostate cancer and highlight complexities in Dkk-3 regulation of TGF-β signaling.

(5)**Mapping a functional cancer genome atlas of tumor suppressors in mouse liver using AAV CRISPR mediated direct in vivo screening**:-

Cancer genomics have potrayed numerous human cancers. Whereas some mutations were found in classical oncogenes and TSGs other haven't functionally studied in vivo. Using Adeno Associated Viruses(AAVs) carrying a single guide RNA (sgRNA) library targeting putative tumour suppressor genes are mutated in human cancers, scientists directly pool-mutagenized the livers of Cre-inducible CRISPER-Cas9 mice. All mice that received the AAV-mTSG library developed liver cancer and died in 4 months. A molecular probe sequencing of the sgRNA target sites to chart the mutational landscape of these tumours, revevealing the functional consequence of multiple variants in driving liver tumorigenesis in immunocompetent mice. AAV mediated autochthonous CRISPER screens provide a powerful means prowerful means for mapping a provisional functional cancer genome atlas of tumour suppressors in vivo. Christopher D. Guzman et al. 2018

APPLICATIONS OF CRISPER AGAINST CONGENTIAL LUNG DISEASE-

Congenital genetic lung diseases includes cystic fibrosis and inherited surfactant protein syndromes(SPs). Monogenic lung diseases caused by mutations in SP genes of the pulmonary epithelium show perinatal lethal respiratory failure death or chronic diffuse lung disease with few therapeutic options. Using CRISPR fluorescent reporter system, scientists precisely timed intra-amniotic delivery of CRISPR/Cas9 components into a prenatal mouse model with the human SP gene SFTPC mutation to inactivate mutant SFTPC gene through NHEJ. Prenatal gene editing in SFTPC mutant mice rescued lung pathophysiology, improved lung development, and increased survival rate to an approx 22.8%. For intra-amniotic delivery, the amniotic cavity of embryonic day 16 mouse fetus, in which fetal breathing movements are optimal for fetal lung editing, was injected. After prenatal CRISPR delivery, embryonic day 19 fetus achieved up to 32% SFTPC wild-type airway and alveolar epithelial cells in SFTPC mice, rescued lung pathophysiology by immunohistology, improved lung development by reducing the synthesis of mis trafficked SFTPC mutant proprotein, and increased survival rate to 22.8%.

Cystic fibrosis is another life threatening monogenic lung disease caused by mutations in CFTR gene. Researchers applied CRISPR to precisely corrected CFTR carrying homozygous F508 deletion(F508del) in exon 10 in the induced Pluripotent Stem Cells(iPSC) separated from cystic fibrosis patients and the overall correction efficiency is up to 90% using piggyBac transposase as selection marker. Xu group applied the electroporation of CRISPR/Cas RNP and achieved more than 20% correction rate in patient derived iPSC cell line with F508del mutation. As expected, CRISPR-induced genetic correction leads to the recovery of CFTR function in airway epithelial cells or proximal lung organoids derived from iPSC.

RNAi-mediated control of CRISPR functions-

CRISPR-Cas9 has become a versatile tool for genome editing, gene regulation, and live imaging in a wide range of organisms. The engineered CRISPR-Cas9 system contains two components: the Cas9 endonuclease and a single guide RNA (sgRNA), which recruits the Cas9 protein to a target DNA sequence. However, in some cases, the technology has not been proven to be accurate enough, with various unexpected off-target effects. To render CRISPR based editing more precise and safer, strategies to control CRISPR-Cas9 activity are highly desired. The tools to effectively and reversibly control the activity of CRISPR systems can alleviate safety concerns related to their accidental misuse. The newly developed CRISPR switches based on small molecules or light have attracted much attention. By adding exogenous inducers, the expression of the CRISPR system or the recombination of the Cas9 functional domains can be controlled to achieve the spatiotemporal specificity of gene editing. However, approaches necessary for inducing gene expression may have problems such as low induction efficiency and delayed gene expression. The strategy of splitting Cas9 protein may reduce the background efficiency of the system. The weak penetration of light into tissues is also a problem worth considering. Synthetic circuits controlling CRISPR expression are another area of active interest. These methods may be limited by the complexity of the genetic circuits and the rare types of control nodes that can be rewired to control CRISPR systems. Recently, phage-encoded "anti-CRISPR" proteins have been shown to block Cas9-mediated gene editing and regulation in bacterial and human cells. However, this approach requires the introduction of a foreign protein, which would broadly inhibit all sgRNA/Cas9 complexes within cells. To this end, methods for controlling CRISPR systems have been developed, but there is still a need for new tools for more specific, precise, and efficient control of the CRISPR system.

RNA interference (RNAi) is a well-conserved mechanism that uses small noncoding RNAs, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs), to silence gene expression post-transcriptionally. Mammalian miRNAs are endogenous 20-25nt RNA guides that mediate mRNA degradation by pairing with the

mRNAs of protein-coding genes. The most conserved motif pairs with nucleotides between 2 and 7 at the 5' end of the miRNA, which is called the 'seed' region, and the mRNA binding site. The functional unit of miRNA-mediated RNAi forms a complex with Argonaute proteins known as the miRNA-induced silencing complex (miRISC). The mammalian miRNAs can target not only protein-coding mRNAs in the cytoplasm, but also regulate noncoding RNAs in the nucleus. A small-molecule enoxacin (Penetrex) was found to enhance RNAi by promoting the processing and loading of miRNAs onto the miRISC. Besides natural miRNAs, artificial miRNAs (amiRNAs) were also developed by several laboratories using natural miRNA scaffolds to target unusual RNAs. amiRNA-based approaches do not disrupt native cellular processes and may provide safer RNAi expression vectors compared with short hairpin RNAs (shRNAs).

RNAi and CRISPR systems cleave their targets using nucleases mediated by the guide sequences. Although RNAi and CRISPR-Cas9 have many similarities in terms of their mechanisms of action, few studies have suggested a direct relationship between the two in mammalian cells. Considerable evidence has suggested a role of mammalian miRNAs in restricting exogenous viral nucleic acids. Therefore, it would be interesting to study whether mammalian miRNAs can block the foreign CRISPR system. Two studies have suggested that control of the CRISPR system using mammalian miRNAs can be achieved by inserting the miRNA binding sequence into the 5'UTR of Cas9 mRNA or the 5' and 3' ends of the sgRNA. However, previous studies have ignored the potential impact of RNAi on the wild-type sgRNAs in mammalian cells. Although it is known that Cas9/sgRNA are localized in the nucleus and that miRISCs are mainly localized in the cytoplasm, miRNAs may regulate sgRNAs directly by returning to the nucleus to act on noncoding RNAs.

It’s hypothesized that miRNAs could exert an inhibitory effect on the CRISPR system's function by binding to sgRNAs. We studied the quantitative inhibition of the CRISPR system by miRNAs combined with the RNAi enhancer enoxacin and attempted to improve the targeting specificity of the CRISPR system. Furthermore, we studied the feasibility of improving the efficiency of gene editing and regulation by blocking the effects of natural intracellular miRNAs on sgRNAs. The results revealed the competitive relationship between the RNAi pathway and the CRISPR system at the sgRNA level. Thus, the study represents a novel approach for resolving key issues of CRISPR research, including spatio-temporal specific regulation of gene editing or regulation, improving sgRNA targeting specificity, and enhancing the function of the CRISPR system.

II. CONCLUSION

CRISPR/Cas has shown a great potential in generating disease models and correcting monogenic disease mutations. The CRISPR disease models can accelerate the discovery and development of drug targets. In addition to the widely used type II CRISPR/Cas systems, continued discovery and development of CRISPR systems from prokaryotic species has generated new technologies. For example, DN1S-SpCas9 fusion protein blocks local NHEJ events and increases HDR frequency. Moreover, Cas13a based RNA-targeting tools enable RNA changes that are temporally and spatially controllable, and will broaden and facilitate the application of RNA therapy in human diseases. Before the application of CRISPR for human disease correction, efforts are needed to optimize and maximize the editing efficiency as well as minimize offtargets and develop novel tools to specifically deliver the CRISPR components to the target tissue for gene editing. As CRISPR/Casbased gene therapy enters clinical trials, this technology holds great potential for treating genetic diseases particularly for the present incurable ones and enhancing cell therapies.

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